Protein Tyrosine Phosphatase Pez:

Its role in the regulation of cell-cell adhesions

A thesis submitted in fulfilment of the requirement for the award of the degree

DOCTOR OF PHILOSOPHY

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by

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Thesis Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person except where due reference has been made in the text.

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Abstract

The balance of tyrosine phosphorylation in the cell is maintained by the opposing actions of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs). Investigation into tyrosine phosphorylation was initially focused on the action of PTKs. However, research over the past decade has revealed that PTPs also play a key role in signal transduction.

The multi-protein complexes that constitute the cell-cell adhesions in endothelial and epithelial tissues are dynamically restructured in response to extracellular and intracellular signalling. Tyrosine phosphorylation is involved in the regulation of both adherens junctions and tight junctions. Inhibitors of PTPs have been shown to disrupt cell-cell adhesions indicating that PTPs are important in maintaining adhesion integrity.

The maintenance of a selectively permeable barrier is an essential function of endothelial cells, which are the cells that line the lumen of blood vessels. Therefore, it is important to understand the normal functioning of the proteins in the cell-cell adhesion complexes. The aims of this research were to ascertain which members of the PTP family are expressed in human umbilical vein endothelial cells (HUVEC) and to characterise a PTP that may potentially be involved in the regulation of cell-cell adhesions.

A homology screen identified a cytosolic phosphatase, PTP-Pez, to be highly expressed in HUVEC. The presence of the protein-protein interaction FERM domain (band 4.1, ezrin, radixin and moesin) at the N-terminus of Pez predicted its localisation to the plasma membrane. Specific antibodies showed that in confluent monolayers Pez is cytoplasmic and concentrated at intercellular junctions but the protein is nuclear in sub-confluent cells. The adherens junction protein β-catenin and the tight junction protein occludin were both identified as potential substrates of Pez using a "substrate-trapping" approach. Data showing that Pez bound to and dephosphorylated β-catenin *in vivo* further substantiated this. A truncated form of Pez lacking the catalytic domain acted as a dominant negative mutant inhibiting the dephosphorylation of its

substrates at intercellular junctions and enhancing cell motility. Canine epithelial cells overexpressing Pez underwent an apparent epithelial to mesenchymal transition (EMT), a process typified by downregulation of cell-cell contacts. These findings indicate that Pez plays a role in the regulation of cell-cell adhesion.

Abbreviations

Ab	Antibody
AJ	Adherens junction
ATP	Adenosine triphosphate
BCR	B cell-antigen receptor
CA	Carbonic anhydrase
Cdc	Cell division cycle
Cdk-2	Cyclin-dependent kinase 2
cDNA	Complementary DNA
CK2	Casein kinase 2
DEP-1	Density dependent PTP-1
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's modified eagle's medium
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EC	Endothelial cell
ECF	Enhanced chemifluorescent
ECL	Enhanced chemiluminescent
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EGTA	Ethyleneglycol-bis- (beta-amino-ethylether)-N,N,N',N'-tetra-acetic acid

ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ERK	Extracellular-signal-related kinase
FAK	Focal adhesion kinase
FAP-1	Fas-associated phosphatase-1
FCS	Foetal calf serum
FERM domain	4.1,ezrin, radixin. moesin
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
FRET	Fluorescence resonance energy transfer
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPI	Glycosylphosphatidylinositol
HEK293	Human embryonic kidney 293
HUVEC	Human umbilical endothelial cells
Ig	Immunoglobulin
IL	Interleukin
IRK	Insulin receptor kinase
ITIM	Immunoreceptor tyrosine-based inhibitory motif
JAK	Janus kinases
JAM	Junctional adhesion molecule
K_{cat}	Enzyme-substrate dissociation rate
kDa	Kilodalton
K_M	Michaelis constant
MAM	Meprins-xenopus A5-mu

MAPK	Mitogen-activated protein kinase
MDCK	Madin-Darby canine kidney
me/me	Motheaten
MQ water	Milli-Q purified water
MW	Molecular weight
NFκB	Nuclear factor κB
NS	Noonan syndrome
NGFR	Nerve growth factor receptor
PCR	Polynucleotide chain reaction
PDGF	Platelet-derived growth factor
PDZ domain	PSD-95,DLG and ZO-1
PECAM	Platelet endothelial cell adhesion molecule-1 (PECAM)
PEST	Region rich in proline, glutamate, serine and threonine
Pez	PTP and ezrin like domains
PI3K	Phosphatidylinositol 3-kinase
PKC	Protein kinase C
PMA	Phorbol myristate acetate
PTB	Phosphotyrosine-binding domain
PTEN	Phosphatase and tensin homologue deleted from chromosome 10
PTK	Protein tyrosine kinase
PTP	Protein tyrosine phosphatase
PV	Polycythemia vera
RACK1	Receptor for activated protein C kinase
RNA	Ribonucleic acid
RPTK	Receptor PTK

RPTP	Receptor PTP
RT-PCR	Reverse transcription PCR
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SH2	Src homology 2 domain
SHP	PTP containing SH2 domains
ST	Substrate-trapping
STAT	Signal transducers and activators of transcription
TCF	T-cell factor
TER	Transepithelial resistance
TJ	Tight junction
TNF	Tumor necrosis factor
TNFR	TNF receptor
VE-cadherin	Vascular endothelial cadherin
VEGF	Vascular endothelial growth factor
VHR	Vaccinia H1-related enzyme
wt	Wild type
ZO	Zonula occludens
ZONAB	Z0-1-associated-nucleic-acid-binding protein

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CHAPTER ONE
Introduction

Chapter 1: Introduction

1.1 Tyrosine phosphorylation

Protein tyrosine phosphorylation plays a central role in the regulation of many cellular processes. Intracellular signal transduction via integral-membrane and soluble tyrosine kinases can determine such diverse fates as cell growth, differentiation, metabolism, cell cycle regulation, apoptosis, cancer and cytoskeletal function (Blume-Jensen and Hunter, 2001; Hubbard and Till, 2000; Kolibaba and Druker, 1997; Schlessinger, 2000). The level of cellular tyrosine phosphorylation is kept under tight control and in the resting cell is maintained at a very low level. Slight increases in the steady-state level of phosphotyrosine can result in hyperproliferation and malignant transformation of cells.

1.2 Protein tyrosine kinases

Protein tyrosine kinases (PTKs) transfer phosphate from ATP to tyrosine residues on specific cellular proteins, thereby altering the function of the substrates. Tyrosine kinases can be divided into two major subfamilies, receptor and non-receptor tyrosine kinases.

1.2.1 Receptor tyrosine kinases

In multicellular organisms, one of the fundamental means of signal communication between cells is the binding of a ligand to the extracellular domain of its cognate receptor that has tyrosine kinase activity. Ligand binding results in the transduction of the signal to the cytoplasm by the phosphorylation of tyrosine residues on the cytosolic domain of the receptor and downstream signalling molecules (for review see Hubbard and Till, 2000). The receptor protein tyrosine kinases (RPTKs) share several structural features. All of them contain an extracellular ligand-binding domain, usually glycosylated, which determines ligand specificity. A hydrophobic transmembrane domain anchors the receptor to the plasma membrane. The cytoplasmic domain contains the tyrosine kinase active site, in addition to sequences that regulate substrate binding

and the activity of the kinase. There are currently 19 classes of RPTKs (Kolibaba and Druker, 1997).

1.2.1.1 Phosphotyrosine binding proteins

Many growth factor and cytokine receptors are phosphorylated at multiple tyrosine residues. Interacting proteins contain binding domains that bind to specific phosphotyrosine motifs on the receptor. Prominent amongst these binding domains is the Src homology-2 domain (SH2) (Waksman et al., 1993). The SH2 domain is a conserved motif of approximately 100 amino acids that binds to phosphorylated tyrosine residues within a defined amino acid sequence (Songyang et al., 1993). Examples of SH2 domains are found in the Grb2-Sos complex, phosphatidylinositol 3-kinases (PI3K) and phospholipase Cγ. These proteins are consequently recruited to the plasma membrane where they then initiate particular signalling pathways. Other phosphoproteins are recognised by phosphotyrosine-binding domains (PTBs), originally identified as modular domains that recognise phosphorylated Asn-Pro-Xxx-pTyr- containing proteins. However, it is now known that there is greater diversity in the recognition sequences (reviewed in Forman-Kay and Pawson, 1999). An example of a protein containing a PTB domain is Shc, which interacts with the tyrosine-phosphorylated insulin receptor following stimulation by the insulin ligand. The Shc protein is subsequently tyrosine-phosphorylated by the insulin receptor enabling the binding of the SH2 domain of the adapter protein Grb2. The protein complex then couples via Grb2 to Sos, a guanine nucleotide exchange factor for p21ras, resulting in downstream signalling. These modular interactions communicate extracellular signals across the plasma membrane to the cytoplasm of the cell and ultimately to the nucleus. In the course of these interactions large molecular complexes transiently form and dissociate in a process that simultaneously transmits and amplifies the signal thus altering the fate of the cell.

1.2.2 Non-receptor tyrosine kinases

Non-receptor tyrosine kinases are cytoplasmic proteins that receive extracellular signals from integral membrane protein receptors (see section 1.2.2.1) and transduce them to downstream intermediates in pathways that regulate cellular growth, activation and differentiation. There are ten different classes of non-receptor tyrosine kinases (NRTKs), grouped according to structural and functional similarity. The largest is the Src sub family which has 11 family members. Src family members participate in a variety of signalling processes including T and B cell activation, growth factor mediated signalling and cytoskeleton remodelling. The members of the Src family all contain SH2 and SH3 protein interaction domains. Members of the Janus kinase (JAK) subfamily are a critical part of the immune system. JAKs are noncovalently associated with the cytoplasmic domain of cytokine receptors and are activated by ligand induced receptor dimerisation. Members of the JAK subfamily of NRTK contain a kinase like domain upstream of the active kinase domain in addition to an SH2 and a FERM (band 4.1 ezrin, radixin, moesin) domain that is involved in phospholipid binding. Other NRTK subfamily-specific domains include the plextrin homology (PH) which binds to phospholipids, actin binding, integrin binding and DNA binding domains (reviewed in Robinson et al., 2000; Hubbard and Till, 2000).

1.2.2.1 Non-receptor tyrosine kinase signalling pathways

NRTKs are activated by extracellular events such as binding of ligands to their cognate receptors resulting in the recruitment and activation of cytosolic kinases. For example in the JAK/STAT (JAK/signal transducers and activators of transcription) signalling pathway the JAKs bound to the cytokine receptor are activated by autophosphorylation following ligand induced receptor aggregation. The activated JAKs then phosphorylate tyrosine residues on STAT molecules causing the rapid nuclear translocation of the activated STAT proteins (Shuai et al., 1993). Signalling by activated T and B cells of the immune system involves multiple NRTKs including the Src family members Lck and Lyn together with NRTKs Zap-70, Syk and Btk. Following

receptor activation these kinases participate in downstream signalling events culminating in the transcriptional activation of cytokine genes (reviewed in Hubbard and Till, 2000). Other NRTKs are activated by events such as cellular adhesion, calcium influx, inflammatory cytokines, or at particular stages of the cell cycle.

1.3 Protein tyrosine phosphatases

Approximately 30% of all cellular proteins are phosphorylated and the inappropriate phosphorylation or dephosphorylation of signalling proteins resulting in the activation or inactivation of signalling pathways is either a cause or a consequence of major diseases such as cancer, diabetes and rheumatoid arthritis. The balance of tyrosine phosphorylation in the cell is tightly controlled by the opposing actions of PTKs and protein tyrosine phosphatases (PTPs), which remove phosphate groups from tyrosine residues. Until recently, most studies of tyrosine phosphorylation focused on the PTKs, many of which were cloned twenty years ago, whereas research on PTPs has lagged behind. In some measure this was due to the technical difficulties involved in characterising PTP function. Obtaining a purified tyrosine-phosphorylated substrate for the measurement of PTP activity was problematic. Moreover, the transient nature of the interaction between a PTP and its substrate meant the identification of substrates was difficult. However, the major obstacle was the prevailing view that the tyrosine kinases were exclusively responsible for the initiation and control of tyrosyl phosphorylation induced signalling. Tyrosine phosphatases were believed to lack the specificity required for signalling and were relegated to the role of "housekeeping" enzymes.

1.3.1 PTP superfamily

The identification of a large and diverse family of PTPs (reviewed in Neel and Tonks, 1997; Tonks and Neel, 2001) has led to the recognition that these enzymes match PTKs in complexity and specificity in intracellular signalling. The PTKs and the PTPs are in a state of dynamic equilibrium. Total phosphotyrosine in normal cells constitutes only 0.1-0.01% of acid-stable

protein-bound phosphate, which is around a thousand-fold less than the levels of phosphoserine and phosphothreonine (reviewed in Mustelin et al., 1999) indicating that PTPs rapidly counter the effects of PTKs. When cells are treated with pervanadate, a potent inhibitor of PTPs, there is a dramatic increase in intracellular tyrosine-phosphorylated proteins, reflecting the finding that PTP activity is several orders of magnitude higher in the cell than PTK activity (reviewed in Mustelin et al., 1999) .

There are at least three families of molecules comprising the phosphatases that can dephosphorylate tyrosyl phosphate:

1) The dual-specificity phosphatases

2) The low molecular weight phosphatases.

3) The tyrosine-specific or "classical" phosphatases

Hereafter in this thesis, the term "PTP" will refer to the tyrosine–specific subfamily unless otherwise specified.

1.3.2 Catalytic mechanism

All members of the PTP superfamily share an active site motif consisting of a cysteine and an arginine, separated by 5 residues (HCxxGxxRS (T) and all exploit the same catalytic mechanism (Figure 1.1 reproduced from (Denu et al., 1996a). The classical PTPs share greater than 30% sequence homology within their catalytic domains. However, there is less than 5% sequence homology between the "classical" PTPs and the dual-specificity PTPs.

The crystal structures of several PTPs including PTP1B, the bacteria *Yersinia* PTP YOP51, the receptor PTPs alpha and Mu and the SH2 domain containing PTP SHP-1 have now been solved at high resolution (Barford et al., 1994;Stuckey et al., 1994; Bilwes et al., 1996; Hoffmann et al., 1997; Yang et al., 1998; reviewed in Neel and Tonks, 1997; Tonks and Neel, 2001). These structures reveal that despite limited sequence homology, which is only slightly higher than would be expected in two randomly selected proteins, the overall structural fold of

the catalytic domain of the dual-specificity phosphatase VHR (Vaccinia H1-related enzyme) is highly similar to that of PTP1B (reviewed in Denu et al., 1996a).

The catalytic motif amino acid residues are found on a single loop at the base of a cleft on the surface of the protein. Substrate specificity of the classical PTPs is limited to phosphotyrosine, due to the depth of the catalytic cleft (Barford et al., 1994). Phosphoserine and phosphothreonine are not long enough to access the catalytic cysteine. The cysteine residue of the catalytic motif (Fig.1.1) stages a nucleophilic attack on the phosphate group of the substrate, forming a cysteinyl-phosphate intermediate. An invariant aspartate residue, situated on a loop upstream of the catalytic cysteine, undergoes a conformational change following binding of the substrate bringing it into position to act as a general acid. The aspartate residue protonates the phenolic oxygen of the tyrosyl leaving group, allowing efficient formation of the thiophosphate intermediate. The aspartate residue subsequently acts as a general base, activating a water molecule to promote hydrolysis of the cysteinyl-phosphate intermediate (Jia et al., 1995; Zhang et al., 1994; Denu et al., 1996b; Pannifer et al., 1998; reviewed in Tonks and Neel, 2001 and Denu et al., 1996a).

1.4 The classical PTP family

The largest family is the classical family of PTPs that are characterised by an approximately 240 amino acid conserved catalytic domain (the PTP domain). The family is further divided into receptor-like PTPs that contain hydrophobic transmembrane domains, and cytosolic PTPs (Fig 1.2).

Figure 1.1

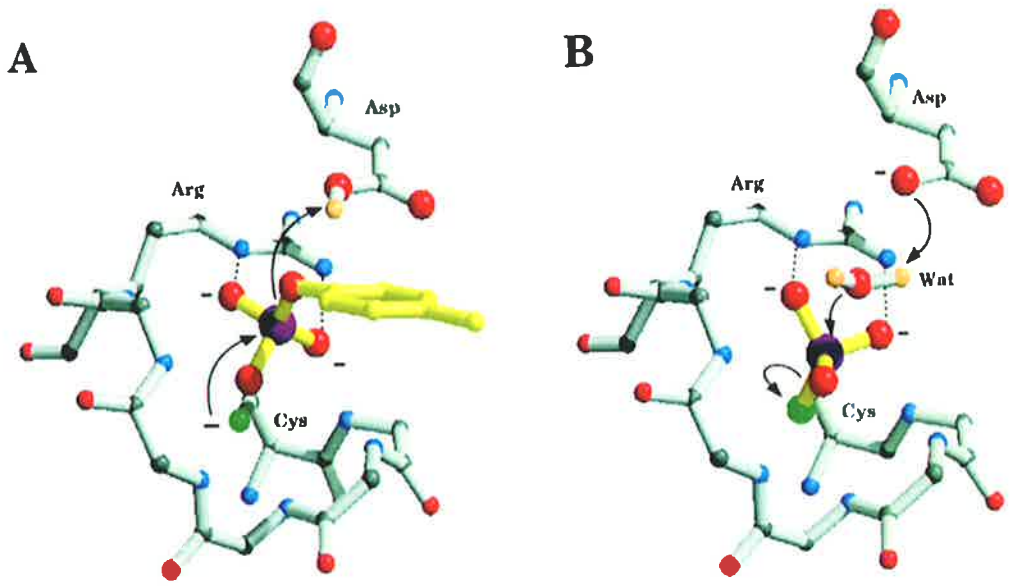


Figure 1.1. Catalytic mechanism of protein tyrosine phosphatases (from Denu et al., 1996a)

(A) A model of the enzyme-substrate complex of PTP derived from two X-ray crystallographic models: Cys-ser mutant of PTP1B complexed with phosphotyrosine (Jia et al., 1995) and Yersinia PTP complexed with vanadate (Denu et al., 1996b) The phosphotyrosine substrate (shown in yellow) is bound to the centre of the catalytic motif.

B) A model of the phosphoenzyme intermediate of PTP. The phosphorus is covalently bound to the cysteine. A water molecule (Wat) positioned so that the aspartic acid can remove the proton. See text for a more detailed explanation.

Figure 1.2

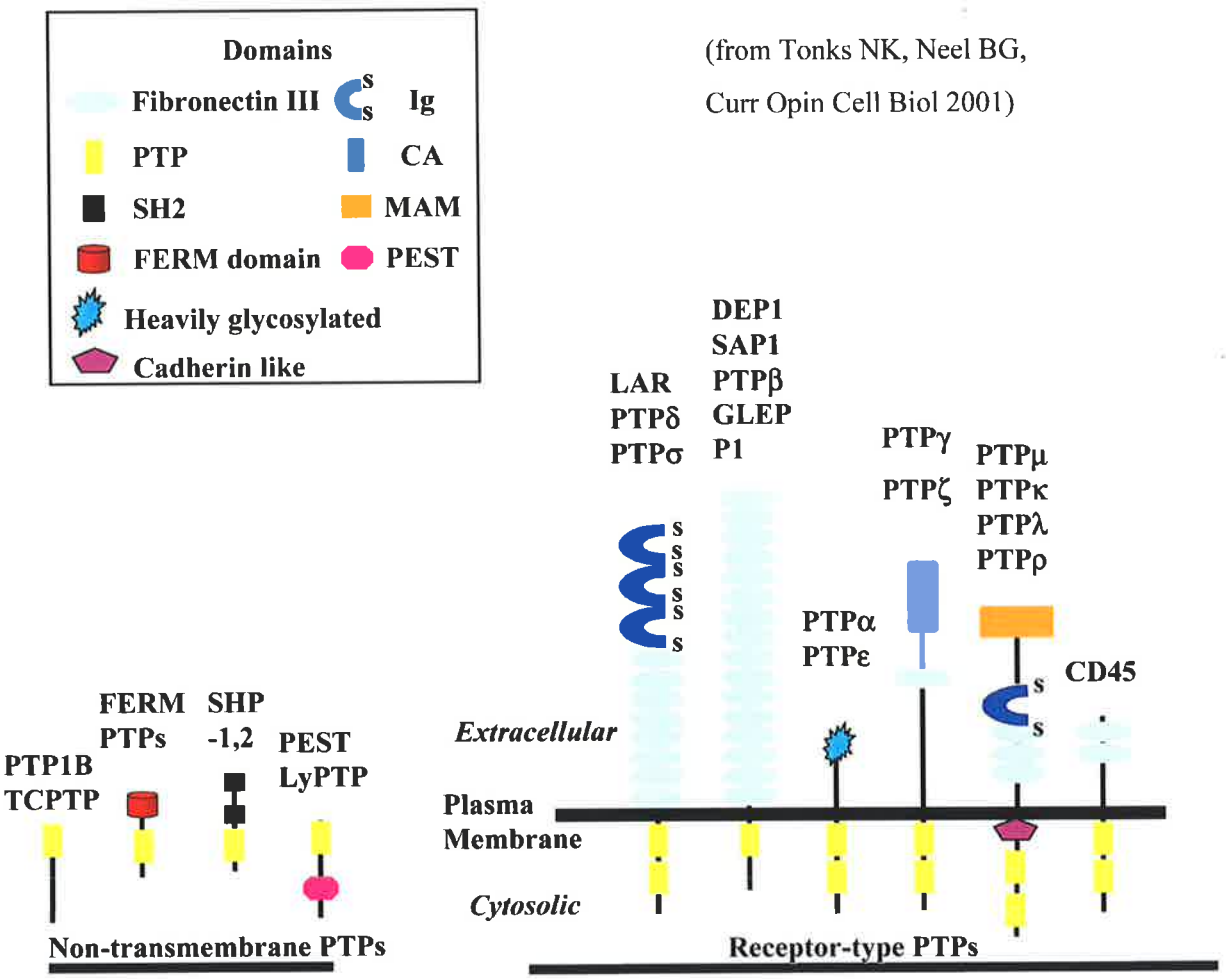


Figure 1.2. The classical phosphotyrosine-specific family of protein tyrosine phosphatases.

1.4.1 Receptor-like PTPs

The receptor-like PTPs consist of an intracellular region containing one or two phosphatase domains, a single transmembrane domain and a variable extracellular segment. Usually only the juxta-membrane phosphatase domain has catalytic activity. The catalytically inactive carboxy-terminal PTP domain may play a role in substrate specificity by binding to regulatory proteins or in the autoregulation of the activity of the PTP (Wallace et al., 1998).

1.4.1.1 RPTP extracellular domains

The extracellular domains of many PTPs contain structural features that are involved in cell-cell or cell-matrix adhesion. The extracellular domain of RPTPζ/β contains a carbonic anhydrase domain and is also rich in fibronectin type III repeats. These structural features are common to cell adhesion proteins, suggesting that this phosphatase participates in cell-cell communication. RPTPζ/β is expressed on the surface of glial cells and recognises contactin, a GPI–anchored protein on the surface of neurons (Peles et al., 1995). This interaction initiates the assembly of protein-complexes resulting in signalling between the cells that are involved in neurite outgrowth. PTPμ and PTPκ have extracellular sequences with homology to immunoglobulin domains. These PTPs engage in homophilic binding, that is, the PTPμ on one cell will bind to the PTPμ on an adjacent cell via its immunoglobulin domain (Brady-Kalnay et al., 1995).

PTPμ and PTPκ belong to the MAM (meprins-*xenopus* A5-*mu*) family of PTPs, which have now been shown to associate with the adherens junction, cadherin-catenin complexes. A recent study has shown that PTPμ recruits the scaffolding protein RACK1(receptor for activated protein C) kinase to the plasma membrane (Mourton et al., 2001). PTPμ is thought to regulate the PKC pathway via its interaction with RACK1, resulting in modification of E-cadherin-dependent adhesion (Hellberg et al., 2002).

Another member of the transmembrane PTPs, PTP LAR, has an extracellular domain that contains immunoglobulin domains and fibronectin type III repeats. LAR localises to focal adhesions where it specifically dephosphorylates and destabilizes p130 (cas) and may play a role in regulating cell adhesion-mediated cell survival (Weng et al., 1999).

1.4.2 Cytosolic PTPs

The cytosolic or non-transmembrane PTPs have a single catalytic domain and various non-catalytic domains that play a role in regulation and subcellular targeting. PTP1B is the smallest member of the cytosolic PTPs comprising only the catalytic domain and a carboxy terminal targeting sequence that localises it to the endoplasmic reticulum (Frangioni et al., 1992).

1.4.2.1 Non-catalytic domains of cytosolic PTPs

PTPs SHP-1 and SHP-2 are highly related cytosolic PTPs, each containing two tandem SH2 domains. The SH2 domains recruit the PTPs to binding proteins within the cell and also regulate the activity of the PTPs. The amino terminal SH2 domain binds to the catalytic site in the absence of ligand, inactivating the PTP (Pei et al., 1994). This auto-inhibition is disrupted by ligand binding which activates the enzyme (Pluskey et al., 1995).

PTP-PEST (region rich in proline, glutamate, serine and threonine) is a cytosolic phosphatase, which is ubiquitously expressed in mammalian tissues (Yang et al., 1993). PTP-PEST contains PEST sequences (regions rich in proline, glutamate, serine and threonine) in its non-catalytic C terminus that are characteristic of proteins with short half-lives in eukaryotes. PTP-PEST specifically dephosphorylates p130 (cas), a protein implicated in mitogenic and cell-adhesion induced signalling and in transformation by a variety of oncogenes. (Garton et al., 1996). The specificity of this interaction is reinforced by a high affinity interaction between the SH3 domain of p130 (cas) and a proline-rich sequence within the C-terminal segment of PTP-PEST (Garton et al., 1997). It has recently been shown that the actin-associated protein PSTPIP recruits both PTP-PEST and c-Abl, a non-receptor tyrosine kinase involved in the regulation of

cell adhesion. In the resulting complex PTP-PEST dephosphorylates and regulates the activity of c-Abl (Cong et al., 2000). PTP-PEST therefore plays a pivotal role in the regulation of cell adhesion and migration.

The emerging picture of dephosphorylation by PTPs involves the highly specific interactions of individual PTPs with their substrates. Although a number of PTPs have multiple substrates and some target proteins may be dephosphorylated by several PTPs, dephosphorylation is substrate specific and subject to tight regulation.

1.5 PTP substrate specificity

As all members of the PTP family employ the same catalytic mechanism, the established view has been that substrate specificity resides in the diversity of the non-catalytic domains of the PTPs. More recently it has been demonstrated that the catalytic domains also show specificity in substrate recognition (reviewed in Tonks and Neel, 2001).

1.5.1 Substrate Specificity of SHP-1 and SHP-2

SHP-1 and SHP-2 are highly related having approximately 60% sequence identity but have distinct biological functions (reviewed in Neel and Tonks, 1997; Tonks and Neel, 2001). They are both cytosolic PTPs with two SH2 protein interaction domains in their N-termini. SH2 domains bind to distinct amino acid sequences surrounding a phosphotyrosine residue (reviewed in Brown and Cooper, 1996), thus targeting the PTPs to specific phosphotyrosine ligands. Both of the SH2 domains are involved in targeting the PTP to its substrates. The N-terminal SH2 domain also binds to and inactivates the catalytic domain in the absence of ligand (Pei et al., 1994; Pei et al., 1996; reviewed in Barford and Neel, 1998). The SH2 domains are therefore involved in regulating both catalytic activity and substrate binding. However, the SH2 domains are not sufficient for determining substrate specificity. SHP-1 binds to and dephosphorylates the epidermal growth factor receptor (EGFR) but SHP-2 does not. Chimaeric PTPs containing the catalytic domain of SHP-2 and the SHP-1 SH2 domains were able to recognise and bind to EGFR

but were unable to dephosphorylate it. However, chimaeric PTPs dephosphorylated the EGF receptor when they contained the catalytic domain of SHP-1. This indicates that the differential interaction of SHP-1 and SHP-2 with the EGFR is due to the specificity of the catalytic domains rather than the SH2 domains (Tenev et al., 1997; reviewed in Tonks and Neel, 2001).

A study of chimaeric SHP- constructs in *Xenopus* also showed that the catalytic domain contributes significantly to SHP-2 substrate specificity. However, the chimaeric constructs were unable to completely rescue impaired development without intact SH2 domains, indicating that both the PTP domain and the SH2 domains are determinants of SHP specificity (O'Reilly and Neel, 1998).

1.5.2 Substrate specificity of PTP1B and TC-PTP

PTP1B and T-cell PTP (TC-PTP) share a high degree of sequence similarity with 75% homology in their catalytic sequences. However, they have different substrate specificities and distinct biological functions. There are two alternatively spliced forms of TC-PTP, TC45 and TC48. TC45 localises to the nucleus whereas TC48 and PTP1B both localise to the endoplasmic reticulum (ER). PTP1B and TC48 will both bind to the EGFR when targeted to the endoplasmic reticulum, but PTP1B specifically interacts with a 68kDa phosphotyrosine protein that TC48 does not recognise (Tiganis et al., 1998; reviewed in Tonks and Neel, 2001).

Their different roles are underscored by the knockout studies. While the $PTP1B^{-/-}$ mice are apparently healthy and live a normal lifespan, deletion of TC-PTP resulted in the death of all homozygous mice at 3-5 weeks of age. The abnormalities seen in these mice included specific defects in bone marrow, B cell lymphopoiesis, and erythropoiesis, as well as impaired T and B cell functions (You-Ten et al., 1997). The different outcomes of knocking out either of the two genes demonstrate that the two proteins have different functions. However, the cause of lethality resulting from TC-PTP deficiency may be due to its absolute requirement in some tissues or the loss of the nuclear TC-PTP isoform TC45.

Substrate specificity is determined in part by preferential dephosphorylation of phosphotyrosines that are adjacent to specific residues (Cho et al., 1993; Hippen et al., 1993; Huyer et al., 1998). A screen of a synthetic peptide library containing malonyltyrosine as a phosphotyrosine mimic confirmed previous findings (Ruzzene et al., 1993) that PTP1B preferentially dephosphorylates substrates that have acidic residues in positions X-2 and X-3 while an aliphatic residue is preferred at X-1 (Pellegrini et al., 1998).

1.6 PTP knockouts

Gene targeting techniques facilitate the investigation of the function of a gene in the context of the development and functioning of the whole animal. The analysis of targeted and naturally occurring deletions of PTPs has revealed much about the high degree of functional specificity of the family members.

1.6.1 SHP-1 deficient "motheaten" mice

A recessive frameshift mutation in the SHP-1 gene results in motheaten (me/me) mice that display multiple haematopoietic abnormalities, most prominently hyperproliferation and inappropriate activation of granulocytes and macrophages. Mice homozygous for the mutation usually do not survive past 8 weeks (Green and Shultz, 1975; Chen et al., 1996). Another naturally occurring point mutation of the SHP-1 gene is the viable motheaten, which causes the expression of a functionally deficient SHP-1 protein. The viable motheaten mutant mice suffer from progressive inflammation due to a deficiency of SHP-1 enzyme activity. The mice die at 3-4 months of age from macrophage and neutrophil accumulation in the lung (Shultz et al., 1993). These two mouse strains have been invaluable in the analysis of the function of SHP-1. The data indicate that SHP-1 acts as an important negative regulator of many signalling pathways in haemopoietic cells including those emanating from cytokine, growth factor, adhesion and antigen receptors. In these pathways, SHP-1 is recruited to the relevant receptor signalling complexes, usually via the SH2 domains.

The analysis of the motheaten mice has also provided evidence for functional differences between SHP-1 and SHP-2, which in contrast to SHP-1, usually has a positive role in signalling. Targeted mutations of the SHP-2 gene have been generated which delete either exon 2 or exon 3. In contrast to the mice lacking SHP-1, early embryonic lethality is the outcome of embryos homozygous for either deletion (Saxton et al., 1997; Qu et al., 1997). The phenotype of SHP-$2^{-/-}$/wt chimaeric animals is characteristic of mice with mutations in the EGFR signalling pathway (Qu et al., 1999), providing evidence that SHP-2 is a positive regulator of EGF signalling.

1.6.2 CD45 deficient mice

The integral-membrane PTP CD45 is one of the most abundant glycoproteins expressed on the surface of all nucleated haematopoietic cells. The spleens of CD45 deficient mice were found to have twice the number of B cells and one-fifth the number of T-cells of wt mice (Byth et al., 1996). Studies of these transgenic mice have revealed CD45 to be both a positive and a negative regulator of immune cell function (Alexander, 2000). CD45 is believed to upregulate signalling through the B cell-antigen receptor (BCR) by dephosphorylating the carboxyl terminus of the Src family tyrosine kinases. Cells from CD45 knockout mice are hyporesponsive to BCR signalling. On the other hand, SHP-1 is thought to be a negative regulator of BCR signalling and SHP-1 knockout mice are hyper-responsive to BCR signalling. SHP-1/CD45 double-knockout mice display normal B cell development, including a normal B-1 cell subpopulation, with no indication of systemic autoimmunity (Pani et al., 1997). This complementation indicates that SHP-1 and CD45 act on a common signalling pathway in a synchronised fashion to couple antigen receptor engagement to B cell activation and maturation.

1.6.3 PTP1B deficient mice

PTPs have been shown to be negative regulators of the insulin receptor. Vanadate and pervanadate have insulin-mimetic properties, enhancing insulin sensitivity and prolonging insulin

action (reviewed in Fantus and Tsiani, 1998). These effects all appear to be related to the inhibition of PTPs. PTP1B has been specifically implicated in insulin resistance, with increased abundance of catalytically impaired PTP1B having been reported in tissue lysates from obese human subjects with and without type 2 diabetes (Shao et al., 1998).

PTB-1B knockout mice were found to have lower blood glucose levels and a 50% decrease in circulating insulin levels than wt mice. In comparison to wt mice, the $PTP1B^{-/-}$ mice after insulin injection showed increased phosphorylation of the insulin receptor in liver and muscle tissue but not fat. Wild type mice fed a high fat diet gained weight and became insulin resistant, the hallmarks of type II diabetes in humans. Mice heterozygous and homozygous for the PTP1B deletion were resistant to weight gain on a high fat diet and remained insulin sensitive (Elchebly et al., 1999). The $PTP1B^{-/-}$ mice showed a decrease in adipocyte size and an increase in metabolic rate, with an increase in insulin-stimulated glucose uptake in skeletal muscle but not in fat (Klaman et al., 2000).

1.6.4 PTP LAR deficient mice

Mice deficient in the receptor type PTP LAR were also found to have an increased sensitivity to insulin resulting in increased glucose uptake, decreased gluconeogenesis and a significant reduction in circulating insulin and glucose levels. The increased sensitivity was correlated with a decrease in the insulin-stimulated activity of PI3 kinase (Ren et al., 1998).

1.6.5 PTP delta deficient mice

Receptor type PTP delta is specifically expressed in specialised regions of the brain including the hippocampus, B-lymphocytes and thymic medulla. PTP delta-deficient mice were found to have a cluster of learning and behavioural deficiencies, including inadequate food intake (Uetani et al., 2000).

1.6.6 PTPβ/ζ deficient mice

PTPβ/ζ is expressed mainly in the nervous system and contains protein domains which indicate that it is involved in cell-cell contact. Results of *in vitro* experiments suggested that this

PTPβ/ζ was involved in neurite outgrowth and neuronal migration. However, RPTPβ/ζ deficient mice showed no obvious abnormalities, indicating that this PTP is not essential and its function can be performed by other PTPs (Harroch et al., 2000).

1.6.7 Analysis of knockout phenotypes

Gene targeting is a powerful tool for the analysis of gene function. However, the technique has several pitfalls, which should be taken into consideration when interpreting the phenotype. Truncated proteins expressed from the targeted locus may introduce effects such as transdominant interactions with other proteins whereas large genomic deletions may lead to the unintended loss of regulatory elements, governing the expression of unrelated genes. A lack of phenotype may be due to functional redundancies at the single gene level or at the genetic pathway level. In several cases, independent groups have knocked out the same gene, but different phenotypes have been reported. The definitive proof that a specific phenotype is caused by a targeted mutation is to revert the phenotype to wild type by reintroducing a copy of the functional gene (reviewed in Muller, 1999). In the absence of such corroboration alternative explanations for the observed phenotype need to be considered. An example is the targeted deletions in SHP-2, which delete exon 2(Arrandale et al., 1996) or 3 (Saxton et al., 1997). These two deletions both give rise to truncated proteins of the SHP-2 domain that lack the intact N-SH2 domain, which inhibits catalytic activity (Pei et al., 1996; Pregel et al., 1995). Hence, the resulting phenotype could be interpreted as an unintended gain of function. However, because the re-introduction of SHP-2 in the $Ex3^{-/-}$ mice restored growth factor and integrin responses, it demonstrated that the effects of the deletion are due to a loss of function (reviewed in Tonks and Neel, 2001).

The complex phenotypes often observed in knockout animals require further dissection by biochemical means. Investigation into the specific substrates, subcellular localisation and regulation of the PTPs is necessary to fully elucidate their function in the cell.

1.7 Identification of PTP substrates

The identification of PTP substrates has been technically difficult as the interaction between the substrate and the phosphatase is transient, preventing the identification of substrates by coprecipitation. Moreover, the biological labelling techniques used to identify the substrates of the tyrosine kinase family are not feasible in a dephosphorylation reaction. However, two-hybrid screens and "substrate-trapping" techniques have been used successfully to identify many substrates of specific PTPs.

1.7.1 Two-hybrid screens

Proteins that interact with PTPs have been identified using yeast two-hybrid screens. This method has identified proline, serine, threonine phosphatase-interacting protein (PSTPIP) a novel member of the actin-associated protein family, as a protein interacting with PTP-PEST (Spencer et al., 1997). PTP-PEST plays a dual role in cell cytoskeletal organization, by promoting the turnover of focal adhesions required for cell migration, and by directly or indirectly regulating the PSTPIP tyrosine phosphorylation level, which may be involved in regulating cleavage furrow formation or disassembly during normal cell division (Angers-Loustau et al., 1999). The two-hybrid screen also identified PTP-PEST as a protein interacting with p52Shc in a tyrosine phosphorylation-independent manner (Habib et al., 1994; Charest et al., 1996).

A yeast two-hybrid screen looking for interacting regulatory proteins of PTP sigma identified the inactive catalytic domain of another LAR family member, PTP Delta (Wallace et al., 1998).

The FERM family member PTP-BL is a large cytosolic PTP implicated in the regulation of the cytoskeleton. A yeast two-hybrid study determined that the second PDZ domain (PDZ2) of PTP- BL binds to the extreme C-terminus of the tumor suppressor protein APC (Adenomatous Polyposis Coli) (Erdmann et al., 2000). Another screen demonstrated that PTP-BL also interacts with the protein kinase C-related kinase 2 (PRK2), a serine/threonine kinase regulated by the G-

protein rho. The third PDZ domain of PTP-BL and the extreme C-terminus of PRK2 mediate this interaction (Gross et al., 2001). The first PDZ domain of PTP-BAS, the human homologue of PTP-BL, associates with the nuclear factor NFκB inhibitory subunit IκBα. The signal-induced serine phosphorylation of IκBα targets it for ubiquitination and subsequent degradation thereby releasing and activating NFκB. This inducible phosphorylation and degradation is negatively regulated by the tyrosine phosphorylation of IκB (Singh et al., 1996). PTP-BAS may therefore play a regulatory role in activation of the transcription factor NFκB (Maekawa et al., 1999).

PTPμ promotes cell-cell adhesion and interacts with the cadherin-catenin complex. Using the membrane-proximal catalytic domain of PTPμ as bait, the scaffolding protein RACK1 (receptor for activated protein C kinase) was identified as a PTPμ interacting protein (Mourton et al., 2001).

A limitation of the yeast two-hybrid technique is that if a protein requires post-translational modification, such as phosphorylation, to permit binding activity then this protein is less likely to be isolated from the screen. Furthermore the screen may fail to detect protein-protein interactions that require a third non-peptide factor such as DNA (Chen and Han, 2000). A yeast two-hybrid analysis failed to demonstrate a physical interaction between the individual casein kinase 2 (CK2) subunits and CD45. However, a yeast three-hybrid assay, in which both CK2 α and β subunits were coexpressed with the cytoplasmic domain of CD45, demonstrated that both CK2 subunits are necessary for the interaction with CD45 (Greer et al., 2001).

The yeast two-hybrid assay has proved a valuable approach for the identification of PTP interacting proteins. However, the multidomain-PTPs are complexed with many proteins including regulatory, scaffolding and targeting proteins. This technique is also known to generate false positives, requiring experimental confirmation of predicted binding. The identification of

potential PTP substrates will be better served by an assay that can demonstrate both binding and dephosphorylation of the target protein.

1.7.2 Substrate-trapping

The elucidation of the catalytic mechanism instigated the construction of a "substrate-trapping" mutant (Flint et al., 1997), which retains the substrate binding capacity of the catalytic domain but is unable to catalyse the hydrolysis of the phosphate group from the substrate. The substrate and the mutant PTP consequently form a stable complex, allowing the isolation and characterisation of the substrate. This method has been used to identify or confirm the identities of many PTP substrates (see Table 1.1).

Table 1.1 PTP substrates identified or confirmed by "substrate-trapping"

PTP	Substrate	Reference
PTP1B	EGFR	(Flint et al., 1997)
PTP1B	p210 bcr-abl	(LaMontagne et al., 1998)
		(LaMontagne et al., 1998)
PTP1B	Insulin receptor	(Walchli et al., 2000)
PTP1B	STAT5a and 5b	(Aoki and Matsuda, 2000)
PTP1B	STAT5a and 5b	(Aoki and Matsuda, 2000)
PTP1B	JAK2 and TYK2	(Myers et al., 2001)
PTP-PEST	P130 (cas)	(Garton et al., 1996)
		(Cote et al., 1998)
PTP- PEST	Cell adhesion kinase β (CAKβ/PYK2/CADTK/RAFTK)	(Lyons et al., 2001)
TC-PTP	Insulin receptor	(Walchli et al., 2000)
TC-PTP	STAT5a and 5b	(Aoki and Matsuda, 2002)
PTPH1	VCP/p97/CDC48	(Zhang et al., 1999)
VE-PTP	Angiopoietin receptor Tie-2	(Fachinger et al., 1999)
RPTPα	P130 (cas)	(Buist et al., 2000)
PTP epsilon	Voltage-gated potassium channel Kv2.1	(Peretz et al., 2000)
FAP-1	IκBα	(Nakai et al., 2000)

This technique, which is described in section 2.9, has proved highly successful in identifying the physiological substrates of many members of the PTP family, which is a necessary first step in the investigation of their *in vivo* function. For example Fas associated phosphatase-1 dephosphorylates the inhibitor of the NFκB signalling pathway IκBα (Nakai et al.,

2000) while the focal adhesion protein p130 (cas) is a specific substrate of both RPTP alpha (Buist et al., 2000) and PTP-PEST (Cote et al., 1998). Multiple substrates have been identified for PTP1B including members of the JAK/STAT signal transduction pathway STAT5a and 5b (Aoki and Matsuda, 2002) and TYK-2 and JAK-2 (Myers et al., 2001) as well as the EGFR (Flint et al., 1997).

1.8 Regulation of PTPs

The regulation of the activity of PTPs occurs at many levels, involving post-transcriptional modification, subcellular translocation and protein degradation.

1.8.1 Transcriptional regulation

Regulation at the transcriptional level has so far been found in only a few PTPs. A member of the MAM domain subfamily hPTP-J mRNA is transiently up-regulated in Jurkat cells 20 min after the addition of PMA, followed by the complete down-regulation in 8 h after PMA addition (Wang et al., 1999). Receptor type PTP, OB-PTP, which is localised to the olfactory bulb, is expressed during embryogenesis in rats, peaking in expression in the first week after birth. It is then downregulated and is barely detectable in the adult (Yahagi et al., 1996).

Transcription of Cdc25C, a dual-specificity phosphatase that regulates the cell cycle, is inhibited by the tumour suppressor p53 (Krause et al., 2001). Protein kinases, Chk1, Chk2 and Cds1, which are essential for the G2 checkpoint, phosphorylate and inactivate Cdc25 resulting in inactivation of Cdc2 (Furnari et al., 1997; Boddy et al., 1998; Blasina et al., 1999). Cdc25 activity is also regulated by its degradation; following UV or ionising radiation Cdc25 is rapidly ubiquitinated and targeted to the proteosome for degradation (Mailand et al., 2000).

1.8.2 Regulation by alternative-splicing

Differential regulation of gene splicing is another mechanism for regulating tyrosine phosphorylation pathways. TC-PTP isoforms generated by alternative splicing recognise distinct targets (Reddy and Swarup, 1995). The two isoforms have the same catalytic domain but differ at

their carboxy termini. TC48 localises to the endoplasmic reticulum whereas the 45 kDa form TC45 has the hydrophobic carboxy terminus replaced by a six- amino acid sequence which unmasks a nuclear localisation sequence resulting in its nuclear localisation (Lorenzen et al., 1995). Although both isoforms specifically dephosphorylate the EGFR, only the substrate-trapping mutant of the TC45 isoform targets two other proteins including a specific isoform of p52Shc (Tiganis et al., 1998). The two isoforms therefore have specific functions despite having identical catalytic domains (reviewed in Ibarra-Sanchez et al., 2000).

Other examples of alternatively spliced PTPs include a PTPRO (PTPU2/GLEPP1) (Aguiar et al., 1999), PTP LAR (Honkaniemi et al., 1998), PTP-sigma (Endo et al., 1996) and PTP epsilon (Nakamura et al., 1996). Alternatively spliced forms are frequently localised to different subcellular compartments. The longer isoform of PTP epsilon is an integral-membrane protein, whereas the shorter isoform is localised to the cytoplasm (Nakamura et al., 1996). PTP LAR is involved in the regulation of neurite outgrowth; expression of the different transcripts is regulated in a developmental and tissue specific manner (Honkaniemi et al., 1998).

1.8.3Regulation by phosphorylation

Serine/threonine phosphorylation is involved in the regulation of other PTP family members. The dual-specificity kinases CLK1 and CLK2 phosphorylate PTP1B on Ser_{50} *in vitro* activating the phosphatase approximately 3-5-fold (Moeslein et al., 1999). This serine residue is also phosphorylated in the presence of overexpressed Akt. Phosphorylation by Akt, in the presence of insulin, resulted in a reduction in the dephosphorylation of the insulin receptor. This is perhaps a positive feedback mechanism for insulin signalling (Ravichandran et al., 2001).

Phosphorylation of another serine residue in PTP1B, between aa 283-364, in response to cell stimulation is also proposed to regulate phosphatase activity (Brautigan and Pinault, 1993). PTP1B is differentially phosphorylated during the cell cycle (Schievella et al., 1993), with

increased serine phosphorylation on multiple serine residues occurring during the transition from G2 to M phase of the cell cycle (Flint et al., 1993). Casein kinase 2 (Ck2) interacts with the cytoplasmic domain of PTP CD45 in B and T-cells resulting in phosphorylation and a 3-fold increase in phosphatase activity (Wang et al., 1999; Greer et al., 2001)

Receptor-type density dependent PTP DEP-1 is constitutively phosphorylated on tyrosine residues in mammary carcinoma cell lines. Phosphorylation increased significantly after treatment of cells with the PTP inhibitor pervanadate. Also, a 64-kDa serine/threonine kinase was found to form a stable complex with DEP-1 in mammary carcinoma cell lines and to phosphorylate DEP-1 *in vitro,* suggesting a possible mechanism of DEP-1 regulation in tumor cell lines involving serine/threonine and/or tyrosine phosphorylation (Jallal et al., 1997).

The tyrosine phosphatase PTP-U2S is associated with PMA-induced differentiation of myeloid cells and has been shown to enhance differentiation and the onset of apoptosis. Serine/threonine kinase Pim-1 phosphorylates PTP-U2S *in vivo* to decrease the phosphatase activity that may be necessary to prevent the premature onset of apoptosis following differentiation (Wang et al., 2001)

PTP-S2 binds to DNA and is localised to the nucleus in association with chromatin where it plays a role in the regulation of cell proliferation. PTP-S2 localises exclusively to the nucleus in interphase cells, during metaphase and anaphase it is distributed throughout the cytoplasm and excluded from condensed chromosomes. At some stage in metaphase PTP-S2 is phosphorylated (possibly by CK2 or a CK2-like enzyme), resulting in its dissociation from chromatin (Nambirajan et al., 2000)

SHP-2 is serine/threonine phosphorylated by p44mapk (extracellular-signal-related kinase(ERK1)) in response to treatment with EGF resulting in inhibition of SHP-2 enzyme activity *in vitro* (Peraldi et al., 1994). SHP-2 is also phosphorylated by PKC isoforms alpha, beta

1, beta 2, and eta, however there is no alteration in phosphatase activity *in vitro* (Strack et al., 2002)

A member FERM domain family of PTPs, PTPH1, associates with the adaptor molecule 14-3-3 beta in a serine phosphorylation dependent manner (Zhang et al., 1997). PTP36 is another FERM family member that is phosphorylated by serine/threonine kinases *in vitro* following attachment of mouse fibroblasts to the extracellular matrix. Detachment of the cells results in dephosphorylation and translocation of PTP36 into the membrane-associated cytoskeletal fraction (Ogata et al., 1999 b).

1.8.4 Regulation by subcellular localisation

The subcellular localisation of PTPs plays an important role in their regulation. PTP1B is localised predominantly to the cytoplasmic face of the endoplasmic reticulum (ER) via a C-terminal 35 residue targeting sequence (Frangioni et al., 1992). Expression of a deletion mutant of PTP1B lacking the targeting sequence results in global dephosphorylation of cellular proteins (Garton et al., 1996) indicating that the substrate specificity of the PTP is determined in part by its restricted localisation. Agonist-induced platelet activation results in proteolytic cleavage of PTP1B at a site upstream from this targeting sequence, causing subcellular relocation of its catalytic domain from membranes to the cytosol and a 2-fold increase in its catalytic activity (Frangioni et al., 1993). However, the subcellular localisation of a PTP does not necessarily indicate where its substrates are to be found. Several receptor tyrosine kinases that localise to the plasma membrane are substrates of PTP1B, despite the paradoxical localisation of the phosphatase at the ER. The way in which the PTP gains access to the RTKs has recently been determined, using fluorescence resonance energy transfer (FRET) methods to monitor the interactions between the epidermal- and platelet-derived growth factor receptors and PTP1B. The activated receptors undergo endocytosis and internalisation resulting in dephosphorylation by PTP1B at specific sites on the ER (Haj et al., 2002).

The FERM-family PTP MEG-1, SHP-1 that is not tyrosine-phosphorylated, RPTP alpha and RPTP epsilon are all subject to agonist-induced cleavage by calpain (Gu and Majerus, 1996; Falet et al., 1998; Gil-Henn et al., 2001). Calpain cleavage is associated with translocation and/or modulation of the catalytic activity of the phosphatases. This form of posttranslational modification represents a mechanism for maintaining tight control of the catalytic mechanism of PTPs that have multiple substrates.

Members of the Src family of receptor PTKs are specific targets of CD45 (Burns et al., 1994; Koretzky et al., 1992; Ostergaard et al., 1989), however the mechanism of specificity is largely unknown. A possible mechanism is the regulated exclusion of PTPs from lipid rafts, subdomains of the plasma membrane that are enriched in PTKs. In support of this hypothesis, it was found by sucrose density gradient sedimentation that the bulk of PTP activity is localised outside of the rafts. Targeting of the cytosolic PTP SHP-1 to the lipid rafts by attaching it to the N-terminal region of Lck profoundly inhibits CD3 tyrosine phosphorylation and T-cell receptor activation.

1.8.5 Regulation by oxidation

PTPs may also be regulated by reversible reduction/oxidation. The active-site cysteine in PTPs is the target of oxidation by cellular oxidants such as hydrogen peroxide (H_2O_2). H_2O_2 is generated during growth factor stimulation and H_2O_2 production is associated with increased tyrosine phosphorylation due to the transient inactivation of PTPs. PTP1B is reversibly inactivated by H_2O_2. (Barrett et al., 1999; Lee et al., 1998) and superoxide-induced oxidation of its catalytic cysteine residue. Multiple PTPs are inactivated following treatment of Rat-1 cells with H_2O_2 and inhibition of PTP function is important for reactive oxygen species-induced mitogenesis (Meng et al., 2002). Stimulation with platelet dependent growth factor (PDGF) also resulted in the transient oxidation and inactivation of SHP-2 (Meng et al., 2002). PTP1, LAR (leukocyte antigen-related), and VHR (vaccinia H1-related) are rapidly and reversibly inactivated

with low micromolar concentrations of H_2O_2 (Denu and Tanner, 1998). Hydrogen peroxide had no apparent effect on serine/threonine phosphatase activity presumably because there is no catalytic cysteine involved in the catalytic mechanism of these phosphatases (reviewed in Denu et al., 1996a).

These findings indicate that there is a co-coordinated response to growth factor stimulation, which results in activation of kinases and the transient inactivation of inhibitory PTPs resulting in a rapid increase in cellular tyrosine phosphorylation (Su et al., 2001).

1.9 Regulation of cellular functions by PTPs

PTPs play a role in the regulation of multiple cellular functions such as cell proliferation, cell adhesion and insulin signalling. This section will focus on the regulation of cytokine signalling and cell adhesion by PTPs as these are of particular importance to endothelial cell biology. Cytokines such as vascular endothelial growth factor (VEGF), tumour necrosis factor α (TNFα) and fibroblast growth factor -1(FGF) have all been characterised as stimulators of angiogenesis (for review see Klagsbrun and D'Amore, 1996). EGF, VEGF and hepatocyte growth factor (HGF) increase the level of tyrosine phosphorylation at the intercellular junctions of epithelial and endothelial cells resulting in a loss of adhesive strength. The results presented in chapters 5 to 8 show that PTP-Pez potentially plays a role in regulating cell-cell adhesion.

1.9.1 PTP regulation of cytokine signalling

Cytokine signalling triggers the activation of intracellular signalling pathways regulated by the opposing actions of kinases and phosphatases resulting in diverse cellular responses. The role of PTPs in specific pathways is beginning to be elucidated.

The finding that SHP-1 deficiency correlated with the systemic autoimmunity and severe inflammation seen in motheaten mice led to the identification of SHP-1 as an inhibitor of activation-promoting signalling cascades. It soon became evident that PTPs such as SHP-1 specifically modulate the downstream effects of extracellular simulation. SHP-1 has since been

implicated in the down-regulation of JAK/STAT signalling activated by cytokines interleukin-2 (IL-2), IL-3, IL-4, IL-13, erythropoietin, and interferon-α/β (David et al., 1995; Klingmuller et al., 1995; Migone et al., 1998; Yi et al., 1993; Yi et al., 1995). In these pathways SHP-1 is recruited to the signalling complex usually via its SH2 domains (Jiao et al., 1996).

The SH2 domains of SHP-1 also bind to phosphotyrosyl residues within the motif known as the ITIM (immunoreceptor tyrosine-based inhibitory motif) present in the cytoplasmic domain of inhibitory receptors on lymphoid and myeloid cells resulting in downregulation of signalling (reviewed in Ravetch and Lanier, 2000). SHP-1 is in general a negative regulator of cytokine signalling pathways, although there have been reports of some positive effects on signalling. For example EGF and IFN-γ-induced STAT activation is increased in HeLa cells expressing SHP-1 (You and Zhao, 1997).

In contrast to SHP-1, SHP-2 plays a positive role downstream of various receptors. Activation of receptors for many growth factors and cytokines (eg. receptors for IL-3, GM-CSF and PDGF has been shown to generate direct binding sites for SHP-2. After stimulation of these receptors, SHP-2 is tyrosine-phosphorylated and binds to the Grb2-SOS complex (Li et al., 1994), resulting in activation of p21*ras* and the MAP kinase cascade. The mechanism of activation by SHP-2 may be to dephosphorylate either an inactive positive regulator, leading to its activation, or an active negative regulator, switching it off. SHP-2 positively regulates signalling from the EGFR as demonstrated by the reduction in EGFR signalling in the fibroblasts of SHP-2 $^{-/-}$/wt chimaeric animals. Moreover, in genetic crosses, a heterozygous SHP-2 mutation accentuated the phenotype of a weak mutant allele of EGFR (*waved-2*), resulting in defects similar to those seen in EGFR knockout mice (Qu et al., 1999). SHP-2 has also been shown to negatively regulate interferon-γ- and IL-6- induced JAK/STAT signalling (Kim et al., 1998; Schaper et al., 1998; You et al., 1999).

Although some of the substrates for SHP-2 have been characterised, it is unclear which molecules are the principal substrates of SHP-2 and whether any of those identified mediate the observed biological effects of SHP-2. Catalytically inactive mutants have in some cases been useful in resolving these issues. For example, expression of an inactive mutant of SHP-2, in which the catalytic cysteine was mutated to serine, resulted in the increased tyrosine phosphorylation of an integral-membrane protein SHPS-1 (Yamauchi and Pessin, 1995; Yamauchi et al., 1995: Fujioka, 1996). SHPS-1 is phosphorylated by the insulin-like growth factor –1 receptor (IGF-1R) following IGF-1 stimulation. The phosphorylation results in the recruitment of SHP-2, which then dephosphorylates SHPS-1 and the IGF-1 receptor (Maile and Clemmons, 2002).

Another putative substrate of SHP-2 is p97/Gab2, a scaffolding/docking protein that was identified as the major binding protein of SHP-2 in haemopoietic cells (Gu et al., 1998). In drosophila Daughter of Sevenless (DOS), which has structural homologies to Gab1 and Gab2, has been identified as a substrate for corkscrew, the drosophila homologue of SHP-2 (Herbst et al., 1996). Gab2 and possibly other Gab proteins provide a key pathway to PI-3Kinase activation in response to some receptors that lack direct binding sites for PI-3K (Gu et al., 2001).

Other PTPs have also been implicated in the regulation of cytokine signalling through the JAK/STAT pathway including PTP1B, which dephosphorylates JAK2 and Tyk2 (Myers et al., 2001). In addition it also dephosphorylates prolactin activated STAT5a and STAT5b, inhibiting their nuclear translocation and transactivation (Aoki and Matsuda, 2000). Ectopic expression of a cytosolic form of PTP epsilon downregulates IL-6, IL-10 and Lif-induced STAT3 signalling by inhibiting JAK activation (Tanuma et al., 2000; Tanuma et al., 2001). JAK1 and JAK3 have also been identified as physiological substrates of TC-PTP (Simoncic et al., 2002).

1.9.2 PTP regulation of cell adhesion

The extracellular domains of many of the receptor PTPs contain structural features such as immunoglobulin domains, MAM domains and fibronectin type III repeats that indicate a potential role in cell-cell or cell-matrix adhesion. Moreover, it has been found that the cytosolic PTPs such as PTP1B (Balsamo et al., 1996), PTP-PEST (Garton et al., 1996) and SHP-1 and 2 (Fujioka et al., 1996; Yamauchi et al., 1995; Yu et al., 1998), along with low molecular weight phosphatases (Chiarugi et al., 2000) are involved in regulation of cytoskeletal restructuring and cell adhesion.

Adhesion to the extracellular matrix (ECM) and to neighbouring cells is a requirement of regulated cell function and one of the key requirements in the formation of a multicellular organism. Cells receive signals via adhesion receptors that regulate cell fate and trigger changes in the cell such as proliferation, cell mobility, differentiation and apoptosis. Loss of adhesion will ultimately result in cell death in a normal cell. The ability to form contacts with neighbouring cells is necessary for the formation of barriers between tissues. Cell adhesion must be sufficiently strong to maintain the integrity of the tissue when subjected to forces exerted from the environment. Yet, the contacts must also be dynamic, allowing the cells to move within the tissue to allow such processes as cell division, wound repair and differentiation. Members of the PTP family are involved in the regulation of both cell-cell and cell-matrix adhesion.

1.9.2.1 Cell-matrix adhesion

Cellular adhesion to the ECM involves specialised structures called focal adhesions. Transmembrane proteins, the integrins, cluster at these sites and interact with components of the ECM. Integrins are important molecules in the transduction of location cues from the ECM to the intracellular signalling machinery. The intracellular domain of the integrins interacts with cytoskeletal proteins that anchor the complex to actin stress fibers of the cytoskeleton. Proteins involved in signal transduction are also clustered at these sites.

The focal adhesions are dynamic structures that are constantly being formed and disassembled during processes such as cell migration and cell division. This process is thought to be regulated by tyrosine phosphorylation (Maher et al., 1985; Lipfert et al., 1992; Kornberg et al., 1992). The binding of ECM proteins to integrins requires tyrosine phosphorylation of focal adhesion kinase (FAK), tensin and paxillin (Bockholt and Burridge, 1993; Burridge et al., 1992) such that inhibition of PTPs with pervanadate leads to an increase in focal adhesions and cell spreading (Kornberg et al., 1992).

PTPs that have so far been found to play a role in cell-substratum adhesion include PTPalpha (Harder et al., 1998; Stoker, 1994), PTP-PEST (Garton et al., 1996; Angers-Loustau et al., 1999; Shen et al., 1998), PTPβ/ζ (Peles et al., 1995), PTP LAR (Serra-Pages et al., 1995) and the murine homologue of Pez, PTP36 (Ogata et al., 1999 a).

1.9.2.2 Cell-cell adhesion

Adhesive interactions between cells are critical in the development and functioning of multicellular organisms. Cell-cell contacts play essential roles in the regulation of cell-shape and orientation, organisation of the cytoskeleton and cell fate. As well as providing the mechanical strength necessary for the integrity of cell tissue layers, the cell-cell contacts are an essential part of the signalling network between cells that allows an integrated response of a tissue to environmental changes. Contact between epithelial cells is maintained through a combination of adherens junctions (AJ), tight junctions (TJ), gap junctions and desmosomes. Only the AJs and the TJs will be discussed here.

1.9.2.2.1 Adherens Junctions

Adherens junctions are contacts between adjacent cells that are mediated by the classical cadherin family of single pass calcium dependent transmembrane glycoproteins. The type I classical cadherin family comprises about 30 members composed of a highly conserved

carboxyterminal domain, a single pass transmembrane domain and five extracellular cadherin-motif sub-domains. Tissue specific cadherins have been identified including epithelial (E) cadherin, neuronal (N) cadherin, placental (P) cadherin and vascular endothelial (VE) cadherin.

Many different cellular processes can affect cell adhesion and the state of the cell junctions. During morphogenesis the strength of cell adhesion is rapidly modulated in response to growth factors and other developmental signals. There is accumulating evidence that post-transcriptional modification of adherens junction proteins is responsible for the rapid changes in cell junctions that are an integral part of morphogenesis and normal tissue function.

Cadherin regulation has been examined in many model systems. All classes of cadherin are engaged in two different types of self-interaction, homophilic interactions between cadherin molecules localised on opposing cells and lateral interactions within the same cell membrane (Angres et al., 1996; Brieher et al., 1996; Yap et al., 1997). The extracellular domains of these molecules bind to the cadherins of neighbouring cells in a homophilic, calcium dependent manner to form the intercellular bonds. However the extracellular domain is insufficient for strong adhesion, which is mediated by the cytoplasmic tail (Nagafuchi and Takeichi, 1988; Ozawa et al., 1990; Kintner, 1992).

The carboxy terminal 25 amino acids of the intracellular domain of the cadherins bind to the C-termini of the armadillo family members β- or γ-catenin (see Fig. 6.1). The N-termini of β- and γ-catenin bind α-catenin forming a bridge between cadherins and α-catenin. α-Catenin binds to the actin cytoskeleton and also to other actin binding proteins such as zonula occludens-1 (ZO-1), ZO-2, vinculin and α-actinin.

Another key mechanism in the regulation of adhesive strength is the lateral clustering of the cadherins within the plane of the plasma membrane, which is mediated by the cadherin juxtamembrane domain (Yap et al., 1997). Another member of the catenin family, $p120^{ctn}$, binds

to the juxtamembrane domain (Thoreson et al., 2000) and plays a direct role in clustering (Yap et al., 1998). In most cells, p120 is extensively phosphorylated on serine residues (and to a lesser extent on threonine residues (Downing and Reynolds, 1991; Ratcliffe et al., 1997; Ratcliffe et al., 1999; Aono et al., 1999; Ohkubo and Ozawa, 1999). There is some evidence to show that serine phosphorylation of $p120^{ctn}$ decreases adhesive strength (Aono et al., 1999; reviewed in Anastasiadis and Reynolds, 2000).

Therefore the formation of cadherin-mediated adhesion involves several steps; the formation of cis-dimers, tethering of the complexes to the actin-based cytoskeleton and actin-driven lateral clustering of the junctional complexes.

1.9.2.2.1.1 Strength of adhesion

Cells attach to and detach from each other as tissues develop during morphogenesis. In addition in adult tissue, processes such as wound repair and the flux of cells through self-renewing epithelial tissue involve the making and breaking of cell-cell adhesions. A fundamental question of adherens-junction biology has been how the adhesive strengths change from weak to strong. Changes in the composition of the adherens junction complex, phosphorylation of its component proteins and alterations in the interactions with the cytoskeleton have all been suggested to play a role in the regulation of adhesion.

Changes in composition are seen following expression of members of the Rho family of small GTPases, Cdc42, Rac-1 and Rho (Kaibuchi et al., 1999). This family of small GTPases are involved in the regulation of such processes as cell shape, cell polarity and cell growth (Braga, 1999; Jou and Nelson, 1998). Activated Rho transiently localises to cell-cell junctions upon induction of calcium dependent adhesion (Takaishi et al., 1995; Kotani et al., 1997). This raises the possibility that it is recruited to junctions and activated following the formation of adhesive dimers. Overexpression of constitutively active Rac results in an increase in the localisation of

E-cadherin, β-catenin and actin at the sites of contact of epithelial cells. IQGAP1, an effector of Cdc42 and Rac1, interacts with cadherin and β-catenin and induces the dissociation of α-catenin from the cadherin-catenin complex leading to disruption of cell-cell adhesion. Activated Cdc42 and Rac1 counteract the effect of IQGAP1 by binding to β-catenin and rescuing adhesion. The relative activities of Rho A, Rac, and Cdc42 are altered by p120ctn (Anastasiadis et al.,2000; Grosheva et al., 2000; Noren et al., 2000).

1.9.2.2.1.2 Tyrosine phosphorylation and cell-cell adhesion

Disruption of the adherens complex has been observed to occur when cells are treated with tyrosine phosphatase inhibitors. Increased tyrosine phosphorylation of β-catenin has been correlated with a decrease in adhesive strength. A direct relationship between the phosphorylation of Tyr-654 on β-catenin by $pp60^{c\text{-}src}$ and loss of E-cadherin binding has been demonstrated *in vitro* (Piedra et al., 2001; Roura et al., 1999). Other members of the adherens junction such as E-cadherin, $p120^{ctn}$ and γ-catenin have also been found to be tyrosine-phosphorylated. There is considerable evidence to suggest that tyrosine phosphorylated $p120^{ctn}$ binds with increased affinity to various cadherins (Kinch et al., 1995; Skoudy et al., 1996b; Papkoff, 1997; Calautti et al., 1998). In keratinocytes tyrosine phosphorylation of $p120^{ctn}$ had a positive effect on cell adhesion (Calautti et al., 1998). However, elevated tyrosine phosphorylation of $p120^{ctn}$ was found to correlate with reduced adhesion in ras-transformed epithelia (Kinch et al., 1995).

1.9.2.2.1.3 PTPs and adherens junctions

The remodelling of the adherens junction complexes in response to tyrosine phosphorylation suggests that PTPs play a role in regulating cell-cell adhesion. This has proven to be the case and several PTPs have been shown to dephosphorylate proteins in the adherens junction.

The protein tyrosine phosphatase PTP1B binds to the cytoplasmic domain of E-cadherin, maintaining β-catenin in a tyrosine dephosphorylated state and so maintaining cell-cell adhesion.

Several other PTPs, LAR, PTPμ, PTP κ and SHP-2, have also been linked to the cadherin-catenin complex function (Brady-Kalnay et al., 1995; Fuchs et al., 1996; Kypta et al., 1996; Ukropec et al., 2000).

Overexpression of E-cadherin in L fibroblasts, which do not express endogenous E-cadherin, results in cadherin dependent cell-cell adhesion. The cells dissociated following exposure to pervanadate, a specific inhibitor of PTPs. Pervanadate treatment resulted in the tyrosine phosphorylation of E-cadherin, β-catenin and γ-catenin but not α-catenin. In the treated cells the linking of α-catenin to E-cadherin was greatly reduced. Additionally, in cells expressing a chimaeric molecule in which E-cadherin was covalently coupled to α-catenin, the cells did not dissociate following pervanadate treatment (Ozawa, 1998). These findings indicate that PTPs are required to maintain the adherens junction complexes in a dephosphorylated and hence adherent state. Moreover, it identifies the binding of β-catenin to either E-cadherin or α-catenin as the potential sites of PTP action.

Data from a study of normal human keratinocytes supports a model in which β-catenin tyrosine phosphorylation reduces binding to both E-cadherin and α-catenin resulting in a loss of adhesion. It was further shown that removal of the phosphate group by the addition of exogenous recombinant *Yersinia enterocolitica* PTP resulted in a recovery of cell adhesion (Hu et al., 2001). It is proposed that the tyrosine phosphorylation of different residues on β-catenin are involved in the regulation of binding to E-cadherin and α-catenin.

1.9.2.2.2 Tight Junctions

Epithelia and endothelia serve to separate compartments with different molecular, ionic and cellular composition within the body. The integrity of the epithelial and endothelial barriers is largely maintained by the TJs. Desmosomes and adherens junctions serve to link adjacent cells

together, whereas the TJs are responsible for sealing the barrier between the cellular compartments.

Plasma membranes of polarised cells are functionally divided into apical and basolateral membrane domains. Integral lipids and proteins are able to diffuse freely within the plane of the plasma membrane; however, in polarised cells the apical and basolateral membranes are biochemically distinct. Tight junctions are composed of integral membrane proteins, occludins, claudins and junctional adhesion molecules (JAMs), which encircle the top of individual cells separating the apical and basolateral membrane domains. It is thought that the TJ acts as a fence, establishing the apical and basolateral domains by limiting the lateral diffusion of lipids and integral membrane proteins (Schneeberger and Lynch, 1992).

1.9.2.2.2.1 Assembly of the tight junction complex

The TJs must be disassembled and reformed for some cellular functions to take place, eg cell division and cell migration. Tight junction assembly and disassembly is a significant feature of epithelial wound repair processes, which disrupt the permeability barrier, as well as during the movement of inflammatory and other cells across tight epithelial and endothelial structures (reviewed in Edens and Parkos, 2000). In addition, during epithelial tissue morphogenesis and development, the permeability barrier must be formed *de novo* (Gumbiner, 1987). The mechanisms involved in the restructuring of the tight junctions are not yet fully understood and are the focus of ongoing investigation.

A number of proteins have been identified at the TJ. Among the better characterised of these are the highly homologous proteins ZO-1, -2 and -3. These cytosolic proteins are part of the submembrane complex of the TJ and may be directly or indirectly associated with the actin-based cytoskeleton. Other identified peripheral TJ proteins include the 7H6 antigen, cingulin, symplekin, and AF-6. Occludin was the first of the integral membrane proteins identified as

localising to the TJ although its role remains elusive as TJs are able to form in the absence of occludin (see section 7.1.4).

1.9.2.2.2.2 Tyrosine phosphorylation and tight junction regulation

The regulation of the assembly and disassembly of the TJ has attracted a great deal of interest in the past decade. A key finding is that the tyrosine phosphatase inhibitor pervanadate causes an increase in paracellular permeability in both MDCK(Madin-Darby canine kidney) and brain endothelial cells (Staddon et al., 1995) (Collares-Buzato et al., 1998).

Occludin, ZO-1, ZO-2, ZO-3, and possibly other TJ proteins are known to undergo phosphorylation on serine-threonine residues (Anderson et al., 1988; Citi and Denisenko, 1995; Stuart and Nigam, 1995; Sakakibara et al., 1997). ZO-1, ZO-2, ZO-3, and occludin are known to be phosphorylated on tyrosine residues as well (Kurihara et al., 1995; Staddon et al., 1995; Takeda and Tsukita, 1995; Tsukamoto and Nigam, 1999; Van Itallie et al., 1995). ZO-1, ZO-2, ZO-3, and occludin are all intensely tyrosine-phosphorylated in the presence of pervanadate (Tsukamoto and Nigam, 1999), indicating that a PTP is involved in regulation of TJ tyrosine phosphorylation.

Tyrosine phosphorylation is required for the release of specific TJ proteins from the insoluble fraction during TJ reassembly (Tsukamoto and Nigam, 1999). Moreover, tyrosine kinase activity is necessary for the reassembly of tight junctions following oxidative stress (Meyer et al., 2001) and during ATP repletion (Tsukamoto and Nigam, 1999).

VEGF, which regulates paracellular permeability in endothelial cells, rapidly increases the tyrosine phosphorylation of ZO-1 (Antonetti et al., 1999). Additionally acetaldehyde, an important factor in alcohol related disease, causes increased paracellular permeability and was found to inhibit PTPs, including almost complete loss of PTP1B activity (Atkinson and Rao, 2001).

Tyrosine phosphorylation is clearly a fundamental component of TJ restructuring and the inhibition data indicates that PTPs are involved in that regulation. However, thus far no specific member of the PTP family has been identified as a regulator of tight junction proteins.

1.10 Tyrosine phosphorylation and human disease

1.10.1 Adherens junctions and cancer

The majority of human cancers originate from epithelial cells. An essential step in cancer progression is the loss of cell-cell adhesion. One of the ways in which this occurs is through the decrease in the amount of functional E-cadherin protein in the cell. Many clinical and experimental studies have shown that E-cadherin function is lost in practically all human-epithelial cancers, correlating with de-differentiation, invasive cell growth and metastasis. E-cadherin is lost during the transition from well-differentiated adenoma to invasive carcinoma in a transgenic mouse model of tumourigenesis (reviewed in Hanahan and Weinberg, 2000; and Christofori and Semb, 1999). Moreover, the exogenous expression of recombinant E-cadherin in tumour cell lines or in mouse models of cancer in which E-cadherin function has been lost can reverse invasive and metastatic phenotypes (Christofori and Semb, 1999).

Several different mechanisms result in the loss of functional E-cadherin, including deletion or mutational inactivation of the E-cadherin gene. Germline inactivating mutations in the E-cadherin gene are present in cases of familial gastric cancers (Guilford et al., 1998). Downregulation of E-cadherin expression also results from suppression of E-cadherin promoter activity due to chromatin rearrangement, hypermethylation and loss of transcription factor binding.

Changes in the expression of the proteins associated with E-cadherin such as the catenins can also result in a loss of cell-cell adhesion. The downregulation of β-catenin is associated with malignant transformation (Takayama et al., 1996). Mutations in the β-catenin gene are present in

many primary tumours, including melanoma, colon cancer, gastric cancer and prostate cancer (reviewed in Christofori and Semb, 1999).

1.10.2 Tyrosine phosphorylation of adherens junction proteins and cancer

E-cadherin function may also be suppressed by epigenetic mechanisms that are a part of normal cell regulation. Tyrosine phosphorylation appears to be a key step in the dissociation of the cadherin-cytoskeleton interaction and increasing tyrosine phosphorylation of β-catenin has been associated with loss of adhesive stability. A study of human colorectal cancers showed that upregulation of β-catenin tyrosine phosphorylation was more frequent in cancerous tissue (Takayama et al., 1998). Activation of the EGFR induces the scattering and invasion of epithelial cancer cells. It is proposed that this might be the result of tyrosine phosphorylation of β-catenin (Moon et al., 2001). Overexpression of the EGFR and the tyrosine phosphorylation of β-catenin are often seen in the dedifferentiated cells in invasive colorectal cancer (Maruyama et al., 1998) (Takayama et al., 1998). Tyrosine phosphorylation of β-catenin has also been seen in gastric cancers (Akimoto et al., 1998) in cervical cancer cells (Moon et al., 2001), a human esophageal cancer cell line (Shiozaki et al., 1996) and a breast cancer cell line (Hazan and Norton, 1998). Tyrosine phosphorylation of β-catenin in lung cancer correlates with poor prognosis (Nishimura et al., 1996). Thus, there is considerable evidence linking the tyrosine phosphorylation of β-catenin with the loss of cell adhesion and metastasis suggesting that PTPs will play a role in tumour suppression.

1.10.3 PTPs and cancer

Although at least 18 PTKs have been identified as oncogenes, the anticipated discovery of tumour suppressors amongst the PTPs has not so far eventuated. On the contrary, there is evidence that deregulation of PTPs may also be tumourigenic. PTP alpha activates Src family kinases by dephosphorylating the inhibitory carboxyterminal phosphotyrosine (Ponniah et al., 1999). Overexpression of PTP alpha results in activation of pp60c-src kinase, with associated cell

transformation and tumourigenesis (Zheng et al., 1992). The dual-specificity phosphatase Cdc25A plays an important role in cell-cycle regulation by removing inhibitory phosphates from tyrosine and threonine residues of cyclin-dependent kinases. It has been shown to transform diploid murine fibroblasts in cooperation with activated Ras H. The overexpression of Cdc25A in primary breast tumours is correlated with higher levels of cyclin-dependent kinase 2 (Cdk2) enzymatic activity *in vivo* (Cangi et al., 2000). Cdc25A is also elevated in nuclei derived from colon cancers (Dixon et al., 1998).

Fas is a member of the TNF receptor (TNFR)/nerve growth factor receptor (NGFR) superfamily that induces apoptosis upon activation. Human tumour cell lines become resistant to Fas-mediated apoptosis when transfected with Fas-associated phosphatase-1 (FAP-1), indicating that FAP- 1 functions as a negative regulator in Fas-mediated death signalling (Sato et al., 1995; Nakai et al., 2000). Another PTP, PTPLAR has also been shown to be upregulated in metastatic cancer (Levea et al., 2000; Yang et al., 2000).

A notable exception is PTEN (phosphatase and tensin homologue deleted from chromosome 10), a highly conserved tumour suppressor that has been implicated in many human cancers. PTEN contains a PTP domain with similarity to the catalytic domains of the dual-specificity phosphatases. However, it is an inefficient protein phosphatase *in vitro,* and is in fact a lipid phosphatase that cleaves the D3 phosphate of the second messenger lipid phosphatidylinositol 3,4,5-trisphosphate (PIP-3) (reviewed in Di Cristofano and Pandolfi, 2000).

1.10.4 PTPs and diabetes

Many diseases associated with malfunctions of signal transduction are characterised by high or low tyrosine phosphorylation. As would be predicted from their integral role in key signalling pathways, the deregulation of PTPs contributes to the pathogenesis of human disease. A number of diseases are due to insufficient receptor PTK signalling including non-insulin-dependent diabetes.

Chapter 1

Insulin maintains tight regulation on glucose metabolism; perturbations of insulin signalling result in diabetes mellitus, which is characterised by an inability to maintain glucose homeostasis. The insulin receptor (IR) is autophosphorylated and activated following ligand binding. The activated receptor phosphorylates tyrosine residues of insulin receptor substrate (IRS) proteins and other intracellular substrates. These substrates then bind to various downstream effectors transmitting the metabolic and the mitogenic signals of insulin (reviewed in Cheng et al., 2002).

The involvement of PTPs in the regulation of IR signalling has long been suspected due to the insulin mimetic effect exerted by vanadium compounds, which are specific inhibitors of PTPs. PTP1B, LAR, PTPα, and TC-PTP have all been reported to be negative regulators of insulin signalling (Li et al., 1996; Norris et al., 1997; Ren et al., 1998; Walchli et al., 2000). However, a screen looking for potential IR PTPs failed to confirm the ability of PTPLAR and PTP-α to bind to and dephosphorylate the IR. It is possible that the poor performance of these two PTPs may have been due to factors such as the absence of other binding factors required for substrate binding. The screen confirmed the earlier findings that PTP1B and TC-PTP are physiological PTPs for the IR and also identified PTPγ and Sap1 as PTPs that are likely to be involved in IR dephosphorylation (Walchli et al., 2000).

The involvement of multiple PTPs in the dephosphorylation of the IR may be explained by a high level of redundancy, due to the requirement for tight regulation of the signalling pathway. Alternatively the different PTPs may be acting at various subcellular localisations. The IR is localised to the plasma membrane but is endocytosed following ligand binding. There is evidence that much of the IR directed PTP activity occurs in the endosomes (reviewed Cheng et al., 2002). Therefore, some of the IR PTPs may be acting at the plasma membrane and others in the endosomes. Additionally the PTPs may be acting on different tyrosine residues. There are

three tyrosine residues phosphorylated on the IR in response to ligand binding, each of which may be a substrate of a different PTP. In support of this, PTP-γ was able to bind to the IR but was only able to partially dephosphorylate it, suggesting that PTP-γ is acting on only one of the three residues (Walchli et al., 2000).

The generation of PTP1B knockout mice has conclusively demonstrated the involvement of PTP1B in the IR signalling pathway. Although the phenotype of $PTP1B^{-/-}$ mice is apparently normal, further analysis of these mice revealed that they remain sensitive to insulin and resistant to obesity when fed a high fat diet. These factors normally induce insulin resistance in wild type mice. This demonstrates that PTP1B has a major role in modulating insulin sensitivity (Elchebly et al., 1999; Klaman et al., 2000). PTP1B negatively regulates insulin signalling by dephosphorylating the phosphotyrosine residues of the insulin receptor kinase (IRK) activation segment. The molecular mechanism underlying this interaction has recently been determined. There are three tyrosine residues situated on the activation loop of the insulin receptor. Following insulin binding the activation loop moves out of the active site and is accessible for dephosphorylation. Extensive interactions are formed between PTP1B and the IRK-sequence encompassing the phosphotyrosine residues at 1162 and 1163. Phosphotyrosine residue 1162, which is the critical autophosphorylation site regulating the activity of the IR, binds to the PTP active site (Salmeen et al., 2000).

The activity of the protein-tyrosine phosphatase (PTP) LAR is increased in insulin-responsive tissues of obese, insulin-resistant humans and rodents. The overexpression of human LAR in transgenic mice resulted in a 2.5 fold increase in plasma insulin levels compared to wild type mice leading to whole body insulin resistance (Zabolotny et al., 2001). Like PTP1B, LAR acts on the insulin receptor signalling pathway but appears to preferentially dephosphorylate tyrosine 1150 of IRK (Hashimoto et al., 1992). Furthermore LAR does not bind to the IR *in vitro*

(Walchli et al., 2000) and its *in vivo* substrate may actually be the insulin receptor substrate IRS-1 (Zabolotny et al., 2001). Therefore, although these PTPs act on the insulin-signalling pathway, they may target different components of the signal transduction machinery.

1.10.5 PTPs and other diseases

PTPs have also been implicated in other diseases. Polycythemia *vera* (PV) is a clonal hematological disease characterised by hyperplasia of the three major bone marrow lineages. The total PTP activity in the PV cells was twofold to threefold higher than that in normal cells (Sui et al., 1997). On the other hand, other evidence suggests that decreased expression of SHP-1 may contribute to the pathogenesis of PV. SHP-1 normally acts as a negative regulator of mitogenic signals induced by growth factors such as IL-3 and erythropoietin, to which the erythroid progenitors of PV patients are hypersensitive (Wickrema et al., 1999).

Noonan syndrome (NS) is a developmental disorder characterised by facial dysmorphia, short stature, cardiac defects, and skeletal malformations. Mutations in PTPN11, the gene encoding the cytosolic PTP SHP-2 cause NS, accounting for approximately 50% of cases of this disorder in one study (Tartaglia et al., 2001).

Reduced SHP-2 and PTP1B activities have been associated with the development of cystic kidneys a mouse model of kidney disease. The downregulation of PTP1B and SHP-2 correlates with sustained phosphorylation of FAK and paxillin (Sorenson and Sheibani, 2002).

1.11 Concluding remarks and objectives

PTPs play a critical role in normal cell physiology and in the pathogenesis of disease. Thus it is important to understand the normal functioning of the members of this family and the signalling pathways which they regulate. Although there has been intensive study of a few members of this large family the cellular function of the majority is unknown. The involvement of PTPs in the regulation of vital cellular pathways suggests that they represent a promising

target for new drug discovery (Zhang, 2001). The increased insulin sensitivity and reduced adiposity seen in PTP1B knockout mice indicates that drugs that reduce the activity of PTP1B could be used to treat diabetes. Similarly, reduction of PTPα activity may be a method for the treatment of some forms of cancer. However, a single PTP may regulate many pathways and it is evident that some key pathways, for example insulin signalling, involve the activity of several PTPs. Therefore, it is first necessary to gain a detailed understanding of how PTPs act at a molecular level. Investigation of the structure, distribution, substrate specificity and regulatory mechanisms of the 100 or so members of the human PTP family will greatly increase our knowledge of the regulation of signal transduction and cellular physiology.

The primary aim of the research presented in this thesis was to identify PTPs that are potential novel regulators of cell-cell adhesion. Cell-cell adhesion plays a critical role in the formation and remodelling of the vasculature during development (reviewed in Bazzoni et al., 1999) and also in wound repair and inflammation. Preliminary evidence from this laboratory indicated that the inhibition of PTPs prevented the organisation of vascular-like structures in an *in vitro* model of angiogenesis (Matthias and Gamble unpublished). Prior to the commencement of this study several PTPs had already been shown to participate in the regulation of cell-cell adhesion. However, the number of potential substrates at the AJs and TJs suggested that there were other PTPs involved that had not yet been identified.

A homology screen of PTP expression in human umbilical vein endothelial cells (HUVEC) identified the cytosolic phosphatase, PTP-Pez as a highly expressed PTP with structural features indicating a possible role in cell adhesion.

The specific aims of this research project were:

-Determination of the subcellular localisation of endogenous PTP-Pez using specific antibodies raised against Pez peptide sequences. Identifying the subcellular localisation(s) of Pez is an

important step in determining the proteins with which Pez interacts and thus the potential role of Pez within the cell.

-Identification of potential PTP-Pez substrates, using a "substrate-trapping" strategy. In order to understand the role of Pez it is necessary to identify its substrates and hence the signalling pathways in which it is likely to play a regulatory role.

-Investigation of the physiological role of Pez by means of catalytically inactive mutants of Pez. The phenotypic changes that may result from inhibition of Pez activity will provide an insight into the functional role of Pez.

CHAPTER TWO
Materials and Methods

Chapter 2: Materials and methods

2.1 Reagents

2.1.1 Antibodies

Anti-Flag epitope (M2) monoclonal antibody was obtained from AMRAD Biotech (Victoria Australia), anti β- catenin, anti γ-catenin and anti E-cadherin monoclonal antibodies were obtained from Transduction Laboratories (Lexington, KY), α-catenin monoclonal antibodies were obtained from either Transduction laboratories or Zymed (San Francisco, CA, USA). Monoclonal anti- $p120^{ctn}$ and polyclonal anti-ZO-1, anti- Occludin, anti-PY20 and anti-ERK1/ERK2 antibodies were obtained from Zymed. Monoclonal anti-phosphotyrosine antibody P-100 was obtained from New England Biolabs (Beverley MA, USA) Anti- PCNA monoclonal antibody was obtained from Novacastra (Newcastle–Upon-Tyne, UK). The generation and characterisation of the antibodies to PTP-Pez are detailed in chapter 3.

2.1.2 Solutions and buffers

Table 2.1 Solutions and buffers

Solution	Composition of Reagents	Preparation and Storage
LB	10g/litre NaCl, 10g/litre trypticase peptone 5g/litre yeast extract	Adjusted to pH 7.0 with 4N NaOH Autoclaved Stored at RT
LB-Agar	1 litre LB 20g bacto-agar	Adjusted to pH 7.0 with 4N NaOH Autoclaved Stored at RT
5X Laemmli	10 mls 3.125M Tris-HCl (pH 6.8)' 50 mls glycerol, 10 g SDS, 25 mls β-mercaptoethanol, water added to 100 mls	Stirred at RT in the fume hood to mix. Stored in 20 ml aliquots at -20°C. Aliquots stored at RT once thawed.
Acrylamide-Bisacrylamide	29.2 g acrylamide, 0.8 g methylenebis-acrylamide, water to make100ml.	Stored at 4°C and protected from light
Separating gel buffer	1.5 M Tri-HCl (pH 8.8)	Adjusted to pH 8.8 with 4N NaOH. Autoclaved for long term storage. Stored at RT
Stacking gel buffer	1.0M Tris-HCl (pH 6.8)	Adjusted to pH 6.8 with 4N NaOH. Autoclaved for long-term storage. Stored at RT

Table 2.1 continued

Solution	Composition of Reagents	Preparation and Storage
10% Ammonium persulphate	1g ammonium persulphate in 10 mls water	Stored in 0.1ml aliquots at -20°C Thawed immediately prior to use.
10X Running Buffer	30g Tris-HCl, 144g glycine, 10g SDS made up to 1 litre with MQ water	Store at RT. pH adjustment not required. For 1X buffer, 100mls made up to 1 litre with MQ water.
10X Transfer Buffer	30g Tris-HCl, 144 g glycine made up to 1 litre with MQ water	pH adjustment not required.Store at 4°C. 100 mls added to 200 mls of methanol and made up to 1 litre with water for 1X buffer, cooled to 4°C prior to use.
50X Denhardt's solution	1% (w/v) BSA (bovine serum albumin), 1% (w/v) Ficoll, 1% (w/v) PVP (polyvinylpyrrolidone)	pH adjustment not required Stored at -20°C
0.5M EDTA	18.62 g EDTA in 100 mls MQ water	Adjusted to pH 8.0 with 4N NaOH Stored at RT
20X SSC solution (blotting buffer)	3M NaCl, 0.3M sodium citrate, pH 7.0 1mM EDTA.	Adjusted pH to 7.0 with 10N NaOH. Stored at RT
1M TRIS-HCL pH 7.2	Dissolved 15.76g Tris-HCL in 100ml MQ water	Adjusted pH to 7.2 with 4N NaOH
RIPA buffer	50mM Tris-HCL pH 7.2, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150mM NaCl, 1mM EGTA, 1mM sodium orthovanadate.	Stored at 4°C. 1X protease inhibitor cocktail (P2714 Sigma) added immediately prior to use
1M HEPES pH 7.5	23.83 g HEPES in 100ml MQ water	Adjusted pH to 7.5 with 4N NaOH Stored at 4°C.
Substrate trapping buffer	50 mM HEPES, pH 7.5 150mM NaCl, 150mM NaF, 10mM sodium pyrophosphate, 5mM EDTA, 1% Triton X-100.	Stored at -20°C. Protease inhibitor cocktail (P2714 Sigma) added immediately prior to use
Nuclear-extract buffer A	50mM Hepes, pH 7.5, 100mM NaF, 300 mM NaCl, 10% glycerol, 10mM sodium pyrophosphate, 10 mM MgCl, 2 mM sodium orthovanadate, 2 mM sodium molybdate, 2 mM EDTA,	1x protease inhibitor cocktail (Sigma)) and 0.2% Nonidet P-40 (Sigma) added immediately prior to use.
Nuclear-extract buffer B	50mM Hepes, pH 7.5, 100 mM NaF, 300 mM NaCl, 10% glycerol, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 2 mM sodium molybdate, 2 mM EDTA,	Stored at -20°C. 1x protease inhibitor cocktail (Sigma) and 0.1% Nonidet P-40 (Sigma) added immediately prior to use.

Table 2.1 continued

Solution	Composition of Reagents	Preparation and Storage
DEPC water (RNase free)	MQ water mixed vigorously with 0.1% Diethylpyrocarbonate for 2 hours	Autoclaved for long term storage and stored at RT.
10X MEN buffer	200 mM MOPS, 10mM EDTA (pH 8.0), 50mM NaAc (pH 4.5).	Adjusted to pH 7.0 with 4N NaOH .Autoclaved for long term storage and stored at RT.
Formaldehyde sample buffer	100µl 10X MEN, 500µl formamide, 178µl formaldehyde, 222µl MQ H2O	Stored at -20°C.
Bicarbonate buffer (ELISA)	0.295g Na_2CO_3 1.465g $NaHCO_3$ made up to 500 ml with MQ water	pH 9.6, no pH adjustment required stored at RT
Citrate buffer (ELISA)	14.7g tri-sodium citrate 6.5ml 0.1M citric acid made up to 500 ml with MQ water	pH to 6.5 with 5M NaOH stored at RT
OPD mixture (ELISA)	10mg OPD 10ml citrate buffer 10µl 30% H_2O_2	Prepared immediately prior to use

2.1.3 Tissue culture reagents

All tissue culture media and other solutions were purchased from the Commonwealth Serum Laboratories (CSL) unless otherwise indicated. Dulbecco's Modified Eagle's Medium (DMEM) and RPMI 1640 were supplemented with 10% v/v Foetal calf serum (FCS), 10mM HEPES, 2mM glutamine, 50 U/ml penicillin G and 50µg/ml streptomycin sulphate.

M199 was supplemented with 20% FCS 20mM HEPES, 2mM glutamine, 1mM sodium pyruvate (Multicel, Trace Biosciences Pty. Ltd Australia), 1% non-essential amino acids (Multicel), 25µg /ml EC growth factors (GIBCO BRL, Life Technologies) and 20µg/ml heparin (GIBCO BRL, Life Technologies).

Analytical grade reagents were used in the preparation of all solutions. Phosphate buffered saline was used to wash cells.

2.1.4 Transfection of cells

HEK 293 and MDCK stable cell lines were generated by transfection using the transfection reagent Lipofectamine 2000 (GIBCO BRL, Life Technologies) according to the manufacturer's instructions (HEK293) or calcium phosphate precipitation (MDCK).

2.2 Tissue Culture

2.2.1 Cell Lines

Table 2.2 Summary of cell lines

Cell Line	ATCC Number	Description
MDCK	ATCC CRL-6253	Normal canine kidney
HEK293	ATCC CRL-1573	Transformed human kidney
HeLa	ATCC CCL-2	Human cervical adenocarcinoma
SW480	ATCC CCL-228	Colorectal adenocarcinoma
NIH3T3	ATCC CRL-1658	Contact inhibited NIH Swiss mouse embryo-fibroblast
A431	ATCC CRL-1555	Human epidermoid carcinoma

All cell lines were maintained in tissue culture flasks (Greiner, Labortechnik, Germany) containing the indicated tissue culture medium and incubated at 37°C in a humidified atmosphere of 5% CO2 in air. Cells were sub-cultured every 3 to 5 days depending on growth rate. Cell densities were calculated using a haemocytometer and exclusion of trypan blue (0.8% w/v in saline) diluted 9/10 was used to determine cell viabilities. Adherent cells were harvested by rinsing briefly in PBS/0.01M Ethylenediaminetetra-acetic acid (EDTA) followed by trypsin treatment. The duration of trypsin treatment was 30 seconds for most cell lines. MDCK cell lines required longer pre-incubation (5-10 minutes) in PBS-EDTA, followed by 5-10 minutes trypsin treatment at 37°C to detach the cells. Detached cells were resuspended in media supplemented with 2% FCS and centrifuged at 1200g for 5 minutes before being seeded into fresh flasks.

2.2.2 Cryopreservation of cells

Cells in culture were harvested at log phase and resuspended at $2x10^7$ cells /ml in medium containing 10% FCS. The cells were incubated on ice and an equal volume of ice-cold cryoprotectant (30% FCS, 20% dimethyl sulfoxide (MERCK Pty. Ltd. Victoria Australia) and 50% medium) was then added drop wise. Cells were placed in cryotubes (Greiner) in 1 ml

aliquots and cooled to-80°C. The cryotubes were then transferred to liquid nitrogen for long-term storage.

For thawing cryopreserved cells, cryotubes from liquid nitrogen were rapidly warmed to 37°C in a water-bath. The cell suspension was transferred to a 10ml tube and an equal volume of appropriate medium containing 10% FCS (pre-warmed to 37°C) was added drop wise, mixed and allowed to stand for 5 minutes. Cells were then centrifuged at 200xg for 5 minutes, resuspended in fresh medium supplemented with 10% FCS before being placed into flasks containing the appropriate medium.

2.2.3 Isolation and culture of human umbilical vein endothelial cells

HUVEC were isolated from umbilical cords by collagenase treatment (Hirschberg et al., 1975). Isolated cells were cultured in 25cm^2 tissue culture flasks (Costar, Cambridge, MA, USA) precoated with gelatin and maintained in endotoxin free M199 supplemented as described (Section 2.1.1). Cells were maintained at 37°C with 5% CO_2 in air. After three days in culture, the medium was aspirated and replaced with fresh medium. On the fourth day, confluent monolayers were harvested by rinsing briefly with PBS/0.01M /EDTA followed by trypsin treatment. Detached cells were resuspended in M199 medium supplemented with 2% FCS and centrifuged at 200xg for 5 minutes before being seeded into fresh gelatin coated flasks in medium supplemented with growth factors and heparin. Cells were cultured for three days before being sub-cultured as described. All experiments were performed using cells from passage 1 to 4.

2.2.4 Culturing stable cell lines

HEK 293 and MDCK stable cell lines were generated by transfection using the transfection reagent Lipofectamine 2000 (GIBCO BRL, Life Technologies) according to the manufacturer's instructions (HEK293) or calcium phosphate precipitation (MDCK). All stable cell lines generated are summarised in Table 2.3.

Table 2.3 Stable cell lines generated

Cell Line	Vector	Insert Name	DNA sequence
HEK293/Pez2	pcDNA3	WT Pez	N-terminally flag tagged Pez $_{1-1187}$
HEK293/Pez14	pcDNA3	ST Pez$_{D1079A}$	C-terminally flag tagged Pez $_{1-1187}$ (D_{1079}- A)
HEK293/Pez16	pcDNA3	ΔFERM-Pez	C-terminally flag tagged Pez $_{337-1187}$
MDCK/Pez 2	pcDNA3	WT Pez	N-terminally flag tagged Pez $_{1-1187}$
MDCK/Pez 4	pcDNA3	ΔPTP-Pez (-NLS)	N-terminally flag tagged Pez $_{1-877}$
MDCK/Pez 14	pcDNA3	ST Pez$_{D1079A}$	C-terminally flag tagged Pez $_{1-1187}$ (D_{1079}- A)
MDCK/Pez 16	pcDNA3	ΔFERM-Pez	C-terminally flag tagged Pez $_{337-1187}$
MDCK/Pez 18	pcDNA3	WT Pez	C-terminally flag tagged Pez $_{1-1187}$
MDCK/Pez 19	pcDNA3	ΔPTP-Pez(+NLS)	N-terminally flag tagged Pez $_{1-930}$
MDCK/Pez 20	pcDNA3	DN Pez	C-terminally flag tagged Pez $_{1-1187}$ (R_{1127}-M)

Cells were seeded overnight onto fibronectin coated tissue culture plates to achieve a cell density of 90-95% (Lipofectamine 2000) or 40-50% confluent (calcium phosphate) at the time of transfection. The cells were transfected the following day. Following 48 hours incubation in transfection medium the cells were replated at a 1:10 ratio. The cells were cultured in selective medium containing geneticin (G418, GIBCO BRL, Life Technologies) at a concentration of 400μg/ml (HEK293) or 500μg/ml (MDCK). Following selection isolated colonies were picked and transferred to a 96-well tissue culture plate containing selective medium. Twenty clones of each stable transfectant were selected and expanded up. Expression of exogenous proteins from transfected DNA was assayed by Western blotting with an anti-Flag Ab to detect the Flag-epitope included in all constructs. Where the expression of exogenous protein was too low to detect by Western blotting anti- Flag immunoprecipitation followed by Western blotting with anti-Flag Ab was used. All cell lines were stored by cryopreservation.

2.3 RNA isolation

2.3.1 Cell lysis

Total RNA was extracted from HUVEC by the acidic guanidium isothiocyanate phenol-chloroform extraction method TRIZOL reagent (Life Technologies, Victoria Australia). HUVEC were grown to confluence on a 10 cm diameter culture dish and then lysed directly by the addition of 1ml of TRIZOL reagent. The lysate was passed through a pipette several times.

2.3.2 Phase separation

The homogenised samples were incubated at RT for 5 minutes to permit the complete dissociation of the nucleoprotein complexes. 0.2 ml of chloroform was added per ml of TRIZOL reagent. The samples were shaken vigorously by hand for 15 seconds and incubated at room temperature for 2 to 3 minutes. The samples were centrifuged at 13,000 rpm for 15 minutes at 4°C.

2.3.3 RNA precipitation

The aqueous phase containing the RNA was transferred to a fresh tube. 0.5 ml of isopropyl alcohol was added per 1 ml of TRIZOL reagent. The samples were incubated at room temperature for 10 minutes and then centrifuged at 13,000 rpm for 10 minutes at 4°C. The supernatant was discarded and 1 ml of 75% ethanol added to the pellet. The sample was vortexed and centrifuged at 5000 rpm for 5 minutes at 4°C. The pellet was then air-dried and redissolved in RNase-free water by passing the solution several times through a pipette and incubating for 10 minutes at 55°C.

2.4 Northern blotting

Total RNA (10-20μg) from passage 2 HUVEC was electrophoresed on 1% agarose gel.

2.4.1Formaldehyde agarose gel for RNA

1% agarose gel- 100 ml final volume
1g agarose
10ml 10X MEN buffer (Table 2.1)
72ml MQ-H2O
Melt agarose in microwave
Cool to approx. 55°C
Add 18ml of formaldehyde solution (37% w/v)
Cast gel

2.4.2 Sample preparation

10-20μg total RNA dried in speed vac.
Redissolved in 10μl formaldehyde sample buffer
Heated at 65°C for 5 minutes
2μl of loading dye added sample loaded onto gel and the gel electrophoresed in 1X MEN buffer (Table 2.1)at 80V

2.4.3 Gel staining

The gel was rinsed 2X in MQ-H2O, stained in 10μl of ethidium bromide (EtBr) in 100ml water for 10 mins. The gel was destained in several changes of water until the bands were clearly visible (~30 mins). The gel was then checked and photographed under UV light.

The gel was soaked in a glass dish with 300 ml 10 X saline sodium citrate (SSC) buffer for 30 minutes to remove excess formaldehyde. 2 sheets of dry blotting paper were placed over the platform to act as a wick. The wick was moistened with 10 X SSC and all air bubbles were removed by rolling with a pipette. The gel was placed face down, taking care to avoid air bubbles the edges of the gel were surrounded by cling film. A Nylon membrane (Hybond-N Amersham, Buckinghamshire England) was cut to the same size as the gel and soaked in10 X SSC for 2 minutes. One of the corners from the gel and membrane was cut off to help in identification later. This was placed on top of the gel followed by sheets of pre-wet blotting paper, 2 sheets of dry blotting paper and a large pile of dry paper towels to absorb the buffer. Finally, a weight (about

500g) was placed on the top of the paper towels, and the transfer was allowed to proceed overnight. Once completed, the blot was disassembled and the wells were marked with a pencil. Successful transfer of RNA was detected by visualisation of ethidium bromide stained rRNAs on the membrane under UV light. The Nylon membrane was then placed on the UV box RNA side down for 5 minutes to crosslink the mRNA to the membrane. The membrane was stored in a sealed plastic bag at 4°C or used directly for hybridisation.

2.4.4 Radioactive labelling of cDNA probes

Double stranded cDNA in 0.1 M EDTA (pH8.0) was denatured by heating to 95°C for 5 minutes, chilled quickly on ice

Reaction mix:
1µl (25-50ng) denatured DNA
3µl forward primer (10ng/µl)
3µl reverse primer (10ng/µl)
6µl buffer 2A (Bresatec/Geneworks Thebarton Australia)
5µl α-^{32}P dATP (50 µCi, 3000 Ci/mMol) (Bresatec/Geneworks)
5µl MQ-H2O
1µl klenow polymerase (Bresatec/Geneworks)

The reaction was incubated at 37°C for four hours. The reaction was stopped by adding 1µl 0.5 M EDTA (pH 8). Non-incorporated deoxyribonucleoside-triphosphates were removed prior to hybridisation using a Microspin™ G-25 spin column (Pharmacia Biotech) 2900 rpm (735xg) for 2 minutes

2.4.5 Hybridisation

2.4.5.1 SSC hybridisation method

The membrane was prehybridised in 10 mls of hybridisation buffer: 50% formamide, 5X SSC, 5X Denhardt's solution, 1% SDS, 0.05% NaPPi, 100 µg/ml denatured salmon sperm DNA at 42°C for more than 4 hours in a hybridisation oven (Hybaid, Middlesex UK). The volume was reduced to 5ml and the probe added to the hybridisation bottle. The membrane was incubated overnight in the hybridisation oven at 42°C. The membrane was washed in 50ml of 2X SSC with

0.1% SDS at room temperature for 10 min. and the wash step repeated. The membrane was then washed for 20 minutes in 50 mls of 1X SSC with 0.1% SDS that had been preheated to 42°C.

2.4.5.2 ExpressHyb™ method

ExpressHyb™ (BD Biosciences, NJ, USA) solution was dissolved at 68°C. The membranes were prehybridised in 7mls of 1X ExpressHyb™ + 50µl denatured salmon sperm DNA for 30 minutes at 68°C. The probe was then added and incubated at 68°C in the hybridisation oven for 50 minutes. The membranes were then rinsed with 2X SSC containing 0.05% SDS and then washed in the same buffer overnight at RT.

Following hybridisation the membrane was wrapped in cling film and exposed overnight on a phosphorimaging screen. The amount of cDNA probe hybridising to specific mRNA was quantified by phosphorimaging using the Imagequant software (Molecular Dynamics, Amersham Biosciences, Piscataway USA).

2.5 cDNA synthesis and sequencing by reverse transcription and PCR amplification

2.5.1 First strand cDNA synthesis

The following reagents were added to a nuclease free microcentrifuge tube:

1µl random primers p(dN)6 (500 µg/ml)(Boehringer)
2 µg total RNA
Sterile MQ water to 12µl

This mixture was heated to 70°C for 10 minutes and then incubated on ice for 2 minutes. The contents of the tube were collected by brief centrifugation (10000g) and then the following reagents were added:

4µl First strand buffer (250 mM Tris-HCl), pH 8.3, 375 mM KCl, 15 mM $MgCl_2$)
2µl 0.1 M DTT
1µl 10 mM dNTP Mix (10 mM each dATP,dGTP, dCTP and dTTP at neutral pH)

The contents were then mixed gently and incubated at 42°C for 2 minutes.

1µl (200 units) of Superscript II reverse transcriptase (Promega corporation Annandale NSW Australia) was added to the mixture and mixed by pipetting up and down. The reaction was incubated at42°C for 50 minutes and then inactivated by heating at 70°C for 15 minutes

2.5.2 PCR reaction

The 2nd strand synthesis was performed with Taq polymerase (Roche, Basel, Switzerland), which generates a 5' A overhang.

The following reagents were added to a PCR reaction tube for a final reaction volume of 50µl.

1.25 µl 10 x Taq PCR buffer
1 µl dNTP Mix (10 µM)
1µl 25mM $MgCl_2$
1 µl Amplification primer 1 (10 µM)
1 µl Amplification primer 2
MQ water to 12.5 µl
1 bead of AmpliWax® PCR Gem 50 (Applied Biosystems, CA USA)

The mixture was then heated at 80°C for 5 minutes to melt the wax. When the wax had cooled and solidified the following reagents were added:

3.75 µl 10x Taq PCR buffer
3µl $MgCl_2$
1 µl Taq DNA polymerase
5 µg first strand cDNA
Sterile MQ water to total reaction volume of 50µl.

Reactions were heated to 94°C for 2 minutes to denature and then cDNAs were amplified with 25 cycles of PCR:

1 min at 94°C
1 min at 55°C
1 min at 72°C
7 mins at 72µC in the final cycle

The PCR product was electrophoresed in a 0.7% low melting point agarose gel at 70V (the gel tank was stood in a container of saline sodium citrate

ice to prevent overheating during electrophoresis). The PCR product was visualised under UV light and excised from the gel and the DNA purified by phenol chloroform extraction. The precipitated DNA was resuspended in 40µl of TE buffer. The DNA recovery was then checked by electrophoresing 5µl of the DNA on a 2% agarose gel. The gel was then stained with ethidium bromide and visualised under UV light.

2.5.3 Specific amplification of PTP sequences from first strand cDNA

Two degenerate oligonucleotide sense and antisense primers corresponding to the amino acid sequences (H/D)FWRM(I/V)W (5'-A(C/T)T(C/T)TGG(A/C)GIATG(A/G)TITGG-3')and WPD(F/H)GVP (5'-GGIAC(G/A)(T/A)(G/A)(G/A)TCIGGCCA-3') respectively(Cheng et al., 1997), were used to specifically amplify PTP sequences by PCR from first strand cDNA (see 2.5).

2.5.4 Ligation of PCR product into pGEM –T easy vector (Promega)

1µl T4 DNA ligase 10X buffer
50ng pGEM®-T easy vector (Promega)
12.5 ng PCR product
1µl T4 DNA ligase
sterile MQ DNA to final volume of 10µl
Incubated at 4°C overnight

2.5.5 Transformation of competent JM109

A 200µl aliquot of competent JM109 *E. coli* was thawed on ice.2µl of the PCR product ligation was added to50µl competent JM109 *E. coli* in a10 ml tube and incubated on ice for 20 minutes. The *E. coli* were then heat shocked at 42°C for 45 seconds followed by a 2-minute incubation on ice. 950µl LB was then added and the cells shaken at 190rpm at 37°C for 45 minutes. The volume was then reduced by centrifuging at 2000 rpm for 5 minutes and aspirating 800µl of the supernatant. The *E. coli* were then resuspended in the remaining medium and plated on Amp/IPTG/X-Gal LB agar selection plates for blue/white colour screening. The pGEM®-T Vectors carry a segment of the *lac*Z gene that encodes the amino terminal fragment of beta-galactosidase. Expression of this fragment will complement certain *E. coli lac*Z mutations. The multiple cloning site of the pGEM®-T Vectors is within the *lac*Z coding region on the

plasmid and inserted fragments will interrupt the expression of the *lac*Z sequences. In the presence of an inducer of the *lac* operon (IPTG) and an indicator substrate (X-Gal) colonies expressing *lac*Z sequences from the plasmid are blue, while those containing plasmids with inserts are white. Clones that contain PCR products can be identified by blue/white colour screening on indicator plates. Positive colonies were picked and grown in Luria Broth (LB) (Table 2.1). Restriction digests of the plasmid DNA were screened for inserts of around 270bp, as this is the size of the PTP DNA sequence flanked by the two degenerate primer sequences. Positive clones were then sequenced using an automatic sequencer (ABI PRISM models 377 and 373) using a dideoxy terminator cycle sequencing kit (Perkin Elmer, Boston, MA. USA).

2.6 cDNA probes

cDNA probes to PTPD1, PTPMEG-1 and PTPH1 were generated from 5µg of HUVEC first strand cDNA (see 2.5), which was amplified using primers specific to the variable regions of PTPD1 bp $_{2527-2901}$ (sense 5'-CGCGCGAGCCTCGGCCC-3', antisense 5'- GGCAGCCAGTTTAAGAGG-3'), PTPMEG-1 bp $_{1014-1480}$ (sense 5'-GGCCTCGCCTCTCGCG-3', antisense 5'CGACCCGGAGCCGCCAGC-3') and PTPH1 bp $_{1076-1524}$ (sense 5'-CGGGATGGTGTGGAACCC-3', antisense 5'-TTGTCACAGTAGTACTGGC-3').

A cDNA probe to PTP-Pez was generated by PCR using pBsk/PTP-Pez/N-Flag (see 2.8) as the template and primers specific to the variable region of Pez bp $_{2403-2737}$ (sense 5'-CCGGGAGAAGATGGAGTACA-3', antisense 5'-CTGATCATCATCTCTCTCTC-3'). The PCR products were cloned into pGem 4Z vector (Promega, Madison WI USA) and the sequence verified as in 2.5.

2.7 Pez DNA constructs

All PCR amplifications of Pez constructs (Fig 2.1) were carried out using Pfu DNA Polymerase (Promega, Madison WI USA). The primers used in the PCR amplifications are listed in Table 2.4

Figure 2.1

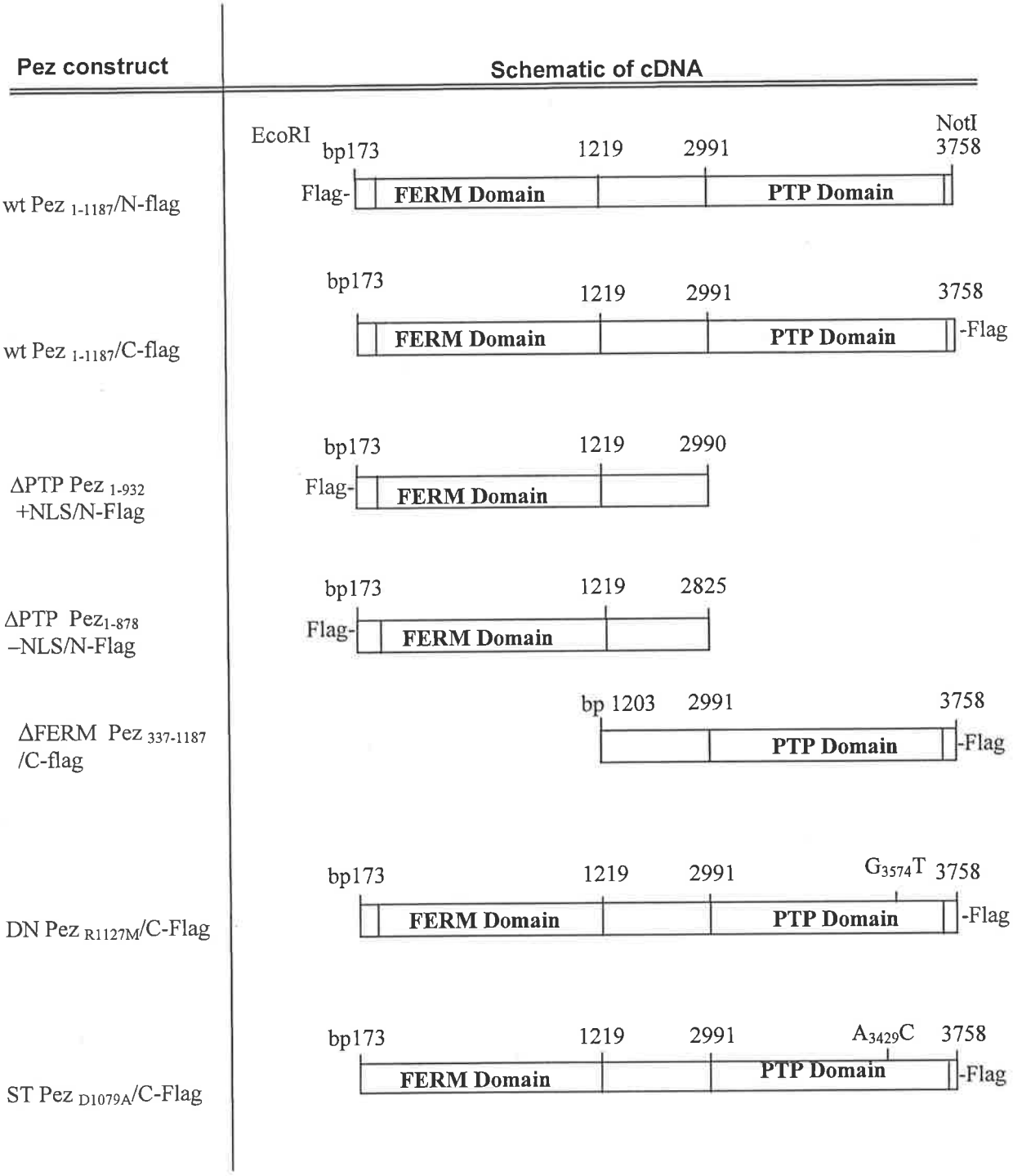


Figure 2.1 Schematic representation of Pez deletion and point mutation constructs. The cDNAs were inserted into EcoR1 and NotI restriction sites in a pcDNA3 expression vector.

Table 2.4 PCR primers used for making PTP-Pez constructs

Pez construct	Sense primer	Antisense primer
Pez/N	5'-ACGTGAATTCATGGACT ACAAGGACGACGATGACA AGCCTTTTGGTCTGAAGCT C-3'	5'-TGTATGCAGGATCCC CC-3'
Pez/C	5'-AGAGGGGGATCCTGCA TAC-3',	5'-ACGTGCGGCCGCTTAAA TGAGTCTGGAGTTTTGG-3'
Pez $_{337-1187}$/C-Flag (Δ FERM Pez)	5'-ACGTGAATTCACCATGC TGCCTCCCGTTCACGTC-3';	5'-ACGTGCGGCCGCTTACT TGTCATCGTCGTCCTTGTA GTCAATGAGTCTGGA GTTTTGGAGGAACTG-3'
Pez$_{1-932}$/N-Flag (ΔPTP Pez +NLS)	5'-AGAGGGGGATCCTGC ATAC-3'	5'-ACGTGCGGCCGCTTATT CTGGCAGAGCTGCTG-3'
Pez$_{D1079A}$ /C-Flag (ST Pez) Fragment 1	5'-CTGACTGGCCAG***C***TCAC GGCTGTCC-3'	5'-ACGTGCGGCCGCTTACT TGTCATCGTCGTCCTTGTA GTCAATGAGTCTGGA GTTTTGGAGGAACTG-3'
Pez$_{D1079A}$ /C-Flag Fragment 2	5'-CTGCAGGCGGCCCTG GC-3'	5'-CTGGCCAGTCAGTATAT TG-3'
Pez$_{D1079A}$ /C-Flag Overlapping PCR	5'-CTGCAGGCGGCCCTG GC-3'	5'-ACGTGCGGCCGCTTACT TGTCATCGTCGTCCTTGTA GTCAATGAGTCTGGAGTT TTGGAGGAACTG-3'
Pez $_{R1127M}$/C-flag DN Pez)	5, -CAACAGCATGCTGGA AGGCACCAAGAACCGGCA CCCGCCCATCGTGGTCCA CTGTAGTGCTGGGGTGGG AA***T***GACCGGC-3'	5'-ACGTGCGGCCGCTTACT TGTCATCGTCGTCCTTGTA GTCAATGAGTCTGGA GTTTTGGAGGAACTG-3'

Table 2.4 Oligonucleotide primer sequences used to generate the Pez deletion and point mutation constructs. Point mutations are indicated by bold italics. The Flag epitope sequence is underlined.

N-terminal flag epitope tagged full length Pez cDNA (Pez$_{1-1187}$/N-flag) was generated by RT PCR (see 2.3) from passage 1 HUVEC total RNA. Amino terminus (bp197-1552) PCR amplification was carried out for 35 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 4 mins. The PCR products were digested with restriction enzymes and ligated into the EcoR1/BamHI restriction sites of pBluescript SK$^+$ this construct was named pBsk- Pez-N. The carboxy terminal end (bp1552-3758). PCR amplification was carried out for 35 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 4 mins. The PCR products were digested with

restriction enzymes and ligated into the NotI/BamHI restriction sites of pBluescript SK^+ this construct was called pBsk-Pez-C. The N-terminal fragment was then cut out of PTP-Pez-N and ligated into the EcoR1/BamHI restriction sites of PTP-Pez-C to make the construct pBsk-Pez/N-Flag). The sequence was verified by dideoxy cycle sequencing from both the sense and the antisense directions. Sequence comparison was performed using the database in NCBI. The full length Flag-epitope tagged insert was cut out and inserted into the Not1/EcoR1restriction sites of the expression vector pcDNA3 (Invitrogen, Groningen, The Netherlands) (pcDNA3-Pez/N-Flag).

The FERM domain deleted Pez construct (pcDNA3-Pez $_{337\text{-}1187}$/C-Flag) was generated by PCR amplification using pBsk-Pez as the template. PCR amplification was carried out for 25 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 5mins 20 secs. The PCR product was restriction digested and ligated into the EcoR1/Not1 restriction sites of pcDNA3 expression vector. The ΔPTPPez $_{1\text{-}878}$/N-Flag construct (which lacks a putative NLS sequence) was made by digesting Pez$_{1\text{-}1187}$/N-flag with XhoI which excised the sequence bp 2825- 3758 (aa 879-1187) the religated construct utilises a stop codon in the XbaI restriction site of the pcDNA3 vector. A second construct Pez$_{1\text{-}932}$/N-Flag lacking the phosphatase domain but including the putative nuclear localisation and nuclear export sequences was generated by PCR amplification of full length Pez. PCR conditions were 1 min at 94°C, 1 min at 50°C and 2 mins at 72°C for 35 cycles. The PCR product was restriction digested with BamHI and NotI and ligated into the pBsk shuttle-vector and subsequently excised with and ligated into the EcoRI/NotI restriction sites of pcDNA3.

The substrate-trapping construct pcDNA3/ Pez$_{D1079A}$/C-Flag was generated by PCR amplification of two fragments. The two fragments generated from these PCR reactions were then used as the template for overlapping The final 1.39 kb PCR product encompassed the region bp 2403 – 3758. PCR product 3 was restriction digested with XbaI and NotI and ligated into

pBsk+/Pez/C-Flag. Following sequence verification the insert was cut out with the restriction enzymes EcoRI and NotI and ligated into pcDNA3.

The pcDNA$_3$/ Pez $_{R1127M}$/C-flag mutation (putative dominant negative) was generated by PCR amplification. PCR cycling of 1 min at 94°C, 1 min at 50°C and 2 mins at 72°C for 35 cycles generated a 285bp product which was ligated into the SphI/NotI restriction sites in pBsk/Pez. The insert was subsequently excised with and ligated into the EcoRI/NotI restriction sites of pcDNA3.

The deletion construct pcDNA3/Δ FERM/Pez$_{D1079A}$/C-Flag was generated by restriction digest of pcDNA3/Δ FERM with BamHI which produced a fragment bp $_{1203-1558}$ that was ligated into BamHI digested pcDNA3/ Pez$_{D1079A}$/C-Flag (bp $_{1559-3755}$).

2.8 SDS-PAGE and Western blotting

2.8.1 Cell lysis

Total cell lysates were prepared by several alternative methods: -

1. Trypsinising cells and then washing in PBS prior to boiling in Laemmli sample buffer (Laemmli, 1970) containing 300mM NaCl
2. Washing cell in PBS then incubating in ice-cold RIPA buffer (50mM Tris-HCL, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150mM NaCl, 1mM EGTA, 1mM sodium orthovanadate, 1X protease inhibitor cocktail (Sigma)), The cells were scraped after 15 minutes and clarified by centrifugation (10 minutes at 13000 rpm). The lysates were then boiled in Laemmli sample buffer.
3. As in 2. but in substrate-trapping (ST) Buffer (50 mM HEPES, pH 7.5, 150mM NaCl, 150mM NaF, 10mM sodium pyrophosphate, 5mM EDTA, 1% Triton X-100, and protease inhibitor cocktail (P2714 SIGMA, Saint Louis, Missouri, USA))

2.8.2 Subcellular fractionation

Subcellular fractionation was carried out according to published protocols (Mui et al., 1995). The cells were harvested by trypsinisation, washed 1x in PBS and resuspended in buffer A (50mM Hepes, pH 7.5, 100mM NaF, 300 mM NaCl, 10% glycerol, 10mM sodium pyrophosphate, 10 mM MgCl, 2 mM sodium orthovanadate, 2 mM sodium molybdate, 2 mM EDTA, 1x protease inhibitor cocktail (Sigma)) containing 0.2% Nonidet P-40 (Sigma) for one minute on ice. The nuclear pellet was collected by brief centrifugation (10000g) for 15 seconds and washed twice with buffer A. The supernatant was retained as the cytosolic fraction. The nuclear pellet was then either lysed in Laemmli sample buffer to obtain a total nuclear fraction or resuspended in Buffer B (50mM Hepes, pH7.5, 100 mM NaF, 300 mM NaCl, 10% glycerol, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 2 mM sodium molybdate, 2 mM EDTA, 1x protease inhibitor cocktail (Sigma)) containing 0.1% Nonidet P-40 and rocked vigorously to obtain nuclear extract (supernatant after centrifugation at 13000 rpm, 5 minutes at 4°C) or nuclear pellet fraction.

2.8.3 Determination of protein concentration of cell lysates

Bradford reagent (Biorad) was diluted 1:5 in MQ water and 200 µl of the diluted reagent added/well on a 96-well microtitre plate. 1µl of each lysate sample was added/well in duplicate and the absorbance measured on a microplate reader (Biorad) at a wavelength of 595nm. The protein concentration was determined against a standard curve generated from known concentrations of BSA (in triplicate) using Microplate Manager software (Biorad)

2.8.4 SDS-PAGE

The cell lysates were electrophoresed on an 8% SDS- polyacrylamide gel (SDS-PAGE) using Biorad Protean II electrophoresis apparatus assembled according to the manufacturers instructions

For two 0.5 mm thick gels of 8% acrylamide:

Separating gel
4.6 ml MQ water
2.7 ml 30% acrylamide
2.5 ml 1.5M Tris pH 8.8
100µl 10% SDS
100µl 10% ammonium persulphate
6µl TEMED (*N,N,N',N'*-tetramethylethylenediamine)
TEMED and ammonium persulphate were added immediately prior to pouring the gel.

The separating gel was mixed and then poured between the assembled glass plates to within a few millimeters below where the wells are formed by the comb. The gel was the overlaid with MQ water. When the acrylamide had polymerized the water was poured off and the stacking gel added

Stacking gel
2.1ml MQ water
0.5ml 30% acrylamide
0.38ml 1M Tris pH 6.8
30µl 10% SDS
30µl 10% ammonium persulphate

The comb was then inserted, ensuring that there was no introduction of air bubbles.
Once the gels had polymerised they were placed in the electrophoresis tank. The combs were removed and the wells rinsed out with 1x running buffer (Table 2.1) to remove any unpolymerised acrylamide. The tank was filled with 1x running buffer. Lysates were boiled for 5 minutes in laemmli buffer (Table 2.1)(Laemmli, 1970) and equal amounts of protein (30-100µg/well) loaded onto the gel. Prestained protein MW standards were included in each gel (GibcoBRL). The gel was electrophoresed at 200V for approximately 45 minutes.

2.8.5 Western blotting

The transfer buffer (1x) (Table 2.1) was chilled to 4°C at least 1 hour prior to transfer. The electrophoresis apparatus was disassembled and the gel removed from the glass plates and soaked in transfer buffer for 10 minutes. Four pieces of blotting paper per gel and 1 piece of Hybond-P PVDF membranes (Amersham, Buckinghamshire, England) were cut to the size of the gel. One

corner of the PVDF membrane was excised, the membrane rinsed briefly in methanol and soaked in transfer buffer for 10 minutes. Gel transfer pads and blotting paper were soaked in transfer buffer for 10 minutes.

The transfer cassette was assembled starting on the black side (the side that faces the negative electrode): pad, blotting paper, gel, pre-wetted PVDF membrane, blotting paper, pad. The apparatus was assembled, immersed in transfer buffer and each layer rolled with a pipette to exclude bubbles between the layers. The cassettes were inserted in the transfer tank and the tank ¾ filled with transfer buffer. An ice block was inserted to prevent overheating. Electrophoresis was carried out at constant 100 V for 1.25 h (130-180 mA) with constant stirring on a magnetic stirrer to prevent localised overheating..

The membrane was removed and blocked with either 5% milk or 5% BSA or 5% blocking reagent (Roche), 0.1% Triton-X 100 in phosphate buffered saline (PBS). Crude antisera against Pez were used at 1:500 dilution, affinity purified antibody were used at 1:50 or 1:100 dilution. Commercial antibodies were used at the concentrations recommended by the supplier. The blots were developed using an HRP-conjugated secondary antibody (Immunotech, Marseille, France) and enhanced chemiluminescence (Amersham). For quantitative Western blotting analysis, the blots were developed using an alkaline phosphatase-conjugated secondary antibody fluorescent Vistra ECF substrates (Amersham) and the resulting band intensities quantitated using a fluorimager and the Imagequant software (Molecular Dynamics). In experiments in which the Pez antibody was preincubated with peptides prior to use in Western blots, preincubation was carried out in 1ml of PBS containing 100μg/ml of peptide for 1 hour at room temperature or overnight at 4°C.

2.9 Tyrosine-phosphatase assay

Protein tyrosine phosphatase activity was assayed using a non-radioactive assay kit from Boehringer/Mannheim (Germany). HEK293 cells were plated on fibronectin coated 10cm dishes and transiently transfected with Flag-tagged wt-Pez, Flag-tagged Pez_{D1079A} or empty vector. The cells were lysed 48 hours post transfection with RIPA buffer (50mM Tris-HCL, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150mM NaCl, 1mM EGTA, in the presence of 5mM DTT and 1x protease inhibitor cocktail (Sigma)). The lysate was precleared with protein A-sepharose. The flag tagged Pez proteins were then immunoprecipitated with M2 anti-flag antibody (Sigma) pre-coupled to protein A-sepharose beads for 1 hour at 4°C. The Pez complexed beads were washed 2x with RIPA buffer and 2X with PTPase assay buffer (25 mM Hepes, pH 7.5, 5mM DTT 0.1% BSA, 1mM EGTA 1x protease inhibitor cocktail (Sigma-Aldrich). The beads were then resuspended in 50µl of PTPase assay buffer. The phosphatase assay was then carried out in a 96-well plate according to assay protocol 1 of the manufacturer's instructions. Briefly 10µl of the resuspended beads were added to 10µl of a 3µM solution of biotin labelled phosphatase substrate 1(PPS1) (PPS1=corresponding to the hirudin 53- 65 C-terminal fragment Biotin-DGDFEEIPEEY (PO^4) LQ-NH2, phosphorylated on tyrosine 63) diluted in PTPase assay buffer together with 10µl of water. The phosphatase reaction was stopped after 2 hours by adding 1/3 of assay volume of inhibitor solution (400µM sodium vanadate). The sample was then stored on ice. 30µl of the sample was then added to the wells of a streptavidin coated microtitre plate and incubated at RT for 1 hour or at 37°C for 20 minutes. The solution was then discarded and the plate washed three times with 300µl of PBS for at least 30 seconds/wash. After the final wash the plate was tapped on a lint-free absorbent cloth. 75µl of anti-phosphotyrosine antibody conjugated to peroxidase was added to each well, the plate was covered with foil and

incubated at 37°C for 1 hour. The solution was discarded and the wells washed three times as above. 100µl of ABTS® (2,2'-Azino-di- [3-ethylbenzthiazoline sulfonate) substrate solution was added to each well and incubated at room temperature until the green colour developed. The absorbance was then read on a microtitre plate reader at 405nm (reference wavelength 490 nm). The level of substrate remaining in the sample was inversely related to the phosphatase activity. The assays were performed in triplicate.

2.10 Enriching for tyrosine phosphorylated proteins

Newly confluent HUVEC and MDCK cells 48 h post-confluence were used as sources of tyrosine-phosphorylated proteins. Generally the HUVEC cells were harvested at passage 2 or 3. The cells were plated on gelatine coated (HUVEC) or uncoated (MDCK) 10cm tissue culture plates and incubated in a 37°C incubator until the cells were just confluent. Cell lysates were prepared as described in Flint *et al* (Flint et al., 1997). Briefly, the cells were incubated for 30 minutes with 50µM sodium pervanadate to enrich for tyrosine-phosphorylated proteins, and harvested by trypsin treatment. The cell pellet was washed 1x in ice cold phosphate buffered saline (PBS) and lysed in (1ml/10 cm dish) substrate-trapping buffer (ST buffer) 50 mM HEPES, pH 7.5, 150mM NaCl, 150mM NaF, 10mM sodium pyrophosphate, 5mM EDTA, 1% Triton X-100, and protease inhibitor cocktail (P2714 Sigma) at 4°C. The lysates were incubated on ice for 30 minutes in the presence of 5mM iodoacetic acid (IAA) to irreversibly inactivate the endogenous PTPs. Unreacted IAA was inactivated with 10mM dithiothreitol (DTT). The lysates were then clarified by centrifugation for 10minutes at 13000rpm. The lysates were then frozen on liquid nitrogen and stored at-70°C.

2.11 Substrate-trapping *in vitro*

2.11.1 PTP-Pez-coupled protein A-sepharose

HEK293 cells were transfected with either WT Pez or the substrate-trapping mutant ST-Pez$_{D1079A}$ both of which were tagged with the Flag-epitope, or the vector alone. The cells were incubated for 48-hours at 37°C and then trypsinised, washed 1x in PBS and lysed in ST buffer at 4°C. The lysate was sonicated and precleared for 30 mins with 20µl of packed protein A-sepharose (Pharmacia) beads/ml of lysate. The flag tagged Pez proteins were then immunoprecipitated with M2 anti-flag antibody (SIGMA) and 20µl of protein A-sepharose beads for 1 hour at 4°C.

2.11.2 Substrate-trapping

The Pez-complexed protein A-sepharose beads were washed three times in ST buffer (Table 2.1) and added to the phosphotyrosine enriched HUVEC lysates for two hours then rocked at 4°C. The beads were washed three times with substrate-trapping buffer and boiled in 1x laemmli sample buffer (Laemmli, 1970). Bound proteins were resolved by 8% SDS-PAGE and blotted onto PVDF membrane (Hybond-P, Amersham Pharmacia Biotech) The blots were blocked with 5%milk, 0.1% Triton X-100 in PBS then incubated with anti-phosphotyrosine antibodies and developed using HRP-conjugated secondary antibody (Immunotech) and enhanced chemiluminescence (Amersham Pharmacia Biotech). The tyrosine-phosphorylated proteins identified by this assay were then counter blotted with antibodies to proteins localising to the cell-cell junctions.

2.11.3. Scale Up for Identification of Substrate by Mass-Spectrometry

The substrate-trapping procedure was scaled up to identify the putative substrate by mass spectrometry. Several pooled HUVEC cell lines were used as the source of phosphorylated proteins yielding a total of around $5x10^6$ cells / immunoprecipitation. The proteins were separated on a Nupage 7% Tris-Acetate polyacrylamide gel (Novex). The dried gel was then sent to the laboratory of Richard Simpson at the Ludwig Institute for Cancer Research, Victoria Australia, for analysis.

2. 12 Co-immunoprecipitations

Cells were washed 1X in PBS and incubated in ice-cold ST buffer containing 1mM orthovanadate. The cells were scraped after 15 minutes and pre-cleared with 20µl of protein A-sepharose for 30 mins at 4°C. Protein concentration was assayed using Bradford Reagent from BioRad (California USA) (Bradford 1976). Equal amounts (2-5 mg) of protein were immunoprecipitated with 2µg of primary antibodies for 1 hour at 4°C supplemented with 20 µl packed protein A-sepharose for 1 hour. The beads were subsequently washed three times in ST buffer. Bound proteins were eluted by boiling in Laemmli sample buffer for 5 mins, separated by 8% SDS-PAGE, transferred to PVDF membrane, blocked with 5% milk, 0.1%Triton X-100 in phosphate buffered saline and blotted with the indicated antibodies

2.12.1 Co-immunoprecipitation with Pez

PezR1 antiserum was incubated with protein A-sepharose for 1 hour or overnight. The antibody-coupled beads were then washed extensively in ST buffer and then incubated with pre-cleared HUVEC lysates (20µl beads/ml of lysate) for 1 hour at 4°C with rocking. The beads were washed three times in ST buffer. The immunoprecipitates were separated by SDS-PAGE, transferred to PVDF membrane and probed with the indicated antibodies. The blots were stripped and counter blotted with PezR1 antiserum.

2.13 *In vivo* tyrosine phosphorylation in transiently transfected A431 cells

A431 cells were transfected with wt-Pez, ΔPTP-Pez or vector using Lipofectamine 2000 , and the cells were lysed *in situ* after 48 hours incubation. 50µg of total protein from each lysate was subjected to SDS-PAGE, transferred to PVDF membrane and then immunoblotted with anti-phosphotyrosine antibody.

2.14 *In vivo* tyrosine phosphorylation

2.14.1 In vivo tyrosine phosphorylation in stable MDCK cell lines

Stable MDCK cell lines expressing either vector, WT PEZ or ΔPTP-Pez were plated at confluence and incubated for three days in the presence of 10 % foetal calf serum (FCS) then serum-starved for 24 hours. The cells were stimulated with 10% FCS for ten minutes. The cells were then lysed *in situ* in the presence of 1mM orthovanadate. Equal amounts of protein were separated by 8% SDS-PAGE, transferred to PVDF membrane and blotted with anti-phosphotyrosine antibody. The blot was subsequently stripped and reblotted for β-catenin.

2.14.2 In vivo tyrosine phosphorylation in stable HEK293 cell lines

Stable HEK 293 cell lines expressing Pez WT or vector were plated at confluence and incubated overnight in the presence of 10 % foetal calf serum (FCS) then serum-starved for 24 hours. The cells were then stimulated +/- 10μg/ml EGF for 24 hours. The cells were incubated in 0.1mM pervanadate for 5 mins prior to lysis. The cells were then lysed *in situ* in the presence of 1mM orthovanadate. Equal amounts of protein were immunoprecipitated with anti-phosphotyrosine antibodies and the immunoprecipitates and whole cell lysates were separated by 8% SDS-PAGE, transferred to PVDF membrane and blotted with anti-phosphotyrosine antibody. The immunoblot was subsequently stripped and counter-blotted with antibodies against α,β and γ-catenin (Transduction Laboratories), E-cadherin (Transduction Laboratories) and occludin (Zymed).

2.15 Microscopy

2.15.1 Confocal microscopy

HUVEC were plated onto fibronectin (50μg/ml) coated glass LabTek chamber slides (Nalge, Nunc Int.) at the indicated densities. Prior to staining the cells were washed 1X in PBS and fixed in 4% paraformaldehyde/PBS for 10 minutes, quenched with 10mg/ml sodium borohydride in PBS pH 8.0 for 15 minutes (x2) and then permeabilised by treatment with 0.1% Triton X-100 in PBS pH 7.0. Affinity purified antibody to Pez was used at 1:10 dilution (whole sera was used at 1:250) and indirect immunofluorescence was detected by incubation with biotinylated anti-rabbit

secondary antibody (Rockland) followed by FITC-conjugated streptavidin (Dako A/S, Denmark). Confocal microscopy was carried out using a BioRad MRC-600 confocal microscope. For comparison of treatments, the laser power, confocal aperture and contrast settings were kept constant.

2.15.2 Immunofluorescence

Stable MDCK cells lines expressing WT Pez, ΔPTP-Pez or Δ4.1 Pez were plated at confluence onto fibronectin coated glass LabTek chamber slides (Nalge, Nunc International) at the indicated densities. The cells were then incubated for between 1 to 5 days as indicated. Prior to staining the cells were washed 1X in PBS and fixed in 4% paraformaldehyde/PBS for 10 minutes, quenched with 10mg/ml sodium borohydride for 15 minutes (x2) and then permeabilised by treatment with 0.1% Triton X-100. The cells were incubated with the indicated primary antibodies for 1 hour at 1:100 dilution and indirect immunofluorescence was detected by incubation with either fluorophore coupled secondary antibodies (anti- rabbit Alexa Fluor 350 (Molecular Probes, Leiden, The Netherlands), anti-rabbit Alexa Fluor 594 (Molecular Probes), anti-mouse FITC (Rockland), anti-rabbit FITC (Rockland) or with biotinylated secondary antibodies (Rockland) followed by either FITC-conjugated streptavidin (Dako) or texas red conjugated streptavidin (Rockland). The coverslip was mounted using fluorescent mounting medium (Dako).
The cells were imaged by epifluorescent microscopy on an Olympus BX-51 microscope equipped with excitation filters for Alexa Fluor 594/texas red, fluorescein (494 nm), and Alexa Fluor 390, acquired to a Cool Snap FX, charge-coupled device (CCD) camera (Photometrics). Images were adjusted for brightness and contrast with V^{++} software (Digital Optics Ltd. Auckland New Zealand). The line-profiling feature of this software was used to plot the intensity vs. position of different fluorophores along a path through the cell monolayer, in cells that had been co-stained for two or more proteins.

2.16 Permeability assays

MDCK stable cell lines were plated at $5x10^4$ or $1x10^5$ on fibronectin coated 0.4μm or 3μm polycarbonate transwells (Costar Corp., Cambridge, MA, USA) in 150μl of medium in the top well with 600μl of medium added to the bottom well. The next day the medium was changed to 0.5% FCS and the cells incubated in this medium for 3 days with the medium replaced daily. Alternatively the cells were incubated for 3-7 days in complete medium (10% FCS), the medium was then changed to 0.5%FCS two hours prior to the assay. The cells were then stimulated in duplicate or triplicate with 0.1mM sodium vanadate, 100 μg/ml EGF or thrombin (0.5, 1 or 2 units/ml). FITC-dextran (SIGMA) (0.5 mg/ml) was added to the top well at T=0 and 20 μl aliquots removed from the bottom well at T=2mins and at five minute intervals up to 1 hour. The samples were then analysed on a luminescence spectrometer (Perkin Elmer Model LS50B) and the amount of fluorescence compared to a standard curve generated from a serial dilution of FITC-Dextran.

2.17 Luciferase reporter assays

Cells were seeded onto 24-well or 96-well plates at the indicated densities and incubated overnight at 37°C. The next day the cells were transfected with either the pTopflash reporter construct which contains three optimal TCF binding sites or the pFOPflash reporter construct in which the TCF sites have been mutated (inactive motif) obtained from Upstate Biotechnology (Lake Placid NY USA) together with pCMV/Renilla luciferase (Promega) using Lipofectamine 2000 (Life Technologies Inc.) according to the manufacturers instructions. The cells were harvested 48 hours after transfection in passive lysis buffer (Promega). Luciferase activities were measured using the dual-luciferase assay system (Promega). The experimental LEF-luciferase reporter activity was controlled for transfection efficiency and potential toxicity of treatments

using the constitutively expressed pCMV/*Renilla* luciferase. Luminescence was measured on a TD-20/20 luminometer (Turner Utah USA)

2.18 Wounding assay

MDCK stable cell lines were plated onto 6-well trays at densities that would give confluent monolayers after 24h. Confluent monolayers were incubated a further 48h to allow intercellular junctions to mature before being serum-starved for 24h. A linear wound was generated on the monolayers by scraping with the edge of a cell scraper. Unattached cells were washed off with agitation. Cells were photographed at the same point on a grid at the time of scraping and again 24h later. The difference in width of the wound between the 2 edges at the time of scraping and 24h later was measured and represents the distance migrated. Each line was plated and wounded in triplicate.

Chapter Three

RT-PCR analysis of PTPs expressed in an endothelial cell line

Chapter 3: RT-PCR analysis of PTPs expressed in an endothelial cell line

3.1 Introduction

The aim of this project was to identify PTPs that are potential regulators of cell-cell adhesion. This question arose from the observation that treatment of HUVEC with vanadate, a specific inhibitor of PTPs, resulted in a breakdown in the cell-cell contacts (Matthias and Gamble unpublished). In the presence of PTP inhibitors selective cleavage and redistribution of the TJ protein, occludin, occurs in conjunction with an increase in permeability of endothelial cells (Wachtel et al., 1999).

Endothelial cells (EC) are the basic building blocks of the vascular tree in vertebrates. They are central to normal angiogenesis during embryonic development, wound healing and the oestrous cycle. Angiogenesis is also critical for the development of certain pathological situations such as in the growth of solid tumours. The endothelium forms a barrier between blood cells and the tissue space but allows the passage of leukocytes into the tissue space when necessary, such as during inflammation. The passage of leukocytes through the endothelium is a tightly regulated process and it is essential that it be so. Deregulation can lead to chronic inflammatory diseases including atherosclerosis.

Many changes occur in the EC during the processes of angiogenesis and inflammation. During angiogenesis, tightly packed EC have to 'disengage', migrate and proliferate in response to growth factors and other external stimuli. This requires the activation of signals that lead to changes in the junctional properties and cytoskeletal rearrangements in addition to signals that lead to cell proliferation. During inflammation, leukocytes move through the blood vessel wall into inflamed tissue. This process of extravasation requires the modification of endothelial cell-cell adhesions to allow the passage through the endothelium. It is important that we understand

the signalling processes that lead to these changes, which are essential for the EC to function properly.

3.1.1 Protein tyrosine phosphatases in endothelial cells

When this project commenced in 1998 it was predicted that there are approximately 500 PTPs in the human genome (Neel and Tonks, 1997). This figure was based on the number of PTPs expressed in *C elegans,* which is known to be 108 (Lander et al., 2001). This estimate was reduced to 100 at the end of 1998, based on a detailed analysis of the human cDNA databases. At this time approximately half the human genome, including just 48 full-length PTPs had been sequenced (Hooft van Huijsduijnen, 1998). An initial sequence analysis of the nearly completed human genome revealed a total of 112 PTPs including the dual-specificity phosphatases (Lander et al., 2001).

There is a high degree of structural diversity in the non-catalytic domains of PTPs (see Figure 1.2). These non-catalytic domains have a regulatory role in substrate specificity and subcellular localisation. It is well established that tyrosyl phosphorylation is involved in the dynamic processes of cell-matrix interaction, cell-cell contact and cell migration (Maher et al., 1985; Birchmeier et al., 1993; Volberg et al., 1992 reviewed in Daniel and Reynolds, 1997). There is growing evidence of the importance of PTPs in the regulation of these processes.

Tyrosyl phosphorylation of signal transducing molecules is one of the major pathways for conveying messages from the cell surface to other parts of the cell in many cell types, including EC. For example, VEGF and angiopoietin, both potent angiogenic factors, bind to receptors that have intrinsic tyrosine kinase activity. Tyrosine phosphorylation is also integral to the dynamic regulation of cell adhesion, both to the cell matrix and to adjacent cells. The extracellular domains of many RPTPs contain structural features that suggest a role in cell-cell adhesion. The receptor type PTPμ and PTPκ have been shown to be involved in homophilic cell-cell interactions; PTPμ interacts with the scaffolding protein RACK1 and may be involved in

recruitment of RACK1 to points of cell-cell contact (Mourton et al., 2001). Members of the LAR family co-localise with β-catenin and γ-catenin at the adherens junctions (Muller et al., 1999) and the RPTP, DEP-1 has recently been found to form a specific interaction with $p120^{ctn}$ suggesting that it too has a role in regulating cell-cell adhesion (Holsinger et al 2002). The cytosolic PTP1B modulates cadherin-mediated cell-cell adhesion through dephosphorylation of β-catenin (Pathre et al., 2001). Loss of N-cadherin mediated cell-cell adhesion results from the overexpression of a dominant negative catalytically inactive form of PTP1B (Rhee et al 2001).

Some PTPs shown to be involved in cell-cell adhesion are also involved in modulating adhesion to the extracellular matrix. These include RPTPs of the LAR family, which co-localise with proteins at the focal adhesions (Serra-Pages et al., 1995), and PTP1B which also appears to modulate both integrin-mediated adhesion to the cell matrix through regulation of Src activation (Pathre et al., 2001). PTP1B is a regulatory component of the molecular complex required for both N-cadherin and β1-integrin- mediated axon growth (Pathre et al., 2001). Another non-receptor type PTP, PTP-PEST is also involved in the regulation of cell-matrix adhesion. PTP-PEST specifically dephosphorylates the focal adhesion-associated adaptor-type protein, p130(cas) (Garton et al., 1996.) PTP-PEST may be necessary for the breaking of focal adhesions, a process essential for cell mobility. The number and size of focal adhesion complexes was greatly increased in $PEST^{-/-}$ fibroblasts, which were also found to be defective in motility (Angers-Loustau et al., 1999).

Because regulation of the cell-cell junctions of endothelial cells is integral to the function of the vasculature, it is important to understand which of the many PTPs are involved in this process, to identify their physiological substrates and characterise their specific functions.

Therefore the primary aim of this project was to identify the PTPs involved in regulating cell adhesion in HUVEC

3.1.1.1 Approach:

1. Analysis of the PTPs expressed in HUVEC using a reverse-transcription-PCR (RT-PCR) amplification homology screen.
2. Selection of likely candidates from the homology screen for further characterisation (see 3.1.1.2).
3. Verification of expression of the selected PTPs.

3.1.1.2. Criteria used for the selection of PTPs for further characterisation:

1. PTPs containing MAM domains, as this family are already known to be involved in homotypic cell-cell adhesion (Brady-Kalnay et al., 1995; Brady-Kalnay and Tonks, 1994; Sap et al., 1994).
2. RPTPs such as the DEP-1 family (Ostman et al., 1994) or members of the LAR family (Kypta et al., 1996) that have extracellular domains, including immunoglobulin domains and fibronectin type III repeats, that are likely to mediate cell adhesion.
3. Cytosolic PTPs containing domains predicted to localise the phosphatase to the plasma membrane and /or interact with the actin cytoskeleton, such as the FERM domain (Marfatia et al., 1995).

3.2 Results

3.2.1 PTP homology screen of HUVEC mRNA

Although the sequences of PTPs can differ substantially, all known classical PTPs have a conserved catalytic domain, within which there are several highly conserved regions interspersed with regions of less conservation that can be used to distinguish between different PTPs. This enables the use of PCR amplification with primers to 2 highly homologous regions flanking a less conserved region to specifically isolate PTP cDNAs expressed by different cell types.

The homology screen to identify PTPs expressed by endothelial cells was carried out using cDNA reverse-transcribed from HUVEC mRNA. To enable unambiguous identification of the amplified PTP, the 2 degenerate primers (sense {(H/D)FWRM(I/V)W} antisense {WPD(F/H)GVP }) chosen for the PCR amplification flanked a region of DNA in which there is minimal sequence homology between family members. These sequences were used successfully by another group in a homology screen that identified the MAM PTP family member PTPλ (Cheng et al., 1997).

3.2.2 Analysis of the PTP clones

The clones obtained by RT-PCR of HUVEC mRNA were analysed to determine if they contained inserts and whether the inserts were PTPs. The data is summarised in Table 3.1.

Table 3.1 Summary of homology screen

Selected colonies	237
Clones containing inserts	190
Sequenced PTP clones	74
Non-specific inserts	69
Unsequenced clones	47

The majority of the selected colonies contained inserts, indicating that the blue/white colour screening for insert containing clones was effective and did not produce a high number of false positives. More than 50% of the clones that were sequenced, contained sequences of known PTP family members. The homology screen identified twelve members of the classical PTP family in HUVEC. The frequency of representation of the individual family members was quite variable. The results obtained from RT-PCR are not quantitative and are subject to bias due in part to differential levels of homology between the degenerate primers and the corresponding sequences in the individual target mRNAs. However, a comparison between the sequences of the identified PTPs and the degenerate primer sequences showed that PTPs with 100% homology to the primers were isolated in lower frequencies than other PTPs with lower homology to the primers. This indicated that the highly represented PTPs, i.e. PTPα and PTP-Pez, were not selectively amplified due to preferential binding to the PCR primers (Table 3.2).

3.2.3 Selecting a PTP to characterise

In order to select a PTP for further characterisation the identified PTPs were considered against the criteria outlined in section 3.1.1.2. There were representatives of most of the PTP subfamilies isolated in the homology screen (Table 3.2). They included PTP LAR, DEP-1and the MAM-domain family member PTP kappa, which are all known to be involved in cell-cell adhesion. PTP LAR has an extracellular domain that contains immunoglobulin domains and

Table 3.2 PTPs expressed in P1 HUVEC

			Homology to PCR Primers	
PTP Name	**No. of clones**	**Frequency**	**FWRM(I/V)W**	**WPD(F/H)GVP**
PTP-PEZ	26	35.6	FWRMVW	WPDHG*C*P
PTP α	16	21.9	FWRMIW	WPD*Y*GVP
PTP1b	9	12.3	FW*E*MVW	WPDFGVP
PTP sigma	5	6.8	FWRMVW	WPDHGVP
T-cell PTP**	5	6.8	FW*L*MVW	WPDFGVP
PTP ETA	4	5.5	FWRMVW	WPDHGVP
PTP LAR	2	2.7	FWRMVW	WPDHGVP
PTPG1	2	2.7	FWRMVW	WPDHGVP
PTP epsilon	1	1.4	FWRMVW	WPDHGVP
PTP kappa	1	1.4	FWRMIW	WPDHGVP
DEP-1	1	1.4	FWRMVW	WPDHGVP
PTPMEG2	1	1.4	FWRMVW	WPD*Y*GVP

(** One of the T-cell PTP clones (clone 72) contained a 23aa insert
NH_3...Y_{156}TVHLLQLENIN**YIENLWITLYLKLLMLDVKRSLK**SGETRTISH$_{176}$ ...COOH)

Table 3.2 PTPs expressed in a confluent passage 1 HUVEC cell line, showing the total number of clones of each PTP isolated and the frequency with which each PTP was represented. The amino acids in italics indicate variation from the degenerate PCR primer sequences used to isolate the clones.

fibronectin type III repeats. LAR localises to focal adhesions where it dephosphorylates p130(cas) (Weng et al., 1999) and also to the adherens junctions where it associates with β-catenin (Muller et al., 1999). RPTP DEP-1 has an extracellular domain containing eight fibronectin type III repeats suggesting a role in cell adhesion. The recent finding that DEP-1 forms a complex with the adherens junction protein $p120^{ctn}$ (Holsinger et al., 2002) supports this supposition. Furthermore the expression of this PTP is enhanced with increasing cell density and is thought to play a role in the contact inhibition of cell growth (Ostman et al., 1994). The extracellular domain of PTPκ also contains fibronectin type III repeats as well as a single immunoglobulin domain and an N-terminal MAM domain. The MAM domain mediates homophilic cell-cell interactions (Zondag et al., 1995). The identification of these PTPs in the homology screen indicates that they may all be participants in the regulation of cell-cell adhesion in endothelial cells. However, the aim of this project was to identify and characterise a PTP with potential to regulate cell-cell adhesion that had not been previously characterised. Therefore, the PTPs that were already the focus of active research by other groups were not characterised further.

3.2.3.1 PTP-Pez

The PTP that was most highly represented in the screen was the cytosolic PTP-Pez. PTP-Pez is a 130kDa cytosolic phosphatase with homology to the FERM family of proteins. The FERM domain was first identified in the band 4.1 protein isolated from erythrocytes. The 298 amino acid domain mediates the localisation Flag-tagged of the band 4.1 protein to the plasma membrane by binding to the cytoplasmic domains of the transmembrane proteins glycophorin A, glycophorin C, band 3 and CD44 (Jons and Drenckhahn, 1992; Marfatia et al., 1995; Nunomura et al., 1997). The FERM domain of the ezrin, radixin and moesin (ERM) family of proteins

Figure 3.1

PTP-Pez amino acid sequence

1 mpfglklrrt rrynvlsknc fvtrirllds nviectlsve stgqecleav aqrlelreth
61 yfglwflsks qqarwvelek plkkhldkfa neplffgvm fyvpnvswlq qeatryqyyl
121 qvkkdvlegr lrctldqvir laglavqadf gdynqfdsqd flreyvlfpm dlaleeavlc
181 eltqkvaqeh kahsgilpae aelmyineve rldgfgqeif pvkdnhgncv hlgiffmgif
241 vrnrigrqav iyrwndmgni thnkstilve linkeetalf htddienaky isrlfatrhk
301 fykqnkicte qsnspppirr qptwsrsslp rqqpyilppv hvqcgehyse thtsqdsifh
361 gneealycns hnsldlnyln gtvtngsvcs vdsvnslncs qsfiqaspvs snlsipgsdi
421 mradyipshr hsaiivpsyr ptpdyetvmr qmkrgilhtd sqsqslrnln iinthaynqp
481 edlvysqpem rerhpytvpy gpqgvysnkl vspsdqrnpk nnvvpskpga saishtvstp
541 elanmqlqgs hnystahmlk nylfrpppppy prprpatstp dlashrhkyv sgsspdlvtr
601 kvqlsvktfq edsspvvhqs lqevseplta tkhhgtvnkr hslevmnsmv rgmeamtlks
661 lhlpmarrnt lreqgppeeg sgshevpqlp qyhhkktfsd atmlihsses eeeeeeapes
721 vpqipmlrek meysaqlqaa laripnkppp eypgprksvs ngalrqdqas lppamararv
781 lrhgpakais msrtdppavn gaslgpsise pdltsvkerv kkepvkerpv semfsledsi
841 ieremmirnl ekqkmaglea qkrplmlaal nglsvarvsg reenrvdatr vpmderfrtl
901 leegmvf teyeqip angifstaal penaersrir evvpyeenrv eliptkennt
961 gyinashikv vvggaewhyi atqgplphtc hdfwqmvweq gvnviamvta eeeggrtksh
1021 rywpklgskh ssatygkfkv ttkfrtdsvc yattglkvkh llsgqertvw hlqytdwpdh
1081 gcpedvqgfl syleeiqsvr rhtnsmlegt knrhppivvh csagvgrtgv lilselmiyc
1141 lehnekvevp mmlrllreqr mfmiqtiaqy kfvyqvliqf lqnsrli

FERM domain

PTP domain

Potential nuclear export sequences

Potential nuclear localisation signals

Proline rich sequences

Acidic sequence

P2 peptide sequence (PezR1 Ab)

P1 peptide sequence (PezR1 Ab)

Potential CK2 sites

localise them to the plasma membrane where they connect integral membrane proteins such as CD43, CD44, ICAM-2 and ICAM-3 to the actin cytoskeleton. The FERM domain is also found in merlin, a brain tumour suppressor that is the neurofibromatosis 2-gene product. The FERM domain is located in the N-terminus of most of the FERM family proteins.

The Pez cDNA was first isolated from normal breast tissue (Smith et al., 1995) (Fig 3.1). It is also expressed in other tissues including skeletal muscle, kidney placenta and lung. There are five mammalian PTPs that carry the FERM domain, PTPH1 (Yang and Tonks, 1991), PTPMEG (Gu et al., 1991) PTPD1/PTP-RL10/rPTP2E (Moller et al., 1994; Higashitsuji et al., 1995), PTPpez/PTP36/PTPD2 (Moller et al., 1994; Sawada et al., 1994; Smith et al., 1995) and PTP-BAS/hPTP1E/PTPL1/FAP-1 (Maekawa et al., 1994; Banville et al., 1994; Saras et al., 1994). Based on their structure the FERM family of PTPs can be divided into 3 subgroups:- 1) PTPH1 and PTPMEG; 2) PTPD1 and PTP-Pez; 3) PTP-BAS (Fig 3.2). PTP-BAS is more distantly related to Pez than the other FERM domain PTP family members, with isoforms ranging from 250 to 270 kDa predicted molecular weight (MW); it is the only family member with a FERM domain in the middle of the molecule and containing five PDZ domains (Maekawa et al., 1994).

The presence of a FERM domain indicated that Pez was likely to bind to integral membrane proteins thereby localising the phosphatase to the plasma membrane. A homology screen of PTPs expressed in insulin target tissues also identified Pez as a PTP highly expressed in endothelial cells (Norris et al., 1997). However, very little was known about the role of Pez when this project commenced. Its predicted localisation at the PM indicated that this phosphatase was a good candidate for further investigation. As conventional PCR is not quantitative, PTP-Pez expression was analysed by Northern blotting in order to verify the level of expression in HUVEC.

Figure 3.2

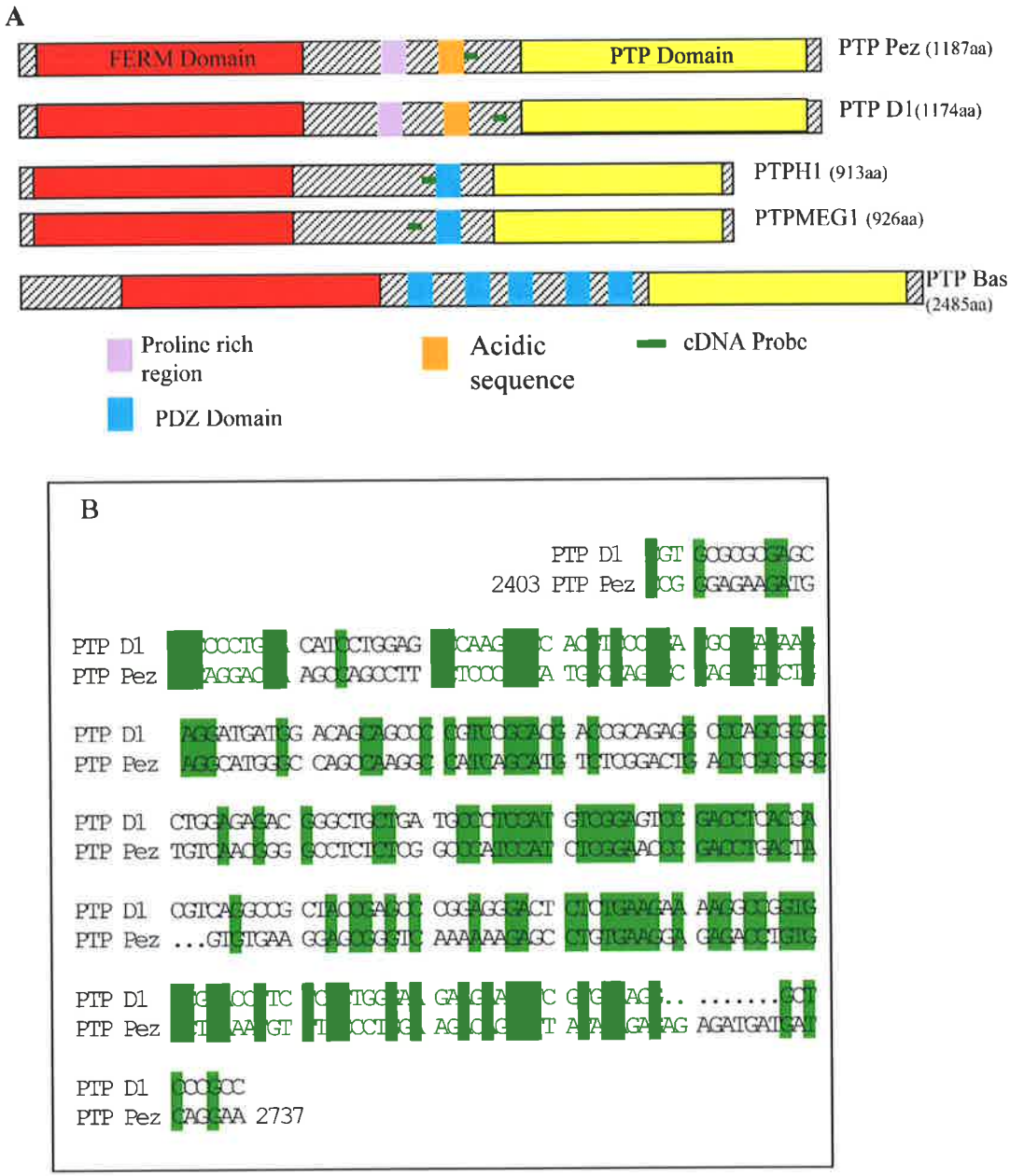


Figure 3.2 A Schematic representation of PTP-Pez and the other members of the FERM family of PTPs showing the FERM domains, the intervening sequences and the PTP domains. The green lines within the variable region (hatched) of the FERM family PTP sequences used to prepare cDNA probes for analysing mRNA expression by Northern blotting.
NCBI accession numbers: - PTP-Pez (NP_005392); PTPD1 (NP_009870); PTPH1 (P26045); PTPMEG1 (NP_002821); PTP-BAS (Q12923).
B Comparison of the cDNA probe sequence to the corresponding region in PTPD1,the FERM PTP with the highest sequence similarity to Pez.
Regions of sequence similarity are highlighted.

3.2.4 Confirmation of high PTP-Pez expression in HUVEC

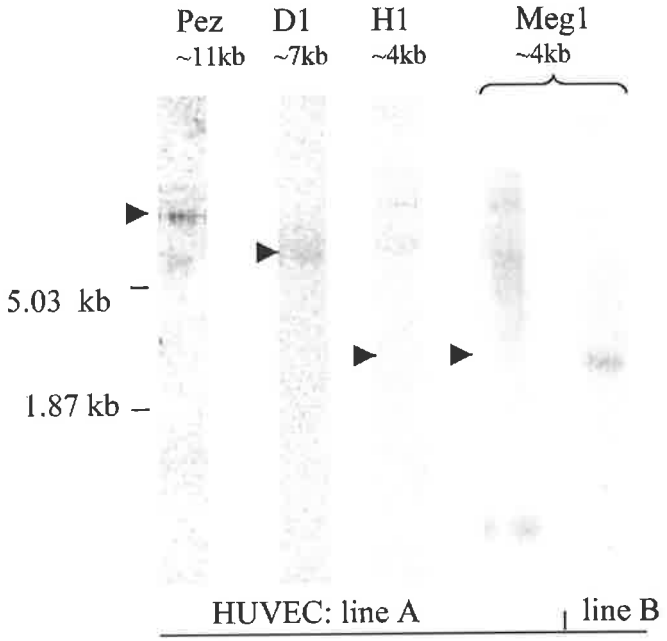
3.2.4.1 Selection of sequence as a probe for Northern analysis

The predicted amino acid sequence of PTP-Pez is shown in figure 3.1 (Smith et al., 1995). There are 5 members of the FERM family of PTPs (Fig 3.2 A). All members of the family have a single phosphatase domain and a FERM domain. The degree of sequence similarity between the family members is high in these regions, however there is lower similarity in the intervening sequences. PTP-BAS is more distantly related to Pez, with isoforms ranging from 250 to 270kDa predicted MW, it is the only family member that has a FERM domain in the central portion of the molecule and the only member that has five PDZ domains. The closest family member to Pez is PTPD1 (see section 3.3.3.1); which has 65.6 % sequence similarity to Pez at the level of mRNA. Both PTPs contain a proline-rich putative Src homology 3 (SH3) domain binding site, and an acidic sequence that may bind positively charged target sequences on other molecules. Pez and PTPD1 also share quite high overall similarity within their variable regions (Fig. 3.2 A). The location of the sequence selected for the generation of a cDNA probe is indicated on the diagram of Pez in figure 3.2.A; the cDNA sequence (bp $_{2403\text{-}2737}$) is shown in figure 3.2B compared to the same region in the PTPD1 sequence. This region was selected to maximise the probability of specific binding to Pez mRNA because it had the lowest degree of sequence identity to PTPD1 and no significant similarity to other proteins in the databases.

The number of Pez clones obtained from the homology screen (see Table 3.2) suggested that it was an abundant PTP in HUVEC. We therefore investigated the relative levels of expression of four of the FERM family PTPs using total RNA isolated from two different lines of P2 HUVEC (Fig 3.3). PTPBAS expression was not analysed in this study. The cDNA sequences (PTPD1 bp $_{2527\text{-}2901}$, PTPMeg bp $_{1014\text{-}1480}$ and PTPH1 bp$_{1076-1524}$) used to probe the northern blots

Figure 3.3

A



B

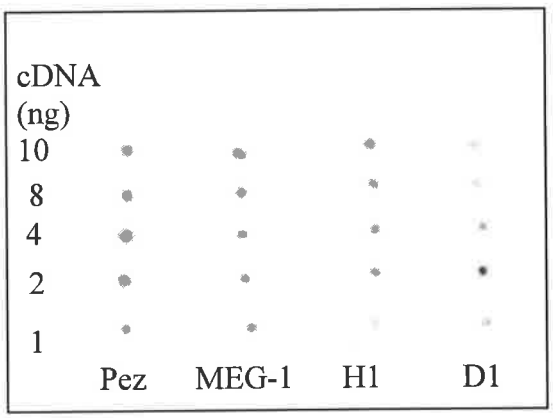


Figure 3.3 A Expression of the FERM PTPs in HUVEC. Northern blots of mRNA isolated from passage 2 HUVEC were hybridised with ^{32}P-radiolabelled probes prepared from the human PTP-Pez, PTPD1, PTPH1 and PTPMEG1 cDNA Northern blots of two separate HUVEC cell lines probed for PTP MEG are shown. The mobilities of the ribosomal RNA subunits are shown in kb. The arrowheads indicate the mobilities of the PTP mRNAs. (blots performed on mRNA from at least 2 cell lines). B Comparison of FERM PTP probes.^{32}P-labelled FERM-PTP probes were hybridised to cDNA spotted onto nylon membrane at increasing concentration from 1ng cDNA.

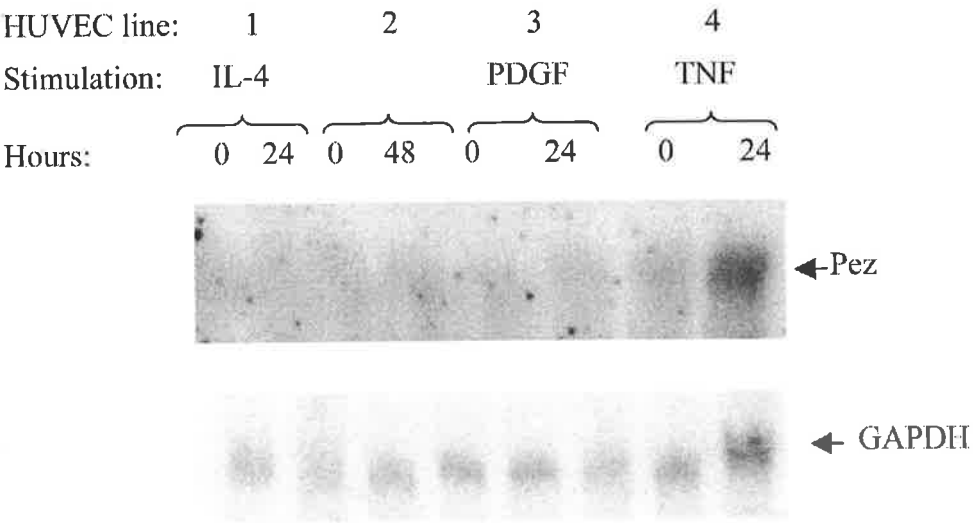
were selected from regions of lowest sequence similarity to the other family members, ie from the central, variable regions of each of the PTP family members (Fig 3.2 A). The probes all detected bands of the appropriate sizes (Fig 3.3 A) indicating that they were specifically detecting the correct mRNAs. Although PTPMEG-1 was not isolated in the homology screen and is almost undetectable in the northern blot of cell line A, it is evidently expressed at high levels in other HUVEC lines (line B). This may be due to a loss of expression of some proteins following passaging of primary cell lines. Expression of Pez mRNA was detected in all cell lines analysed. PTPD1 was also detected although the signal was weaker than PTP Pez, however this was not quantified and may have therefore have been due to loading differences. PTPH1 was not detectable by northern analysis. The variability in detection of the FERM-PTPs may have been due to the relative binding affinities of the different probes rather than a true reflection of the level of mRNA expression. To evaluate this the ^{32}P-labelled FERM-PTP probes were hybridised to cDNA spotted onto nylon membrane at increasing concentration from 1ng cDNA. All probes gave comparable signals indicating that the difference seen in mRNA expression levels is probably real and not due to variation in binding affinities (Fig 3.3B)

3.2.4.2 Analysis of regulation of PTP mRNA expression by growth factors and cytokines

Some PTPs are regulated at the level of transcription. For example, IL-6 stimulation induces the expression of the receptor-like PTP CD45 correlating with an increase in cellular proliferation in myeloma cells (Mahmoud et al., 1998). PTPε and PTP γ mRNA levels are increased in human astrocytoma cells within 4 hours of stimulation by IL-1 or TNFα (Schumann et al., 1998). We therefore investigated whether growth factors and cytokines regulated Pez mRNA expression. However, the level of PTP-Pez mRNA expression did not vary significantly following incubation of HUVEC with TNFα, PDGF-BB, IL-4, oncostatin M (OSM) or VEGF, for periods varying from 24 hours to 48 hours (Fig 3.4). It is possible that the mRNA levels may

Figure 3.4

A.



B

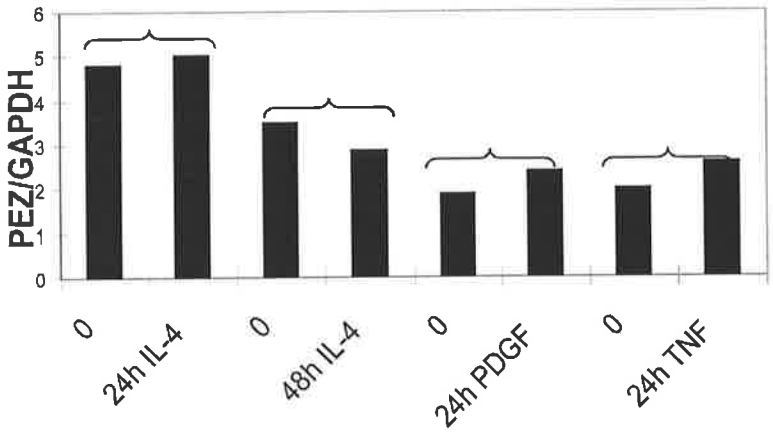


Figure 3.4 A. PTP-Pez expression in different cell lines of P1 HUVEC following stimulation with either IL-4 (10ng/ml), PDGF-BB 10ng/ml or TNF (0.5ng/ml) for the indicated times, the membrane was probed with ^{32}P radiolabelled Pez cDNA probe. The same membrane was then probed for GAPDH.**B.** Quantification of the Pez mRNA expression normalised for GAPDH expression. (The IL-4 data is representative of three experiments, PDGF and TNF data is from one experiment).

have increased and subsequently dropped to basal levels at earlier time points. Future analysis of Pez expression should include earlier time points to evaluate this possibility. The apparent increase in PEZ mRNA following stimulation of HUVEC with TNFα (Fig 3.4A) is due to overloading, since normalisation to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression shows that there is no significant difference compared to the unstimulated control (Fig 3.4B). Therefore, although there was some variation in the level of mRNA expression between cell lines, there is no evidence so far to support regulation of Pez at the level of mRNA expression in HUVEC.

Results from later experiments detailed in chapters 4-8 indicate that Pez plays a role in cell motility and cell proliferation. It would be interesting therefore, to look at Pez mRNA expression during development in an animal model. Analysis by *in situ* hybridisation may show differences in Pez expression during stages of development involving cell migration, such as gastrulation.

3.3 Conclusions

Using RT-PCR and Northern analysis the cytoplasmic PTP Pez was identified as a PTP that is highly expressed in endothelial cells at the mRNA level. This PTP was predicted to localise to the plasma membrane due to the presence of a FERM domain in its N-terminus. Unlike other PTPs identified in the homology screen, little was known about this PTP prior to the commencement of this research. Researching this PTP therefore offered the potential to increase the body of knowledge about the PTP family and the regulation of cell-cell adhesions. PTP Pez therefore represented a good candidate regulator of cell-cell adhesion and was consequently selected for further characterisation.

Chapter 4
Subcellular Localisation of PTP-Pez

Chapter 4: Subcellular localisation of PTP-Pez

4.1 Subcellular localisation of Pez

Subcellular compartmentalisation is an effective means of regulating the physiological function of a protein. Sequestering a protein to an organelle such as the nucleolus or the endoplasmic reticulum or anchoring it to another protein in the cytoplasm may result in its activation or inactivation. Mechanisms such as protein cleavage, phosphorylation or dimerisation can result in the unmasking of a signal sequence or uncoupling from a tether, culminating in the relocalisation of the protein to a different subcellular compartment.

Translocation enables the protein to interact with a different subset of cellular proteins and to participate in processes such as transactivation, cell migration or apoptosis. Examples include the facilitator of glucose transport, GLUT4, which translocates from intracellular compartments to the plasma membrane in response to insulin signalling. The pro-apoptotic Bcl-2 family members Bax and Bak translocate from the cytoplasm to the outer mitochondrial membrane where they facilitate cytochrome-c release during apoptosis (reviewed in Kaufmann and Hengartner, 2001). Calpain cleavage of the carboxy-terminal 11-kDa peptide of PTP1B in activated platelets results in activation of the enzyme and translocation from the cytosol to the plasma membrane (Frangioni et al., 1993).

The subcellular localisation of a protein provides an insight into its role within the cell and suggests possible interacting proteins and potential substrates. If Pez is a regulator of cell-cell adhesion then we would predict that it will be localised to the cell junctions perhaps via interactions with other junctional proteins. Therefore Pez-specific antibodies were generated to determine the subcellular localisation of the endogenous protein. Some of the data presented in this chapter have been published (Wadham et al., 2000).

4.2 Methods

4.2.1 Generation of antibodies

4.2.1.1 Coupling of peptides

The peptides $Pez_{495-510}$ and $Pez_{683-696}$ were cross-linked to ovalbumin (Sigma, St Louis, MO, USA) with EDCI (ethyl dimethylamino propyl carbodiimid-HCl).

Peptide coupling reaction
5 mg peptide
1.5 mg ovalbumin
15mg EDCI
MQ-water to 0.5 ml

The reaction mixture was incubated at RT for 6 hours and then stored at 4°C. The efficiency of the conjugation of the peptides to ovalbumin was verified by SDS-PAGE.

The coupled peptides were dialysed against MQ-water in a centriprep-10 centrifugal concentrator (Amicon, Mass, USA) according to the manufacturer's instructions.

The sample was added to the sample container in the centriprep concentrator and water added up to the fill line. The concentrator was then centrifuged at 3000g until the fluid levels on the outside and the inside of the filtrate collector had equilibrated (approximately 45 mins). The filtrate was decanted. Centrifugation was repeated two more times to reduce the volume of the retentate. Bradford assays carried out on the coupled peptides before and after dialysis showed that there was no significant loss of the peptides during dialysis.

4.2.1.2 Immunisation of rabbits

The coupled peptides were then sent to the IMVS Veterinary Services Division (Gilles Plains SA) where they were used to immunise rabbits. The inoculations were performed sub-cutaneously in several sites. Each immunisation comprised 300μg of peptide in a 1:1 emulsion with Freunds Complete Adjuvant. The immunisation schedule is shown in Table 4.1.

Table 4.1 Immunisation schedule

Timing	Procedure	
Week 0	Pre-bleed	2-5mls serum
Week 0	Primary Inoculation	In Freunds Complete Adjuvant
Week 3	1st Booster	In Freunds Complete Adjuvant
Week 6	2nd Booster	In Freunds Complete Adjuvant
Week 7.5	Test Bleed 1	2-5 mls serum
Week 9	3rd Booster	In Freunds Complete Adjuvant
Week 10.5	Test Bleed 2	2-5 mls serum
Week 12	4th Booster	In Freunds Complete Adjuvant
Week 13.5	Test Bleed 3	2-5 mls serum
Week 26.5	Test bleed 4	2-5 mls serum
Week 28	5th Booster	In Freunds Complete Adjuvant
Week 29.5	Bleed Out	40-80 mls serum

4.2.1.3 Assay of the test-bleeds by ELISA

Antisera generated to $Pez_{495-510}$ and $Pez_{683-696}$ were called PezR1 and PezR2, respectively. Antisera obtained against the peptides were used in an enzyme-linked immunosorbent assay (ELISA) to screen for binding to unconjugated peptide. Serial dilutions of the whole antiserum to PezR1 and PezR2 were incubated with either the specific peptide (P1), the non-specific peptide (P2) or ovalbumin bound to polyvinylchloride (PVC) 96-well plates (Costar).

A solution of 0.1μg/ml of the antigens was prepared in bicarbonate buffer (pH 9.0) (see Table 2.1). 50μl of the antigen solution was added to each well and incubated overnight at 4°C. The

content of the wells was removed by aspiration and the well washed twice with PBS. The wells were filled with blocking buffer (1%BSA, 5% sheep serum/PBS) and incubated at RT for 2 hours. The PezR1 and PezR2 antisera were added to the wells in doubling dilutions starting at ½ (50µl/well), diluted in blocking buffer and incubated for 2 hours at RT. The plate was washed twice with PBS. 50µl of 1/1000 dilution of HRP conjugated goat anti rabbit Ab in 0.5%BSA, 2.5% sheep serum /PBS was added per well and incubated at RT for 1 hour. The plate was washed 3 times in PBS. The reaction was quantitated by the addition of the freshly prepared HRP substrate Orthophenylene diamine (OPD) (Sigma) (Citrate buffer (Table 2.1), 0.1% OPD, 0.03% H2O2) 100µl/well. When the colour had developed (5-15 minutes at RT) the reaction was stopped with the addition of 50µl 1M H_2SO_4. The absorbance was read on a microplate reader (Biorad) at 490nm against a reference of 655nm.

4.2.2 Affinity purification of antibodies

PezR1 and PezR2 antisera were purified on Affi-Gel 15 peptide affinity columns.

4.2.2.1 Coupling peptides to BSA

The peptides $Pez_{495-510}$ and $Pez_{683-696}$ were first cross-linked to bovine serum albumin (BSA) with EDCI (ethyl dimethylamino propyl carbodiimid-HCl). Five mg of Peptides $Pez_{495-510}$ and $Pez_{683-696}$ were added to 1.5mg of BSA and 15 mg of EDCI and dissolved in 0.5ml of water. The coupling reaction was incubated overnight at room temperature.

4.2.2.2 Dialysis

The coupled reaction was dialysed against water using a Centriprep 10 column (Amicon).

Water was added to the 0.5-ml reaction mixture to the fill-line on the column (15 ml). The column was centrifuged at 2000rpm for 45 minutes. This was repeated two more times.

4.2.2.3 Coupling peptides-BSA to affigel

The dialysed peptide-coupled BSA was added to 1ml of Affi-Gel 15 (Bio-Rad Labs, Hercules, CA, USA) matrix, which had been washed in cold MQ water and suspended in 100mM Mops buffer, pH 7.5 (total volume 10 mls). The coupling mixture was rocked on a Clay Adams Nutator mixer (Becton Dickinson NJ USA) overnight at 4°C.

1. The peptide/BSA coupled Affigel-15 mixture (1-ml) was poured into a 10-ml chromatography column (BioRad). The column was washed with 10ml 10mM Tris pH 7.5 followed by 10 ml 100mM glycine pH2.5 and 10ml 10mM Tris pH 8.8. When the column was equilibrated at pH 8.8 10 ml of freshly prepared TEA pH 11.5 was added followed by 10 ml 10mM Tris pH7.5.
2. 2.5 mls of rabbit serum was clarified by centrifugation and diluted 1/10 with 10mM Tris pH7.5. The diluted serum was loaded onto the column and passed through 3x to ensure maximum binding of the antibodies.
3. The column was washed with 20 ml 10 mM Tris pH 7.5 followed by 20 ml 0.5M NaCl, 10mM Tris pH 7.5
4. The bound antibody was eluted with 10ml 100mM Glycine pH 2.5. The eluate was collected in two 5 ml fractions in tubes containing 0.5 ml 1M Tris pH 8.0.
5. 0.1mg/ml of BSA was added to the purified antibodies and stored at 4°C.
6. The eluate was assayed by Western blotting
7. The remaining eluates were dialysed against PBS using Centriprep 10 filtration columns. The volume of the final filtrates was ½ of the original elution volume.

8. The purified antibodies were then stored in 300µl aliquots at -70°C. The antibodies were used at 1:100 or 1:50 for Western blotting for and 1:10 for immunofluorescence.

4.3 Results

4.3.1 Generation of antibodies against PTP-Pez peptides

Two peptides predicted to be immunogenic by the algorithm 'Antigenic' (GCG, University of Wisconsin, USA), located in the variable region of Pez (Figure 3.1), were synthesised. The peptides, PYTVPYGPQGVYSNKLVSPS (P2) corresponding to amino acids (aa) 495-510 (Pez $_{495\text{-}510}$) and SHEVPQLPQYHHKK (P1) corresponding to aa 683-696 (Pez $_{683\text{-}696}$), do not exhibit significant homology to other proteins in the protein databases, including other members of the FERM-PTPs, suggesting that antibodies (Abs) raised against them would be specific to Pez.

4.3.2 Characterisation of the antisera against Pez

4.3.2.1 The antisera bind specifically to the peptides used for immunisation

The titre of antibodies generated against each of the specific peptides was assayed by ELISA and compared to binding of the antiserum to the non-specific peptides (ie. the Pez peptide not used in the specific inoculation). Binding to the coupling protein ovalbumin was also assayed (Fig 4.1).

In Test bleed 1, specific binding of the R1 antibody to its specific peptide was obtained at 1:400 dilution whereas R2 binding to its specific peptide ws only evident at 1:100 dilution. Binding of the two antisera to their respective non-specific peptides and to ovalbumin was negligible (Fig 4.1a). The standard immunisation protocol involves only three booster inoculations followed by the bleed out at 10.5 weeks. In an attempt to improve the titre of the Pez specific antibodies in the two antisera the rabbits were given two additional booster-inoculations at three-week intervals, each followed by further test bleeds (Table 4.1). The fourth booster inoculation (test bleed 3) did not

Figure 4.1

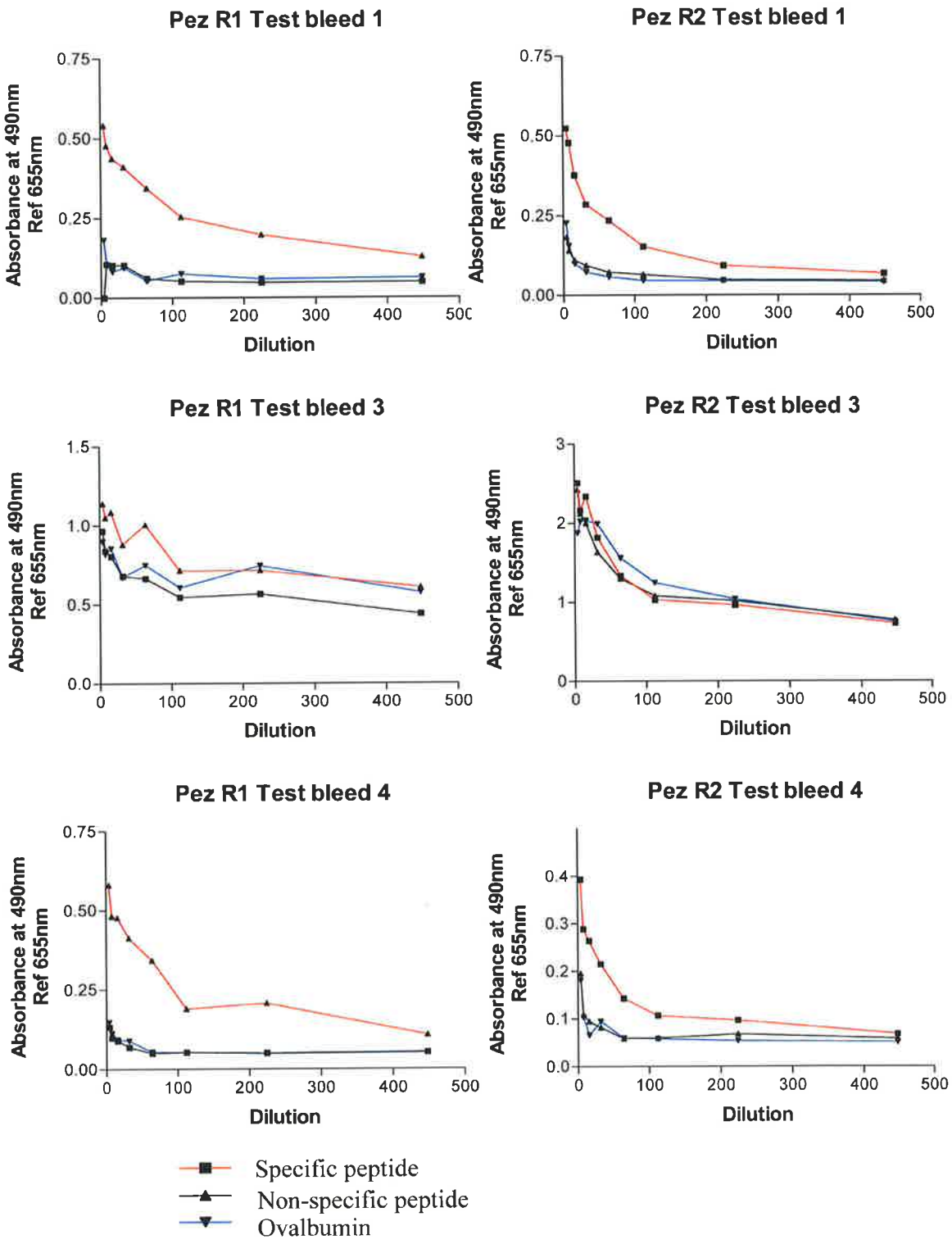


Figure 4.1 ELISA assays of test-bleeds of whole antiserum to PezR1 and PezR2. Serial dilutions of the antisera were incubated with either the specific peptide, non-specific peptide or ovalbumin bound to vinyl
96-well plates (Costar).

significantly improve the titre of either antibody and had the adverse effect of increasing the titre of antibodies raised against ovalbumin (Fig 4.1b). Non-specific binding was also increased in this test bleed as seen by the increased binding to the non-specific peptides. The unusually high background indicated that the rabbits may be stressed and it was decided to rest the rabbits for several weeks to try to improve the specificity of the antisera. Another test bleed 13 weeks later (Fig 4.1c) showed an improvement in the specificity in comparison to test bleed 3 (Fig 4.1b). A final booster inoculation was given at 28 weeks followed by bleeding out of the rabbits at 29.5 weeks. In retrospect, it would have been better to adhere to the standard immunisation protocol, as the additional booster inoculations did not improve the titre of Pez specific antibodies.

4.3.2.2 The antisera bind to the whole Pez protein.

Having demonstrated that the antiserum could specifically detect the relevant peptides used for immunisation we investigated whether they could detect the whole protein either in its denatured or native form by Western blot analysis and by immunoprecipitation. Although each antiserum detected a number of bands in HUVEC lysate, both antisera detected a common band of 130kDa, the correct MW for endogenous Pez (Smith et al., 1995) (Fig. 4.2). The band migrated slower than the 130kDa pre-stained MW standard run on 8% SDS-PAGE. The covalently coupled chromophore in the prestained markers affects protein mobility and this has been calibrated by the manufacturer against unstained markers on a 4-20% acrylamide gel. The migration of the markers on an 8% gel is therefore not precise and has been used as a guide only. However, the size of the protein was verified by comparing its relative migration with that of another 130kDa protein, platelet endothelial cell adhesion molecule-1 (PECAM).

Binding to the 130kDa band was blocked following pre-incubation of the antibodies with the relevant specific immunising peptide, but binding to the other bands was unaffected (Fig. 4.3). Incubation with the non-specific peptide did not block binding of either antiserum to the

Figure 4.2

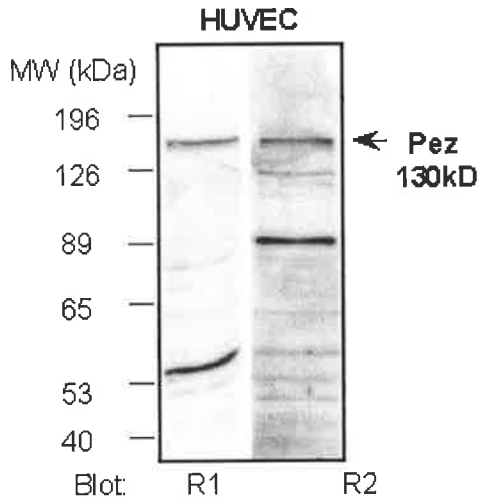


Figure 4.2 Antisera raised against the two Pez-specific peptide sequences both recognise a 130-kDa protein in HUVEC lysates. Western blot of whole cell HUVEC lysate blotted with PezR1 (left) or PezR2 (right) antisera. The migration of the prestained markers (MW) is shown. Each antibody recognises two major bands of which only the 130kDa Pez band is common.

Figure 4.3

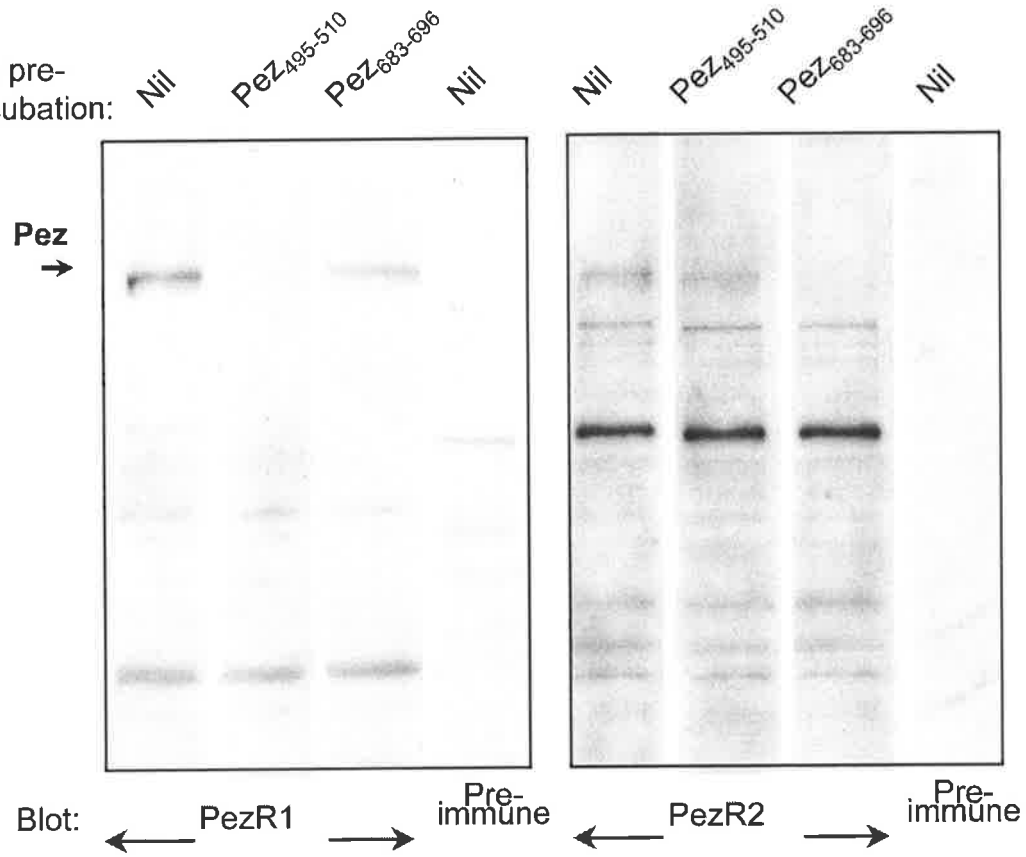


Figure 4.3 Western blot of HUVEC lysate with PezR1 (left) or PezR2 (right) antiserum that was either untreated (Nil), preincubated with the specific immunising peptide (Pez $_{495-510}$ for PezR1, Pez $_{683-696}$ for PezR2) or the non-specific peptide (Pez $_{683-696}$ for PezR1, Pez $_{495-510}$ for PezR2), showing that the immunising peptide specifically blocks the Pez band. The right lane in each panel was blotted with the appropriate pre-immune serum.

130kDa band providing further evidence that this band is Pez. In order to analyse the subcellular localisation of Pez it was necessary to affinity purify the antibodies to remove the antibodies binding to the non-specific bands seen by Western analysis (Fig 4.2). The presence of the non-specific antibodies would be likely to increase background staining in immunofluorescent analysis thereby preventing the determination of Pez localisation. Affinity purification resulted in the PezR1 antibody recognising only the 130kDa band. The affinity purified R2 antibody detected the 130kDa band as well as a minor non-specific low-molecular weight band (Fig 4.4)

Ectopically expressed Pez also migrated as a 130 kDa band (Fig. 4.5, lanes 1-3) providing further evidence that the 130kDa band is Pez. Ectopically expressed Pez tagged with the Flag epitope was immunoprecipitated from a HEK293 cell lysate with the PezR1 antiserum and then Western blotted with an anti-flag antibody. A 130kDa protein was detected. This demonstrates that the Pez antiserum can detect Pez in its native form and provides further evidence that the 130kDa protein is Pez.

4.3.3 Determination of the subcellular localisation of Pez

4.3.3.1 Pez subcellular localisation is cell-density dependent

Having established that the two Pez antisera were specifically binding to Pez in both its native and its denatured form and having successfully affinity-purified the Pez-specific antibodies we then had a tool that we could use to determine the subcellular localisation of Pez.

Primary HUVEC monolayers were incubated with the affinity purified Pez antibodies and then examined by indirect immunofluorescence and confocal laser microscopy. When the cells were confluent, staining was predominantly cytoplasmic (Fig 4.6A). There was also staining of the cell junctions, as predicted by the presence of the FERM domain (Fig 4.6 A). However, when plated at low confluence such that the cells did not form extensive cell-cell contact, the Pez staining was mainly in the nucleus (Fig 4.6 B). The two antibodies both produced the same staining pattern. Pre-immune serum did not stain and pre-incubation of the two antibodies with

Figure 4.4

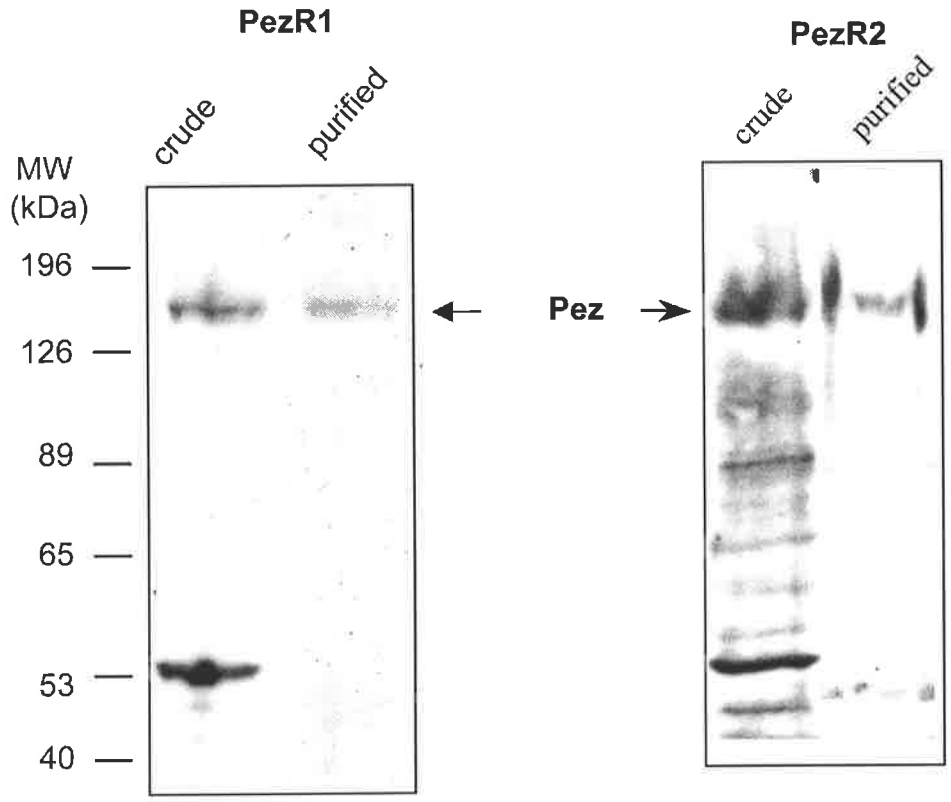


Figure 4.4 Western blots of total HUVEC lysate comparing crude serum and affinity-purified PezR1 and PezR2 Abs. Whole antiserum to PezR1 and PezR2 were purified using peptide affinity columns generated by coupling the peptides Pez495-510 and Pez683-696 cross-linked to BSA to Affigel-15 (Bio-Rad Labs, Hercules, CA, USA) matrix,

Figure 4.5

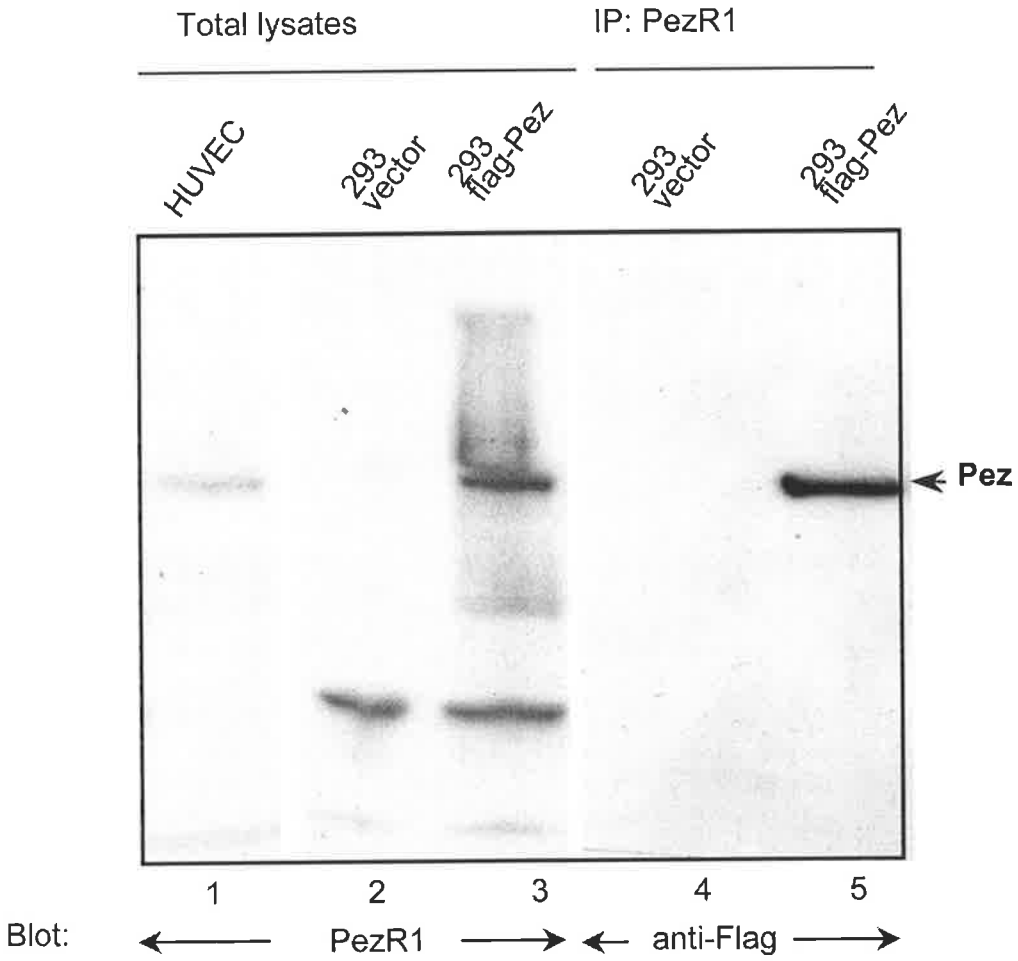


Figure 4.5 Ectopically expressed Pez co-migrates with endogenous Pez Lanes 1-3: western blot of lysates from HUVEC or HEK293 cells transfected with empty vector or flag-tagged Pez using the PezR1 antiserum. Ectopically expressed Pez (Lane 3) co-migrating with endogenous Pez from HUVEC (Lane 1). Lanes 4 and 5: immunoprecipitation using the PezR1 antiserum was carried out using lysates of 293 cells transfected either with empty vector or flag-tagged Pez followed by western blotting with the anti-flag epitope Ab.

Figure 4.6

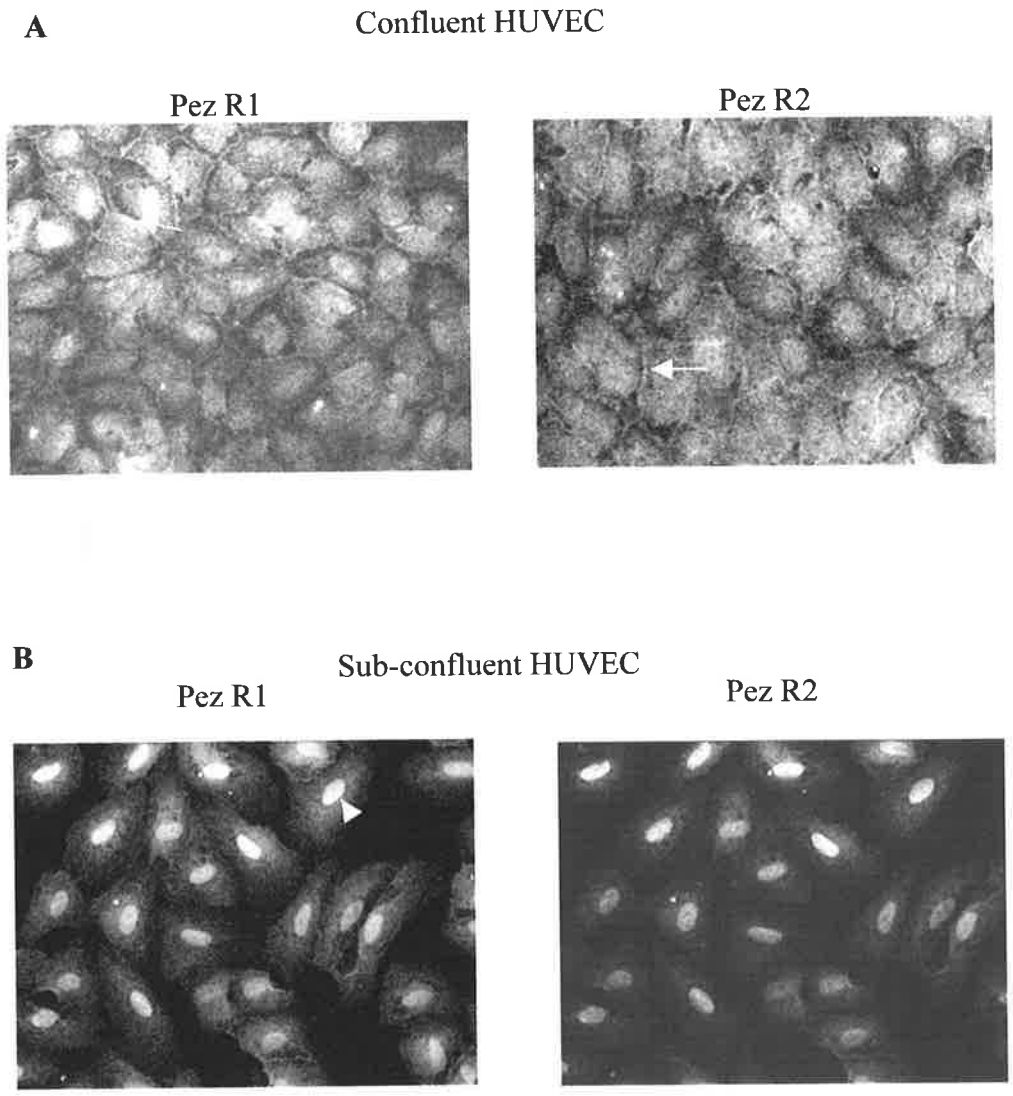


Figure 4.6 Confocal micrographs showing indirect immunofluorescence of HUVEC plated as **A** confluent (9×10^4 cells/well) and **B** sparse (2.5×10^4 cells/well) monolayers stained with either the affinity-purified PezR1 or PezR2 Abs as indicated. The staining with both Abs is predominantly diffuse cytoplasmic with a higher concentration at the intercellular junctions (arrows in A) in the confluent monolayers and nuclear in sparse isolated cells (arrowhead in B). The images are representative of at least three experiments.

their specific peptides resulted in greatly reduced staining (data not shown). The identical pattern of staining observed with the two affinity- purified antibodies supports the conclusion that Pez is localising to different subcellular compartments in a density dependent manner.

Other cell lines were also examined by indirect immunofluorescence using either confocal or epifluorescent microscopy for localisation of endogenous Pez. Endogenous Pez was detectable in the nucleus of HeLa and A431 cells (Wadham et al., 2003) but was not detectable in the other cell lines examined: HEK293, MDCK NIH3T3. Pez may not be detectable in the latter two cell lines, which are canine and murine respectively, either due to lack of expression or to a lack of cross species reactivity of the Ab. Lack of expression in the other human cell lines may be due to cell-type specific expression. Northern analysis of whole tissues (Smith et al., 1995) showed differential expression of Pez in a tissue specific manner, with transcripts undetectable in brain and liver.

HEK293 cells are derived from embryonic kidney cells and although expression of Pez mRNA in kidney was high (Smith et al., 1995) Pez expression was not detectable in HEK293. Lack of expression in HEK293 cells could be due to a loss of expression as a result of immortalisation and culturing. Alternatively Pez may be expressed in only some of the cell types in kidney but not in the cell type from which the HEK293 cell line was derived. It is also possible that Pez is subject to developmentally regulated expression resulting in a difference in embryonal and adult expression. Analysis of the murine homologue of Pez, PTP36, in the developing embryo would be necessary to investigate this possibility.

The nuclear localisation of Pez was unexpected and implied a more complex role for Pez than at first envisaged. To verify the nuclear localisation of Pez, partially confluent monolayers of HUVE cells, in which Pez can be seen to be localising to both the cytoplasm and the nucleus, were fractionated into nuclear and cytosolic extracts which were analysed by Western blotting

with the PezR1 Ab. A 130-kDa band was detected in both the cytosolic and the nuclear fractions (Fig.4.7). Counter blotting with an antibody to the cytosoplasmic MAPK (mitogen activated

Figure 4.7

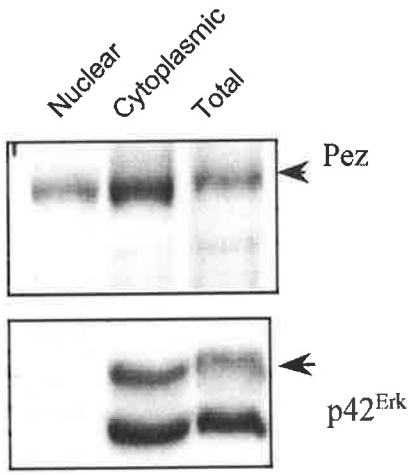


Figure 4.7. Western blot of total HUVEC lysate and after subcellular fractionation into crude nuclear and cytoplasmic extracts, using the PezR1 antiserum and the anti-MAPK Ab. Pez was present in both the nuclear and cytoplasmic fractions whereas the MAPK was detectable only in the cytoplasmic fraction (representative blot from at least three experiments).

protein kinase) proteins, $p44^{Erk}$ and $p42^{Erk}$ showed no contamination of the nuclear fraction by cytosolic proteins. These data confirm that Pez does indeed localise to the nucleus.

4.3.3.2 Nuclear localisation of Pez is serum dependent

When adherent cells in culture are sub-confluent they will continue to proliferate until confluent, provided they are maintained under optimal culture conditions. The density dependent change in subcellular localisation raised the possibility that Pez was localising to the nucleus in proliferating cells and to the cytoplasm and the cell-cell junctions in quiescent cells.

HUVEC plated at low density were grown in reduced serum (0.2% FCS, 48 hours) to induce quiescence and the localisation of Pez examined thereafter. By indirect immunofluorescence, nuclear Pez was significantly reduced in cells made quiescent by serum starvation but was increased following refeeding with 20%FCS for 30 minutes (Figure 4.8 A). To quantitate the amount of Pez in the nucleus following serum starvation and refeeding, nuclear and cytoplasmic fractions of cell lysates were examined for Pez content by Western blotting and fluorimaging (Figure 4.8 B). In cells treated with cycloheximide, 10μg/ml cycloheximide was added 15 minutes prior to refeeding with serum and left in for the entire duration of refeeding (30 minutes). From this analysis, only about 10% of total Pez remained in the nucleus after serum starvation. However, approximately 40-50% of total Pez was back in the nucleus as rapidly as 30 minutes after refeeding with serum, (Fig.4.8 B, C), indicating that Pez is translocated back into the nucleus when quiescent cells (at G_0) are stimulated to proliferate upon the addition of serum. The percentage of nuclear Pez in refed cells compared to serum starved cells was significantly different ($P<0.01$, Student's t-test, $n=2$). The increase in nuclear Pez in this short period of refeeding was unaffected by the presence of the protein synthesis inhibitor, cycloheximide, during refeeding (CH)($P=0.44$, Students t-test, $n=2$) suggesting that the increase in nuclear Pez can occur in the absence of de novo protein synthesis (Fig 4.8, C). In contrast to Pez, there was

Figure 4.8

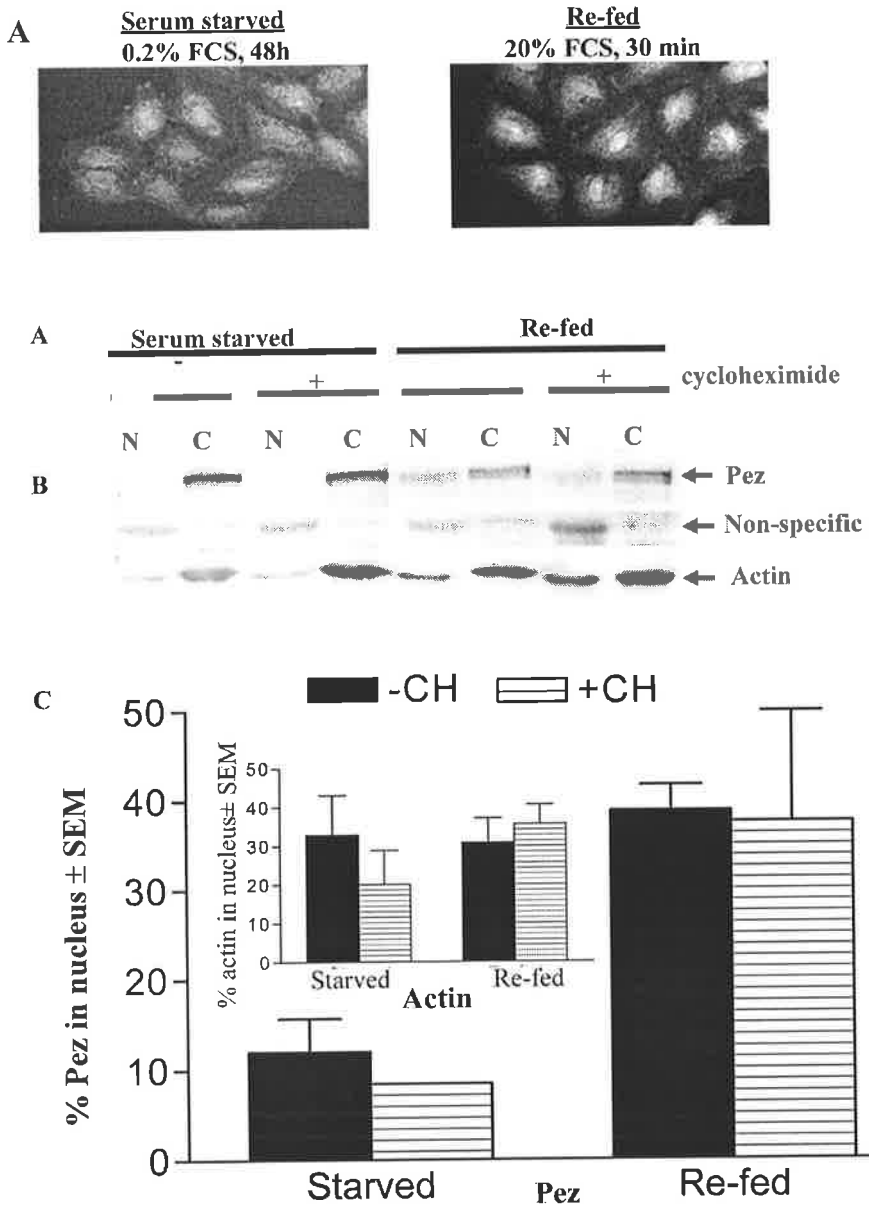


Figure 4.8 Refeeding serum-starved cells with serum increases the fraction of Pez in the nucleus. A Confocal micrographs of cells stained with PezR1 Ab showing loss of nuclear Pez in cells serum starved (0.2% FCS) for 48 hours and increased nuclear Pez following refeeding with 20% FCS for 30 minutes. The figures are representative of three separate experiments, each done in duplicate. **B** A representative western blot detected with the PezR1 Ab (top and middle panels) and reblotted with anti-actin Ab (middle panel) used for quantitating the proportion of Pez in the nucleus. Serum starved or refed cells (as in A) were fractionated into nuclear (N) and cytoplasmic(C) fractions. **C** Quantitation of western blots carried out as described in B using a fluorimager and the Imagequant software.

no significant alteration in the proportion of actin in the nucleus when serum was added back to the cells and this was similarly unaffected by cycloheximide treatment (Fig 4.8 B, C, inset). No significant difference was observed in the percentage of nuclear Pez between refeeding in the presence or absence of cycloheximide Interestingly, the nuclear localisation of the non-specific band also recognised by the crude PezR1 antisera did not alter with serum starvation (Fig. 4.8 B, non-specific) indicating that not all proteins translocate from the nucleus to the cytoplasm when cells become quiescent. The presence of this nonspecific band in the nucleus even after serum-starvation could account for some of the residual nuclear staining detected by immunofluorescence in Fig.4.8A. We have also found that there is no significant alteration in the amount of total Pez in the cells after 30 minutes refeeding with serum.

4.3.3.3 Pez translocates into the nucleus in cells at a 'wound edge': Inhibition by TGFβ.

At sites of vascular injury and during angiogenesis, endothelial cells migrate out of the intact confluent endothelium and proliferate. To model these effects *in vitro*, a confluent monolayer of HUVEC was scraped to generate a linear region denuded of cells mimicking a wound. The cells from the confluent area subsequently migrate into a denuded area and proliferate. Pez localises to the nucleus of cells that have recently migrated into the denuded area, whereas, Pez is cytoplasmic in cells remaining in the confluent area (Fig 4.9 A, B). This finding supports the regulated translocation of Pez in response to an alteration in cell density.

The cells at the wound edge are both migratory and proliferative. Nuclear Pez may therefore have a role in either migration or proliferation or both. To differentiate between these two roles the endothelial cells were treated with TGFβ at the time of wounding. TGFβ is a potent inhibitor of endothelial cell proliferation (Antonelli-Orlidge et al., 1989; Sato and Rifkin, 1989), arresting the cell-cycle progression in G_1 (Iavarone and Massague, 1997). Cell migration however, is unaffected by TGFβ. Following TGFβ treatment of HUVEC, cells at the wound edge

Figure 4.9

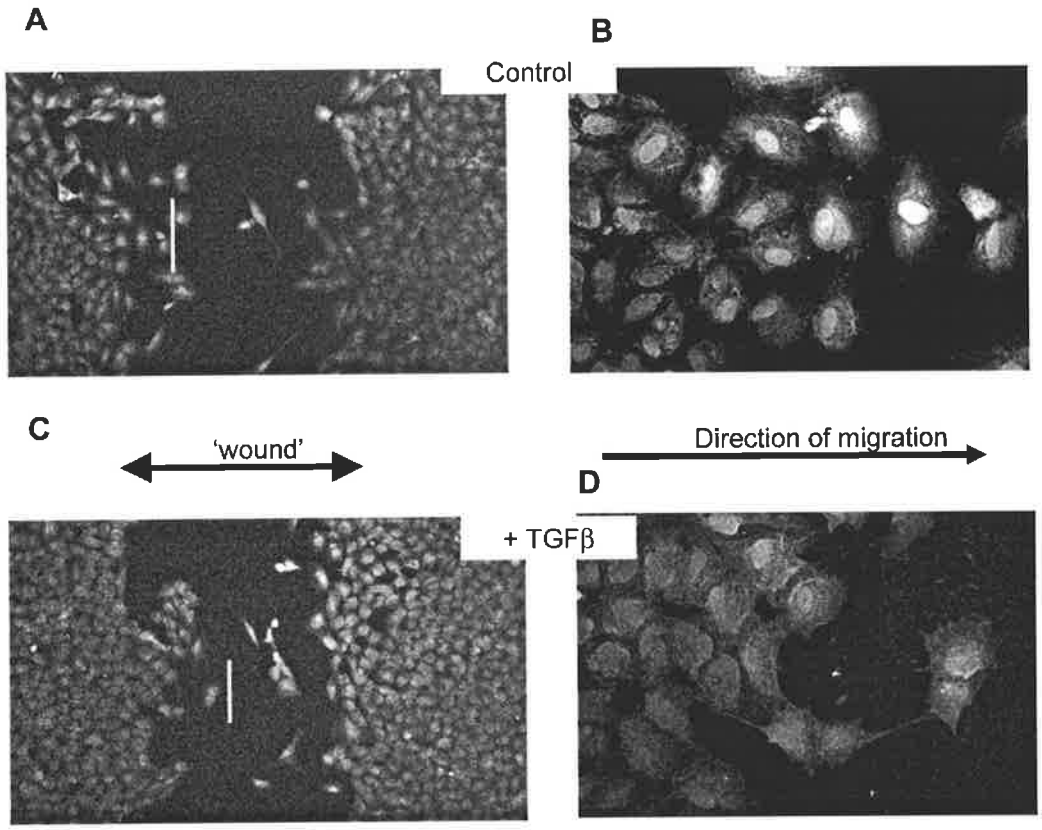


Figure 4.9 TGFβ inhibits translocation of Pez into the nucleus in a 'wound' assay. Confocal microscopy of a confluent monolayer of HUVEC through which a 'wound' was made by denuding the monolayer followed by staining with PezR1 antiserum 17 – 24 hours after wounding. (A,B) Controls, no addition. (C,D) TGFβ (0.4) ng/ml) was added at the time the wound was made. (A and C) show the wound into which a similar degree of cellular migration has occurred in both control and TGFβ cells. (B and D) are higher magnifications of the regions marked with the vertical line in A and C, respectively, showing translocation of Pez into the nucleus in the control but not TGFβ- treated cells that have migrated into the wound. The figures are representative of three separate experiments each done in duplicate.

showed little or no increase in nuclear Pez compared to the cells in the confluent region distal from the wound (Fig 4.9 C, D). This suggests that TGFβ inhibited the translocation of Pez into the nucleus. There was no difference in the distance migrated or the number of cells migrating into the wound between untreated and TGFβ-treated cells, confirming that TGFβ does not inhibit cell migration (Fig 4.9A,C). These data further support the earlier observations that the translocation of Pez into the nucleus correlates with the transition from quiescence to proliferation.

4.3.4 Mechanism of nuclear localisation of Pez

4.3.4.1 Background

Molecules larger than 40 kDa translocate into the nucleus via signal-mediated transport through the nuclear pore complex (NPC). The NPC is a large structure, spanning the nuclear envelope. Signal mediated transport is an active process, which generally requires energy and a nuclear localisation signal on the protein undergoing transportation.

There are two kinds of nuclear localisation signals (NLS) that have been well characterised, the classical NLS and the bipartite NLS. The classical NLS, first described in the Simian Virus 40 large T-antigen (SV40-T) (Kalderon et al., 1984), contains a stretch of basic amino acids, whereas the bipartite NLS (eg nucleoplasmin) (Dingwall et al., 1989; Robbins et al., 1991) is composed of two stretches of basic amino acids separated by a 10 amino acid spacer.

Other bipartite signals have since been identified which include spacers of variable length eg. several PS-IAA4-like proteins from *Arabidopsis thaliana* have a bipartite NLS with a spacer-length polymorphism, X (24- 70), which still targets to the nucleus (Luo and Shibuya, 2001). More than 50% of nuclear proteins have sequences, which match these consensus sequences. Other proteins contain unconventional NLS sequences which have only a few basic and even some acidic amino acids eg c-Myc (Makkerh et al., 1996). The low degree of sequence homology between these non-classical NLS sequences makes identification difficult based on amino acid

sequence alone. A protein carrying a classical or a bipartite NLS binds to a cytoplasmic receptor. There are two receptor subunits: importin-α (Gorlich et al., 1994), which recognises the NLS, and importin β, which targets the complex to the nuclear pore (Enenkel et al., 1995; Moroianu and Blobel, 1995; Moroianu et al., 1995; Rexach and Blobel, 1995) via the GTPase Ran (Melchior et al., 1993; Moore and Blobel, 1993)

Export from the nucleus occurs via a similar process (reviewed in Moroianu, 1999)A nuclear export sequence (NES) located on the protein undergoing translocation is recognised by a soluble nuclear export receptor (Stade et al., 1997). The receptor, exportin, will only bind to its cargo in the presence of Ran GTP. The complex then translocates into the cytoplasm. The best-characterised NES is a sequence rich in leucine (Meyer and Malim, 1994), which binds to the exportin Crm 1 (Fornerod et al., 1997; Stade et al., 1997).

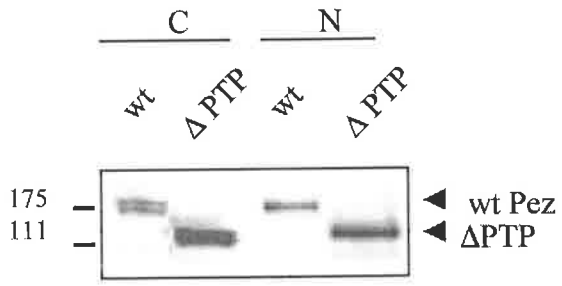
4.3.4.2 Deletion of the FERM domain reduces nuclear accumulation of Ectopic Pez

There are several sequences within Pez that are possible nuclear localisation and export signals (Figure 3.1). Deletion analysis is required to determine the sequences necessary for nuclear translocation. In order to ascertain which of the Pez domains were required for nuclear localisation we made deletion mutants of Pez that lacked either the FERM domain (ΔFERM) or the PTP domain (ΔPTP). Deletion of the PTP domain had no effect on Pez subcellular localisation (Fig.4.10A), however, deletion of the FERM domain resulted in a reduction in the fraction of the truncated protein localising to the nucleus compared to full length Pez (Fig. 4.10 B). Some residual ΔFERM Pez is detectable in the nuclear fraction following longer exposure of the membrane (Fig 4.10B RH panel).

A more detailed deletion or mutational analysis would be required to determine the location of the nuclear localisation sequence and other sequences involved in the subcellular

Figure 4.10

A



B

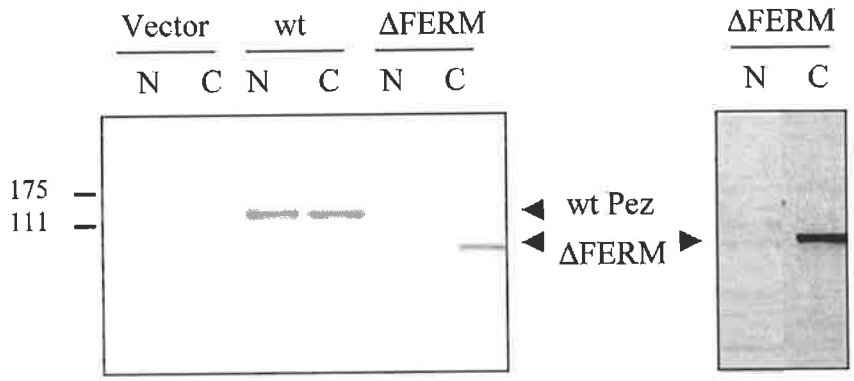


Figure 4.10 Deletion of the FERM domain decreases nuclear localisation. A and B Western blots of HEK293 nuclear (N) and cytoplasmic (C) lysates obtained after subcellular fractionation from cells transfected with either empty vector, wild-type (wt) flag-tagged Pez, C-terminal deletion mutant (ΔPTP) or an N-terminal deletion mutant (ΔFERM). Anti-Flag Ab was used to detect ectopically-expressed Pez (representative blot from at least three separate experiments).

localisation of Pez. Unfortunately time constraints did not allow for this during the course of my candidature.

4.3.4.3 Serine threonine phosphorylation regulates Pez translocation

The manner in which the subcellular localisation of Pez alters in response to the proliferative state of the cell (sections 4.3.3.1 and 4.3.3.2) indicates that translocation is a regulated process. Regulation could occur by several different mechanisms. Pez could be modified so that it is no longer able to bind the import or the export receptors. Alternatively, it may bind to an anchor protein in the cytoplasm or the nucleus, thereby blocking its access to the nuclear pore complex. Phosphorylation is a well-documented mechanism in the regulation of protein shuttling between the nucleus and the cytoplasm; see section 4.4 for discussion.

A small but reproducible shift in the electrophoretic mobility of Pez was detected on high-resolution polyacrylamide gels. This mobility shift could be due to an increased molecular weight resulting from phosphorylation. The higher molecular weight form was mainly found in the cytosolic fraction (Fig 4.11A), whereas the nuclear fraction contained predominantly the lower MW form. This observation is consistent with the finding that PTP36, the murine homologue of Pez, is serine/threonine phosphorylated in a cell-substrate adhesion dependent manner (Ogata et al., 1999 b). PTP36 is rapidly dephosphorylated following detachment from the extracellular matrix and re-phosphorylated upon reattachment to fibronectin.

To determine the effect of phosphorylation on the subcellular localisation of Pez, a confluent monolayer of HUVEC was serum-starved in 5% serum for 48 hours to induce quiescence. Following serum starvation there was little Pez remaining in the nuclei as determined by indirect immunofluorescence (Figure 4.11 B (a)). The cells were then treated for two hours with 70nM staurosporine, an inhibitor of serine/threonine protein kinases. Following staurosporine treatment there was a marked increase in nuclear Pez (Fig. 4.11B (b)), suggesting

Figure 4.11

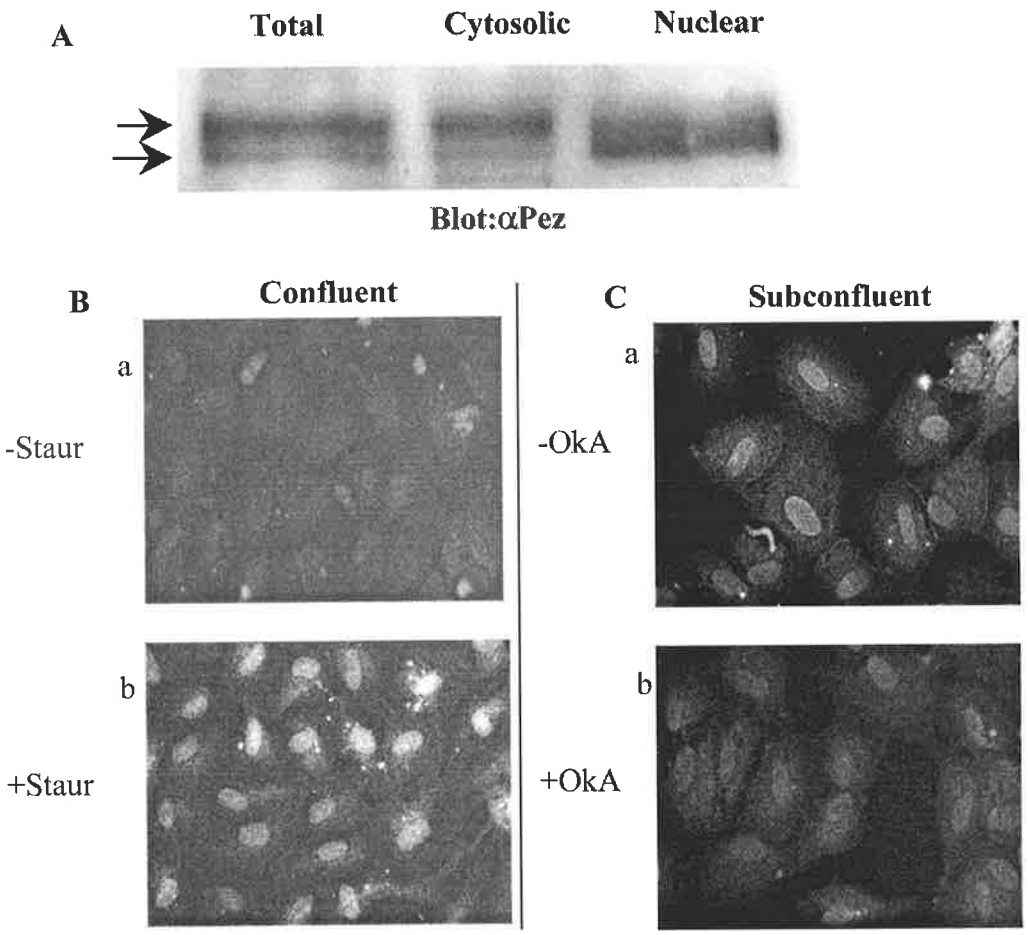


Figure 4.11 Subcellular localisation of PTP-Pez may be regulated by serine threonine phosphorylation. A. Western blot of HUVEC whole cell lysate, cytosolic and nuclear fractions immunoblotted with Pez R1 antiserum. The Pez band migrates as high and low MW forms indicated by the two arrows. **B, C**. Immunofluorescence microscopy using the PezR1 Ab. Confluent HUVEC (B) were serum starved in 5% FCS for 48 hours and treated with either DMSO (a, -St) or protein kinase inhibitor staurosporine (70nM) for 2 hours (b, +St). Subconfluent HUVEC (C) were treated with either DMSO (a +OkA) or the serine/threonine phosphatase inhibitor, okadaic acid (0.2μM)(b +OkA) for 1 hour. After fixation and permeabilisation, Pez was labeled with Pez R1 serum and detected by indirect immunofluorescence using biotinylated anti rabbit antibody followed by FITC-conjugated streptavidin.

that dephosphorylation resulted in nuclear translocation. The relative intensity of the fluorescence in (b) compared to (a) suggests that there is also an increase in the total Pez protein concentration following staurosporine treatment. However, the consistent finding that Pez does not normally localise to the nucleus in confluent cells supports the hypothesis that existing and newly synthesised Pez are localising to the nucleus due to dephosphorylation. To verify this finding, subconfluent HUVEC, which showed strong nuclear localisation (Fig 4.11C (a)), were treated for one hour with 0.2μM okadaic acid (OkA), an inhibitor of serine/threonine protein phosphatases. These data support a model of Pez nuclear cytoplasmic shuttling in which quiescence induces serine/threonine phosphorylation of Pez thereby shifting the equilibrium away from the nucleus. Following OkA treatment Pez had moved out of the nucleus into the cytoplasm (Fig4.11C (b)). localisation. Stimulation of the cells with serum or by culturing at low-density, results in dephosphorylation of Pez, allowing its accumulation in the nucleus.

4.4 Conclusions

The generation of specific antibodies against the endogenous Pez protein has allowed us to determine its subcellular localisation. The predicted targeting of Pez to the plasma membrane due to the presence of an N-terminal FERM domain is supported by immunofluorescence analysis. These data show that Pez is to be found at the cell-cell contacts in confluent cells, suggesting that Pez may be involved in the regulation of cell-cell adhesion. This aspect of Pez function is investigated further in chapters 5-8.

The regulated translocation of Pez between the cytoplasm and the nucleus indicates that Pez has multiple functions within the cell. This pattern of localisation has been demonstrated for other cell-cell adhesion proteins. The Y-box transcription factor, ZONAB (ZO-1-associated nucleic acid-binding protein), binds to the SH3 domain of the tight junction protein ZO-1 at the

cell membrane. ZONAB and ZO-1 relocalise to the nucleus at low cell density and interact in the regulation of the ErbB2 promoter (Balda and Matter, 2000).

Another example is β-catenin, a component of the adherens junction complex that links the transmembrane protein E-cadherin to the actin cytoskeleton. In the absence of a Wnt-signal, cytosolic β-catenin is rapidly targeted for degradation via the proteosome pathway. However, in the presence of a Wnt growth factor signal, β-catenin accumulates in the cytoplasm due to inactivation of the complex that targets it for degradation. Under these conditions, β-catenin is able to translocate into the nucleus where it interacts with the high mobility group transcriptional activator, lymphocyte enhancer binding factor (LEF)/T-cell factor, and directly regulates gene expression (reviewed in Wodarz and Nusse, 1998).

Possible target proteins in the nucleus may be transcription factors or cell-cycle regulators. TC-PTP mRNA fluctuates in a cell-cycle dependent manner reaching maximal levels in late G1 and rapidly decreasing in S phase. TC-PTP$^{-/-}$ murine embryonic fibroblasts proliferate slower than TC-PTP$^{+/+}$ cells and have a slower progression through the G1 phase of the cell-cycle probably due to the delayed induction of cyclin D1 and Cdk2. These effects, which are reversed following the ectopic expression of TC-PTP, are mediated through the NF-κB pathway (Ibarra-Sanchez et al., 2001). Nuclear TC-PTP also dephosphorylates and inactivates STAT5 in mammary epithelial cells (Aoki and Matsuda, 2002)and STAT1 in HeLa and mouse embryonic fibroblast cells (ten Hoeve et al., 2002). IL-6 induced activation of STAT3 is also inhibited by TC-PTP (Yamamoto et al., 2002) in 293T-cells.

Other PTPs have been implicated in regulation of the cell-cycle. The dual-specificity phosphatase cdc25 is a key enzyme involved in cell-cycle progression. In yeast, cdc25 dephosphorylates and activates the cyclin dependent kinase (CDK) Cdk2, initiating the maturation phase (M phase) of the cell-cycle (reviewed in Fauman, 1996). PTP-S2 binds to

chromatin and is localised exclusively to the nucleus in interphase cells, however, during metaphase it is phosphorylated by CK2, leading to its dissociation from chromatin and redistribution throughout the cytoplasm until telophase when it begins to reassociate with the chromatin (Nambirajan et al., 2000). The inducible overexpression of PTP-S2 promotes progression of cells through G1 to S phase (Ganapati et al., 2001).

The presence of Pez in the nuclei of all cells in an asynchronous cell culture indicates that Pez is nuclear in proliferating cells throughout the cell-cycle. Analysis of Pez localisation during the cell-cycle in synchronised cells would be necessary to verify this. There are several potential NLS's within the Pez protein, and deletion constructs lacking either the PTP domain or the FERM domain were expressed in HEK293 cells to determine whether the truncated proteins retained the ability to translocate into the nucleus.

Deletion of the PTP domain does not affect the subcellular localisation of Pez indicating that the NLS does not reside in the PTP domain. However, deletion of the Pez FERM domain results in a reduction in the fraction of the truncated protein localising to the nucleus compared to full length Pez. This may be due to deletion of the nuclear localisation sequence. However, trace amounts of protein are detectable in the nuclear extracts indicating that nuclear translocation was still occurring in the absence of the FERM domain. It is possible that import may depend on multiple sequences located both within and outside of the FERM domain. Alternatively, removal of the FERM domain might alter the conformation of the protein so that a masked NES is exposed thereby altering the equilibrium of Pez localisation. Another possibility is that the FERM domain may be required for anchoring Pez in the nucleus. An isoform of human basic fibroblast growth factor (bFGF), which normally localises to the nucleus, becomes cytoplasmic following deletion of its amino terminus even when coupled to an autologous NLS, suggesting that the amino terminus contains a sequence necessary for nuclear accumulation (Patry et al., 1994). Hog1 (a MAP kinase) in budding yeast transiently increases in the nucleus following activation

by high osmolarity. Two PTPs act as regulators of this translocation. PTP2 acts as a nuclear anchor and is required for nuclear accumulation. PTP3 is localised to the cytoplasm and acts as a cytoplasmic anchor for Hog1 (Mattison and Ota, 2000).

The inhibition of serine/threonine kinases and phosphatases provides preliminary evidence that the relocalisation of Pez is regulated by serine/threonine phosphorylation. Phosphorylation is a well-documented mechanism involved in the regulation of protein-shuttling between the nucleus and the cytoplasm. Mitosis is triggered in vertebrate cells by the cyclinB1-Cdc2 complex. During interphase cyclin B1 shuttles between the cytoplasm and the nucleus due to a strong NES and a cytoplasmic retention sequence (CRS). At the onset of M phase, cyclin B1 is phosphorylated near the CRS allowing its accumulation in the nucleus (Hagting et al., 1999).

Translocation into the nucleus can also be initiated by dephosphorylation. Pho4, a transcription factor in budding yeast, is imported into the nucleus by the importin β homologue Pse 1. Msn5, the export receptor for Pho4, binds exclusively to the phosphorylated form of the protein. When phosphorylated on five serine residues by the CDK-cyclin complex Pho85-Pho80, Pho4 is rapidly exported from the nucleus (Kaffman et al., 1998a). Dephosphorylation of Pho4 causes it to accumulate in the nucleus. Phosphorylation of a site within the NLS of Pho4 also reduces its affinity for the import receptor, importin β homologue, Pse1 (Kaffman et al., 1998b), Komeili and O'Shea 1999). Therefore, phosphorylation of Pho4 both triggers nuclear export and blocks nuclear import. This dual regulation of import and export has been reported for other proteins such as the transcriptional regulator NF-AT (Beals et al 1997a,b; reviewed in Komeili and O'Shea 2000), and NF-κB (Arenzana-Seisdedos et al 1997, Henkel et al 1992).

The preliminary data presented in this chapter suggest that Pez translocates into the nucleus following serine/threonine dephosphorylation and phosphorylation results in movement out of the nucleus. Further analysis of the regulatory mechanism involved in Pez translocation

might include point mutations of putative serine/threonine phosphorylation sites. A good starting point in such a study would be the serine/threonine residues flanking the putative NLS motifs such as the putative CK2 sites. Phosphorylation of the CK2 site in the SV40 large T-antigen enhances the recognition of the NLS by importin (Hubner et al., 1997; Rihs et al., 1991; Rihs and Peters, 1989). Unfortunately there was insufficient time during the course of my candidature to pursue this question, which will hopefully be addressed in the future.

The subcellular localisation of Pez has provided some insight into its potential function. However, in order to characterise its physiological role it is necessary to identify its substrates. Chapter 5 describes the successful implementation of a "substrate-trapping" strategy using a catalytically inactive mutant of Pez to achieve this.

CHAPTER FIVE

Identification of Potential Substrates of PTP-Pez

Chapter 5: Identification of potential substrates of PTP-Pez using a substrate-trapping strategy

5.1 Introduction

An essential prerequisite to the understanding of the function of PTP-Pez is the identification of its physiological substrates. As the interaction between the phosphatase and its substrates is expected to be transient in nature we have employed a "substrate-trapping" strategy based on the method used to identify the substrates of PTP1B (Flint et al., 1997).

PTP catalytic domains consist of around 240 residues containing a number of invariant amino acids including those in the PTP signature motif HCxxGxxRTG (Table 6.1). Structural diversity between the PTPs is found principally in the non-catalytic domains. The crystal structure of the catalytic domains of several PTPs have been solved, including PTP1B (Barford et al., 1994), PTPmu (Hoffmann et al., 1997), PTP alpha (Jiang et al., 1999), PTP-SHP-1 (Yang et al., 1998), Yersinia PTP YopH (Fauman et al., 1996) and PTP-SL (Szedlacsek et al., 2001). The crystal structures showed that the invariant residues are clustered in regions surrounding the catalytic cleft suggesting that they play a role in catalysis. PTP1B, the prototypical PTP, is the smallest member of the classical PTPs. It consists of a single 37kDa domain with the catalytic site located at the bottom of a shallow cleft. Its crystal structure has been determined both in the absence of substrate and also complexed with a peptide substrate (Figure 1.1).

The phosphate recognition site is created from a loop that is located at the amino-terminus of an alpha helix. This site is formed from the 11 amino acid signature motif containing a catalytically essential cysteine residue that acts as a nucleophile in the catalytic reaction. Located 30-40 aa N-terminal of the catalytic motif is a catalytically important aspartic acid residue located on a flexible loop. When substrate is bound to the enzyme the loop undergoes a conformational change. The cysteine residue forms a thiophosphate intermediate with the phosphate group. The

transfer of the phosphate is enhanced by the aspartate residue, which acts as a general acid and protonates the oxygen of the phosphate group (reviewed in Denu et al., 1996a; Neel and Tonks, 1997).

Table 5.1 PTP catalytic domains

PTP1B	RY_{46}	$R_{47}D$	E_{115}	K_{120}	WPD_{181}	HC_{215}	$S_{216}AGIGR_{221}$	Q_{262}
PTP Mu	RY	G N	E	K	WP*D*	HC	S AG AGR	Q
T-CELL	RY	R D	E	K	WP*D*	HC	S AG IGR	Q
PTP-PEST	RY	K D	E	K	WP*D*	HC	S AGCGR	Q
PTPMEG-1	RY	R D	E	K	WP*D*	HC	S AGVGR	Q
PTPH1	RY	K D	E	K	WP*D*	HC	S AG IGR	Q
PTPD1	RF	Q D	E	K	WP*E*	HC	S AGVGR	Q
PTP36	RI	R E	E	K	WP*H*	HC	S AGVGR	Q
PTP-PEZ	RI	R E	E	K	WPD_{1079}	HC_{1121}	S $AGVGR_{1127}$	Q

Table 5.1 Sequence alignment of the catalytic domains of eight PTP family members compared to PTP1B. Members of the FERM family of PTPs are underlined. The catalytic, invariant cysteine residue is shown in bold type. The conserved aspartic acid, which acts as a general acid or the substituted amino acid, is shown in italics. The sequence alignment was extracted from (Hooft van Huijsduijnen, 1998). Comparison was performed using the GCG (Genetics Computer Group) Pileup software (version 8.1), with a gap creation penalty of 3.00 and a gap extension of 0.10.) The alignment of PTPMEG-1 and PTP36 was performed using the MultAlign software from Angis with a gap weight of 1.0.

In order to identify PTP substrates it is necessary to isolate the PTP-substrate complex. However, it is predicted that the nature of the interaction between the wt PTP and its substrate will be transient and will not persist following dephosphorylation of the substrate. Several groups have shown that the mutation of the catalytic cysteine to serine or alanine enables the isolation of

PTPs with their substrates (Furukawa et al., 1994; Jia et al., 1995; Sun et al., 1993). The mutated PTPs bind to their target substrates and remain bound in a more stable complex than the wt PTP, as they are unable to dephosphorylate the substrate. However in some cases the complexes are not sufficiently stable to permit isolation of the substrate.

A mutagenesis study of the invariant residues of PTP1B led to the development of a 'substrate-trapping' mutant (Flint et al., 1997). Mutating the catalytic cysteine to serine or alanine or changing the invariant aspartate residue, Asp_{181} (PTP1B), to alanine results in a mutant able to bind substrates *in vivo*. The latter mutation has a Km that is comparable to the wt PTP but with a 600-fold reduction in the catalytic activity (kcat) rendering the mutated PTP virtually catalytically inactive. Mutation of the aspartate residue results in a stronger interaction with the substrate than altering the catalytic cysteine (Flint et al., 1997). The substrate-trapping strategy has been used successfully to identify substrates of other PTPs (Table 1.1). Accordingly, a 'substrate-trapping' mutant of Pez was generated in which Asp_{1079}, the residue corresponding to Asp_{181} in PTP1B when the catalytic sites of the two PTPs are aligned (Table 5.1), was mutated to alanine (see section.2.7).

5.2 Results

5.2.1 PTP-Pez phosphatase activity

Not all members of the PTP family are catalytically active. PTP NE-6 has no detectable catalytic activity presumably due to the substitution of an aspartate for a critical conserved alanine residue (A_{217} in PTP1B Table 5.1) in the catalytic site (Fitzgerald et al., 1997). Another inactive phosphatase, PTP-NP also has an aspartate residue in place of the catalytic site alanine; mutation of this aspartate residue to alanine resulted in a catalytically active enzyme (Jiang et al., 1998). The Pez catalytic domain does not diverge from the consensus sequence and would therefore be expected to be catalytically active. However, no PTP activity has been detected in

the murine homologue of Pez, PTP36 (Ogata et al., 1999 a). Clearly the 'substrate-trapping' strategy is only viable if the phosphatase has catalytic activity. Therefore, an *in vitro* assay was carried out to determine whether PTP-Pez is an active phosphatase. Full-length Flag-tagged wt or a catalytically inactive'substrate-trapping' mutant ST $Pez_{D1079A,}$ were transiently transfected into HEK293 cells. The wt-Pez or ST Pez_{D1079A} mutants were immunoprecipitated with the Flag Ab and assayed in an *in vitro* colorimetric phosphatase assay. The substrate in this assay was a biotinylated, tyrosine-phosphorylated peptide (PPS-1 see Fig.5.1). Following incubation with the immunoprecipitated PTP, the peptide was bound to a streptavidin-coated 96-well plate and the amount of tyrosine-phosphorylated peptide remaining determined using an HRP-coupled anti-phosphotyrosine Ab and a chromogenic HRP substrate. The assay showed that wt-Pez almost completely dephosphorylated the substrate (Fig 5.1). In this assay the amount of substrate remaining is inversely related to the level of phosphatase activity. The 'substrate-trapping' mutant Pez_{D1079A} retains some catalytic activity compared to the vector control but this is significantly reduced compared to wt. This assay measured the endpoint of the reaction following a two-hour incubation period. It is likely that the substrate was exhausted in the wt-Pez reaction at an earlier time-point. This would result in an overestimation of the comparative activity of the ST Pez mutant. The small amount of dephosphorylation of the substrate seen in the vector control may be due to the immunoprecipitation of other endogenous PTPs that bind non-specifically to the M_2 Ab. The addition of vanadate, a specific inhibitor of PTPs, to the assay completely inhibits the dephosphorylation reaction, verifying that the reduction in detectable substrate is due to PTP activity and showing that the same amount of substrate was added to each reaction (Fig 5.1)

It has previously been reported that PTP36, the murine homologue of PTP-Pez, is catalytically inactive (Ogata et al., 1999 a). The conserved aspartic acid, which acts as a general acid upstream

Figure 5.1

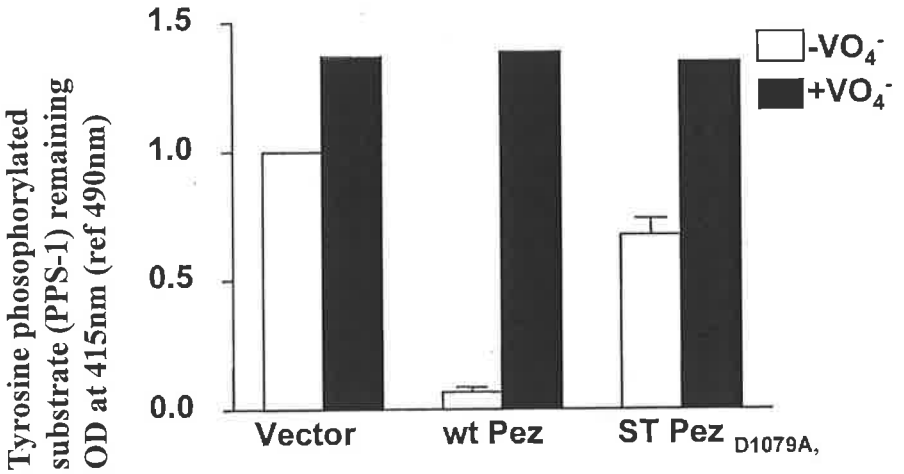


Figure 5.1 *In vitro* tyrosine phosphatase activity of ectopically expressed wt-Pez and ST-Pez D1079A.

IPs from HEK293 cells transfected with Flag-tagged wt-Pez, ST Pez or vector alone using the M2 anti-Flag Ab were assayed for PTP activity according to the method described in Section 2.15. The assays were carried out ± vanadate. The assays were performed in triplicate. Results=Mean ± SEM. Representative results of three assays.

PPS1=corresponding to the hirudin 53 - 65 C-terminal fragment Biotin-DGDFEEIPEEY (PO_4) LQ-NH2, phosphorylated on tyrosine 63
PPS2=corresponding to amino acids 1 – 17 of human gastrin
Bi-EGPWLEEEEEAY(PO_4) GWMDF-NH2, phosphorylated on tyrosine 1

of the catalytic cleft, is substituted by histidine in PTP36 but not in the human PTP-Pez, (Table 5.1). The acidic aspartate residue mediates the cleavage of the P-O bond between the phosphorus atom and the oxygen atom of the tyrosyl side chain of the substrate. Substitution of the basic amino acid histidine for aspartate may suppress cleavage of this bond Figure 5.1and block catalysis. In another member of the FERM family of PTPs, PTP D1, the aspartic acid is substituted by a glutamic acid, resulting in a 600-fold reduction in catalytic activity (Flint et al., 1997). This substitution could account for the difference between the catalytic activities of Pez and PTP36. However, in serine/threonine phosphatases, histidines are able to act as general acids. An aspartic acid is thought to interact with a histidine residue that is near the leaving group and assists in the proton transfer (Denu et al., 1996a). Therefore, the histidine in PTP36 does not necessarily render the phosphatase inactive but may require the appropriate millieu to act as a proton donor. It was also reported that expression of the PTP-Pez phosphatase domain as the PTP-Pez phosphatase domain expressed as a glutathione S-transferase (GST) fusion protein was found to have no catalytic activity when measured using p-nitrophenyl phosphate as a substrate (Ogata et al., 1999 a). It is possible that post-translational modifications, which do not occur in bacteria, are necessary for catalytic activity. Alternatively the absence of the non-catalytic domains might have resulted in incorrect folding of the phosphatase domain resulting in its inability to bind to the substrate. Substrate specificity varies between phosphatases and it is possible that the substrate *p*-nitrophenyl phosphate was not optimal.

5.2.2 Trapping substrates of PTP-Pez

Sequence alignment of the phosphatase domains of Pez and PTP1B indicate that Asp_{1079} of Pez corresponds to Asp_{181} that is essential for catalytic activity of PTP1B (Table 6.1). To generate a substrate-trapping mutant of Pez, Asp_{1079} was mutated to alanine and the reduction in phosphatase activity verified by an *in vitro* phosphatase assay (see Fig 5.1).

We decided to express the substrate-trapping mutant as a full-length protein in mammalian cells rather than as a bacterially expressed GST fusion protein to ensure that the protein was maintained in an active form. The use of the catalytic domain alone may potentially have altered the binding specificity of the Pez or even rendered the phosphatase catalytically inactive. Problems with protein solubility and degradation are often encountered in bacterial expression systems, particularly when the protein being expressed is large. Another consideration was that any post-translational modifications that may be required to produce an active phosphatase would not take place in E coli. Therefore we expressed Pez in mammalian cells and immunoprecipitated the protein using an anti Flag Ab.

Co-immunoprecipitation of potential substrates *in vivo* was not possible due to the necessity of using pervanadate treatment to enrich for tyrosine-phosphorylated proteins. Pervanadate treatment would also inactivate Pez. The substrate-trapping mutant of Pez (ST-Pez_{D1079A}) was therefore expressed in HEK293 cells and immunoprecipitated from the lysates using an anti Flag Ab coupled to protein A-sepharose. The Pez-anti Flag-sepharose complex was then used to "pull down" associated proteins from extracts prepared from newly confluent HUVEC monolayers. In anticipation that Pez might only bind to proteins that are tyrosine-phosphorylated, pervanadate was used to enrich for tyrosine-phosphorylated proteins. This approach resulted in a global increase in the level of cellular tyrosine phosphorylation. As the function of Pez was not known the targeted activation of specific signalling pathways by the

addition of growth factors was impracticable, as this would not necessarily phosphorylate the Pez target. Tyrosine phosphorylated proteins pulled down by wt and ST-Pez$_{D1079A}$ were detected by Western blotting with an anti-phosphotyrosine antibody (Fig. 5.2 panel 2). The Pez immunoprecipitates only pulled down a small number of the tyrosine-phosphorylated bands present in the whole cell lysate (Fig 5.2 panel1) indicating that the interaction with these proteins is specific. Furthermore these proteins are not pulled down from HUVEC lysates that had been incubated with immunoprecipitates from vector transfected HEK293 cells.

The expected outcome of this assay was that the target protein(s) would be dephosphorylated in the presence of the wt-Pez protein. Therefore the target protein would either not be pulled down in the assay or, if remaining complexed with Pez via non-catalytic regions of the PTP protein, then the substrate would not be detected by an anti-phosphotyrosine Ab. However substrate complexed to the ST-Pez$_{D1079A}$ protein would not be dephosphorylated and would therefore be detected on an anti-phosphotyrosine blot. Therefore a tyrosine-phosphorylated band that is detectable only in the ST-Pez$_{D1079A}$ "pull-down" Western blot is a potential Pez substrate.

A number of phosphoproteins pulled down by Pez$_{D1079A}$ were seen at similar levels when wt-Pez was used in the pull-down, suggesting that these proteins associate with Pez but are not dephosphorylated by Pez. However, one phosphoprotein that was prominent in the Pez$_{D1079A}$ (Fig 5.2 band 1) pull-down was barely detectable in the wt-Pez pull-down, indicating that this protein was likely to be a substrate of Pez.

5.2.3 Identification of substrates

Initially an attempt was made to identify the putative substrate by mass spectrometry. However, attempts to scale up the substrate-trapping approach did not yield sufficient quantities

Figure 5.2

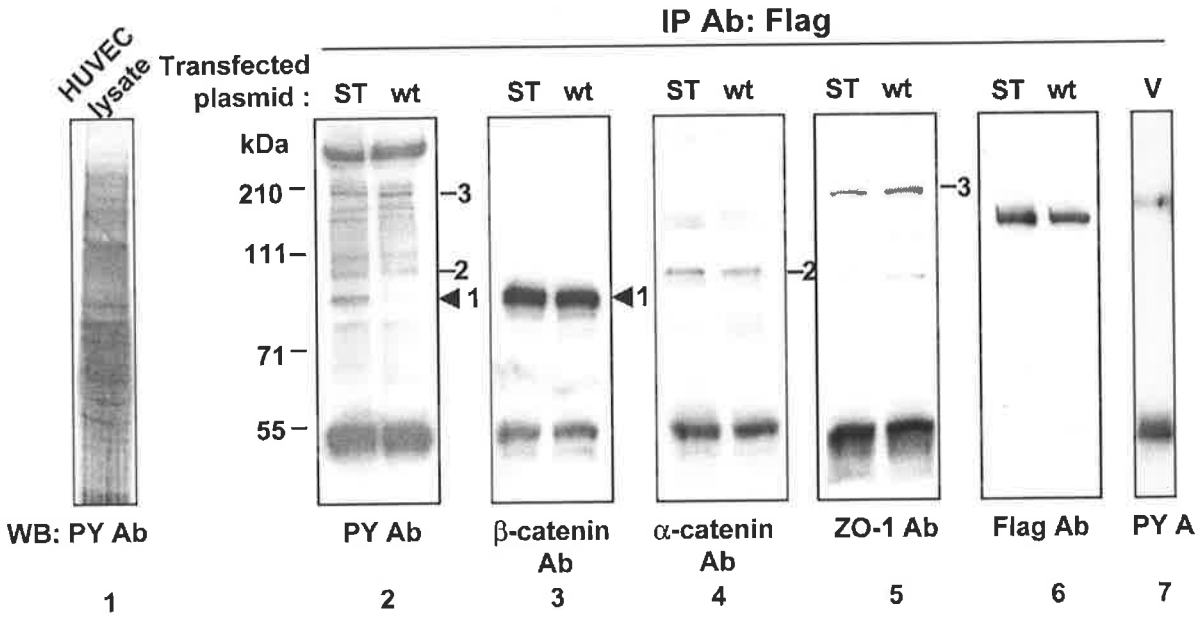


Figure 5.2 Identification of substrates and interacting proteins by 'substrate trapping' from HUVEC lysates. HUVEC lysate enriched for tyrosine phosphorylated proteins (panel 1) as described in section 2.9. was incubated with Flag-tagged wt or ST-Pez$_{D1079A}$ (expressed in HEK293 cells) immunoprecipitates coupled to protein A- sepharose beads via an anti Flag Ab (panel 6). Tyrosine phosphorylated proteins from HUVEC lysate that were bound to either wt or ST-Pez$_{D1079A}$ after washing were separated by SDS-PAGE and Western blotted with an anti phosphotyrosine Ab. Band 1 is the only protein that is tyrosine phosphorylated when precipitated by ST-Pez$_{D1079A}$ but is not phosphorylated when precipitated by wt-Pez (panel 2). The tyrosine-phosphorylated bands were not detected when HUVEC lysates were incubated with immunoprecipitates from vector transfected cells (panel 7). Identical samples electrophoresed on the same gel were Western blotted with β-catenin Ab showing that β-catenin has the same relative mobility as band 1(panel 3) and is pulled down by both wt-Pez and ST-Pez$_{D1079A}$. After stripping, the filter was re-blotted with α-catenin (panel 4) and ZO-1 (panel 5) Abs showing that α-catenin and ZO-1 co-migrate with the tyrosine phosphorylated proteins shown as bands 2 and 3, respectively.

of protein to unequivocally identify the substrate. Two dimensional SDS-Page was also attempted however despite repeated attempts the successful isolation of sufficient protein was not achieved. A second approach based on the rationale below was therefore used for the identification of the putative substrate.

As discussed in chapter four, Pez localises to either the cell-cell junctions or to the nucleus in response to different cell-culture conditions. It can therefore be predicted that these are the locations where Pez interacts with its substrate(s). Although relatively little is known about the role of tyrosine phosphorylation in the nucleus, tyrosine phosphorylation of the proteins involved in cell-cell adhesion has been well-characterised (see section 1.9.2.2.1.2). Components of the cell-cell adhesion complexes are potential substrates of Pez. Antibodies to known cell-cell adhesion complex proteins were therefore employed to investigate whether proteins associated with cell junctions were 'pulled down' by wt and ST-Pez_{D1079A} Pez in the substrate-trapping experiments. Counterblotting the substrate-trapping blots with antibodies to adherens junction and tight junction proteins showed that several junctional proteins were pulled down with both the wt and ST-Pez_{D1079A}.

Counterblotting with an antibody to the adherens junction protein β-catenin showed that β-catenin co-migrated with band 1 (Fig.5.2) suggesting that the putative substrate is β-catenin. Equal amounts of β-catenin were found to be associated with both wt and ST-Pez_{D1079A} indicating that Pez binds to both phosphorylated and unphosphorylated β-catenin. This finding suggests that Pez is either binding to β-catenin via region(s) other than the catalytic cleft or that β-catenin and Pez form part of the same multi-protein complex, which remains intact following dephosphorylation of β-catenin.

Counterblotting with antibodies to other adherens junction proteins revealed that α-catenin (Fig.5.2 panel 4) and γ-catenin (data not shown) were also 'pulled-down' with Pez. The simultaneous precipitation of these adherens junction proteins with Pez indicates that Pez forms part of the adherens junction complex. However, there is no indication from the substrate-trapping data to indicate that α and γ-catenin are substrates of Pez. Only β-catenin was dephosphorylated in the presence of wt-Pez; tyrosine phosphorylation of the bands corresponding to α- and γ-catenin (Fig.5.2 panel 2) was unchanged in the presence of the wt compared to ST-Pez_{D1079A}.

The tight junction protein ZO-1 also binds to wt-Pez and to ST-Pez_{D1079A} (Fig 5.2 panel 5) but is unlikely to be a substrate as its level of tyrosine phosphorylation is the same in the presence of the active and the mutant phosphatase. In epithelial and endothelial cells ZO-1 forms part of the tight junction complex (reviewed in Stevenson and Keon, 1998). Its association with Pez in the substrate-trapping assay suggested that Pez might also play a role in regulating adhesion at TJs. However, ZO-1 can also localise to the adherens junctions in non-epithelial cells (Itoh et al., 1993) Furthermore, prior to the formation of TJs in epithelial cells, ZO-1 initially localises to the adherens junction (Rajasekaran et al., 1996).

In endothelial cells tight junctions are only well formed in the blood vessels that strictly control the exchange between blood and tissues such as at the blood-brain barrier. The organisation of the TJs in HUVEC, where there is not a tight seal between the blood and the surrounding tissue, do not conform to the structure of epithelial TJs. Consequently it is uncertain whether the ZO-1 expressed in HUVEC is associated with the AJs or the TJs. Therefore, to determine if there are other Pez substrates that are components of the TJ complex, 'substrate-trapping' was carried out using extracts from MDCK cells. MDCK cells are a canine epithelial cell line used as a model cell line to study the function of tight junctions. Two tyrosine-

phosphorylated bands that were not detectable in the presence of wt-Pez were pulled down by ST-Pez$_{D1079A}$ (Figure 5.3) from MDCK cell lysates. The migration of these bands corresponded to the MWs of unphosphorylated (65kDa) and hyperphosphorylated (~80kDa) occludin (Sakakibara et al., 1997), an integral membrane component of the tight junctions. Western blots using an anti-occludin Ab on samples run in parallel showed that the higher MW band co-migrated with occludin. However, the occludin Ab did not detect the lower MW band indicating that this band was not occludin.

5.3 Discussion

Two potential Pez substrates have been identified using a 'substrate-trapping' approach, the adherens junction protein β-catenin and occludin, an integral membrane protein that forms part of the TJ complex. The TJ scaffolding protein ZO-1 also bound to both wt and ST-Pez$_{D1079A}$ however it was not dephosphorylated by wt-Pez under the assay conditions used. ZO-1 is therefore less likely to be a Pez substrate but may serve to bring Pez into contact with other tyrosine-phosphorylated proteins at the TJs. On the other hand, the proteins that co-migrated with β-Catenin and occludin were both dephosphorylated in the presence of wt-Pez. These results suggest that β-catenin and occludin are potential substrates of Pez. Both proteins are complexed to Pez in their dephosphorylated form as well as when they are tyrosine phosphorylated suggesting that they may exist *in vivo* as a preformed complex with Pez. The 'substrate-trapping 'strategy involved the use of vanadate to induce the massive phosphorylation of many cellular proteins. The interaction between Pez and the phosphoproteins took place *in vitro* and the phosphatase had access to proteins, which might not necessarily be available in a cellular context. It is therefore important to establish whether Pez can associate with and dephosphorylate these proteins *in vivo*.

Figure 5.3

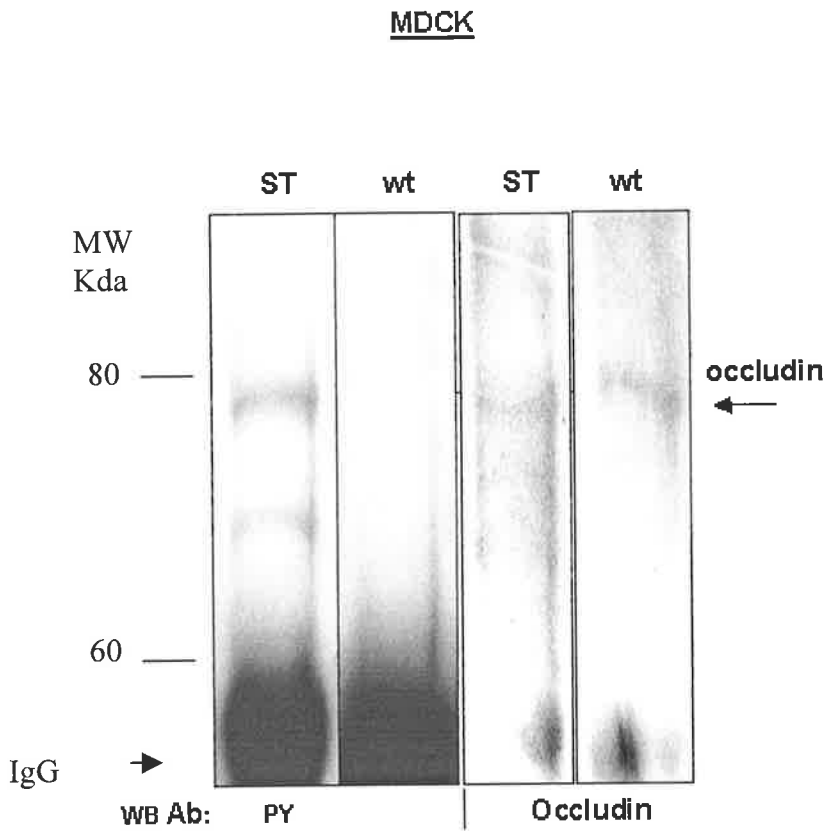


Figure 5.3 Identification of substrates and interacting proteins by 'substrate trapping' from MDCK lysates. As in Figure 5.2 except that MDCK lysate was used as the source of tyrosine phosphorylated substrate. A parallel blot was blotted with occludin Ab showing that the higher MW tyrosine phosphorylated band pulled-down by ST-Pez$_{D1079A}$ co-migrates with occludin.

CHAPTER SIX

PTP-Pez localises with the adherens junction complexes

Chapter 6: PTP-Pez localises with the adherens junction complexes

6.1 Introduction

The substrate-trapping results in chapter 5 provided evidence that PTP-Pez binds to proteins in the adherens junction complex and that β-catenin is a potential substrate of Pez. The adherens junction is one of the complexes involved in establishing and maintaining contact between neighbouring cells.

6.1.1 Cell-cell adhesion

Contact between epithelial cells is maintained through a combination of adherens junctions, tight junctions, gap junctions and desmosomes. Adherens junctions are contacts between adjacent epithelial cells, which are mediated by the cadherin family of single-pass calcium-dependent transmembrane glycoproteins (Figure 6.1) (see section 1.9.2.2). The extracellular domains of these molecules bind to the cadherins of neighbouring cells in a homophilic, calcium dependent manner. The carboxy terminal 25 amino acids of the intracellular domain of the cadherins bind to α-catenin via either β- or γ-catenin (plakoglobin). Alpha-catenin binds to the actin cytoskeleton and also to other actin binding proteins, such as ZO-1, ZO-2, vinculin and α-actinin. All classes of cadherin appear to behave in a similar fashion and require both linkage to the cytoskeleton and dimerisation with cadherins of adjacent cells leading to clustering within the plane of the lipid bilayer of each cell to form strong adhesions.

The ability of cells to detach from each other and to form new cell adhesion complexes is an essential part of development, growth and tissue repair. During morphogenesis cells detach from their neighbours and migrate to new locations where they again form cell-cell contacts. In addition during processes such as wound repair and the flux of cells through self-renewing epithelial tissue such as skin remodelling of cell-cell adhesion complexes is an ongoing process.

Figure 6.1

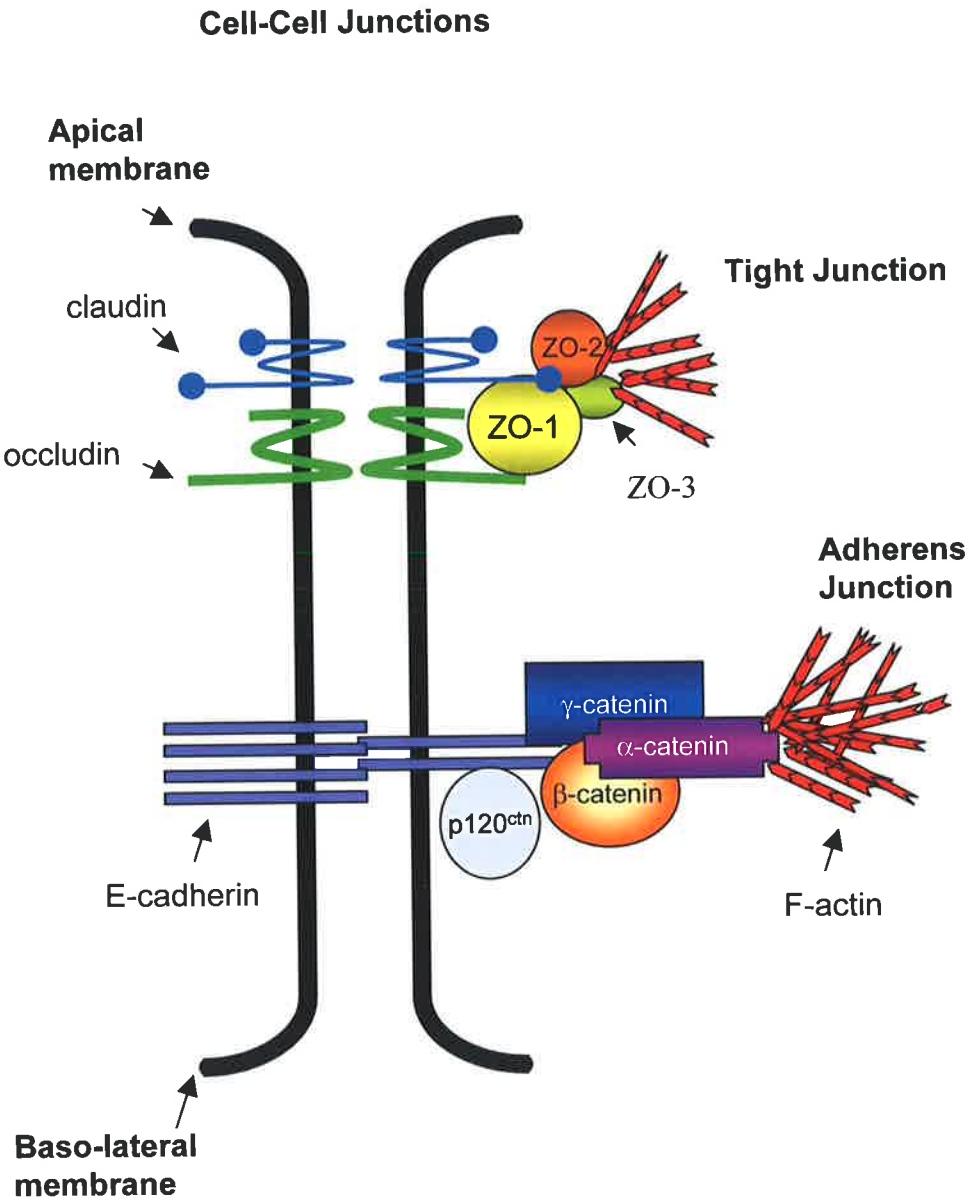


Figure 6.1 Schematic representation of the adherens junction and tight junction complexes between polarised cells

Changes in the organisation of the adherens junction complex, phosphorylation of its constituent proteins and alterations in the interactions with the cytoskeleton have all been suggested to play a role in the regulation of adhesion.

6.1.2 Tyrosine Phosphorylation and the Cell-Cell Adhesion Complexes

Disruption of the adhesion complex has been observed to occur when cells are treated with tyrosine phosphatase inhibitors (Volberg et al., 1992). Tyrosine phosphorylation of β-catenin has been proposed as a mechanism for modulating adhesive strength (Birchmeier et al., 1993; Piedra et al., 2001; Roura et al., 1999). Other members of the adherens junction such as vascular endothelial cadherin (VE-cadherin), $p120^{ctn}$ and γ-catenin have also been found to be tyrosine-phosphorylated (Esser et al., 1998; Lampugnani et al., 1997; Reynolds et al., 1992; Hazan and Norton, 1998). Increased tyrosine phosphorylation of these proteins has been correlated with a decrease in adhesive strength (reviewed in Daniel and Reynolds, 1997; Provost and Rimm, 1999). Tyrosine phosphorylation of β-catenin may result in its dissociation from E-cadherin or α-catenin, either of which would result in a loss of contact between the adherens complex and the actin cytoskeleton. There is considerable evidence to suggest that it is the tyrosine phosphorylation of β-catenin that triggers the dissociation (reviewed in, Daniel and Reynolds, 1997; Lilien et al., 2002) It is possible that the tyrosine phosphorylation of different residues on β-catenin regulates its binding to E-cadherin and alpha-catenin. Tyrosine kinases e.g. EGFR and protein tyrosine phosphatases have all been found in association with the adherens junction complex, (See Sections 1.9 and 1.10 for a more detailed discussion).

6.1.3 Minimum essential criteria to establish that β-catenin is a Pez substrate

In view of the important role played by tyrosine kinases and phosphatases in the regulation of cell-cell adhesion, we were interested in determining whether Pez might be a regulator of β-catenin tyrosine phosphorylation.

To demonstrate that β-catenin is a physiological substrate it must meet several essential conditions; these are as follows:

1. β-catenin must co-localise with Pez.
2. Pez must interact with its substrate *in vivo*
3. β-Catenin must show a decrease in tyrosine phosphorylation in the presence of Pez and/or an increase in tyrosine phosphorylation in the presence of an inactive mutant of Pez

Pez localises to the cell-cell adhesions in confluent cells (Fig 4.6 A) and to the nucleus in subconfluent cells (Fig 4.6B). Therefore it is potentially co-localising with β-catenin at the adherens junctions or in the nucleus (section 4.4) satisfying the first criterion. Since completion of my PhD, co-localisation of Pez at the adherens junctions has been unequivocally demonstrated by confocal microscopy (Wadham et al., 2003). The data in this chapter demonstrate that β-catenin also fulfils criteria 2 and 3 as a substrate of Pez.

6.2 Results

6.2.1 PTP-Pez Interacts With β-catenin *In Vivo*

6.2.1.1 Co-immunoprecipitation of endogenous Pez with β-catenin

To confirm whether endogenous PTP-Pez can associate with β-catenin *in vivo*, co-immunoprecipitation assays were performed using HUVEC, a cell line in which both proteins are co-expressed. Pez was found to co-immunoprecipitate with β-catenin when a β-catenin Ab, but not a control Ab, was used to immunoprecipitate β-catenin from HUVEC lysate (Fig.6.2.A).

Figure 6.2

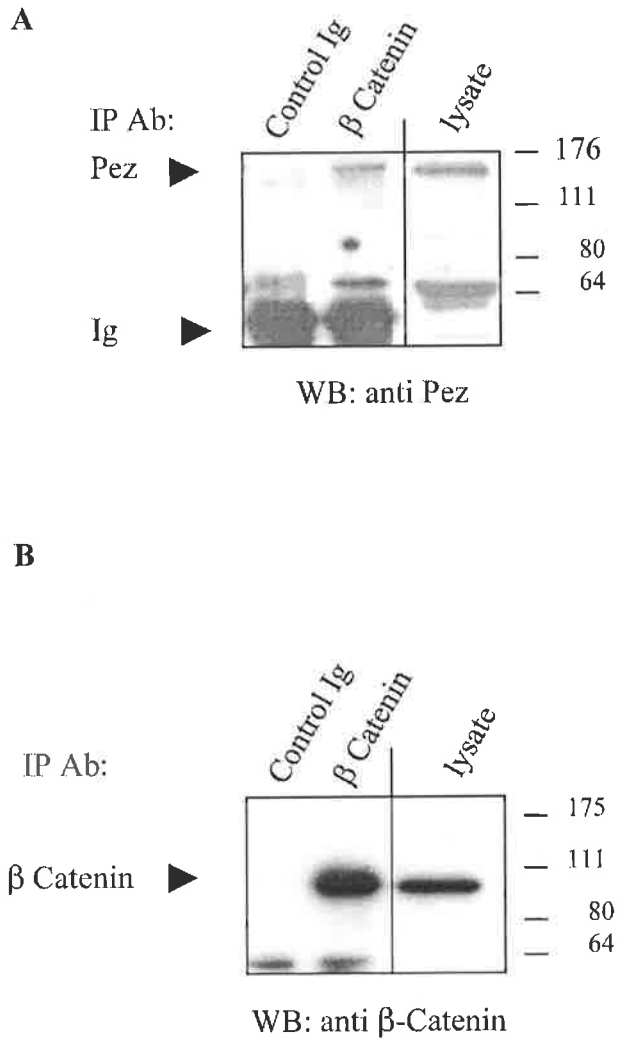


Figure 6.2 Pez co-immunoprecipitates with β-Catenin from HUVEC lysates.
Confluent P3 HUVEC were lysed *in situ* in ST buffer and equal volumes of lysate were immunoprecipitated with either anti β-catenin antibody or an isotype matched control Ig (anti keyhole limpet hemocyanin (KLH) mouse IgG1, 1F11). **A** Western blot of the IPs and total cell lysate using the Pez R1 antiserum. **B.** The membrane was subsequently stripped and re-blotted with anti β-catenin antibody. These data are representative of at least three experiments.

Likewise, when Pez was immunoprecipitated from HUVEC lysates, β-catenin was found to co-immunoprecipitate (Fig 6.3). There was also some non-specific binding of β-catenin to the non-immune control however, in repeated experiments there was consistently more β-catenin co-immunoprecipitating with the Pez Ab.

Only a small fraction of the endogenous pool of β-catenin co-immunoprecipitated with Pez from HUVEC lysates. There are several possible explanations for this. It has been reported that β-catenin exists in four distinct pools (Stewart and Nelson, 1997) indicating that it is segregated within the cell into different protein complexes. Furthermore sub-populations of β-catenin within the cell have distinct binding properties For example only a small proportion of the β-catenin in SW480 cells is able to bind to E-cadherin (Gottardi et al., 2001). The existence of inactive and active pools of β-catenin is supported by several studies showing that β-catenin activity is not regulated solely by protein stability and cytosolic accumulation (Guger and Gumbiner, 2000; Nelson and Gumbiner, 1999)If Pez is binding to β-catenin in only one of these pools then only a proportion of the overall cellular β-catenin is available as a potential substrate. Furthermore Pez is localised to several subcellular compartments (section 4.3.3), the cytoplasm, the cell-cell adhesions and the nucleus. Consequently, only the fraction that co-localises with β-catenin will be able to co-precipitate β-catenin which may only be a small percentage of the total cellular Pez.

6.2.1.2 Truncation mutants of Pez co-immunoprecipitate with β-catenin

The substrate-trapping data (section 5.2.3) suggested that both unphosphorylated and phosphorylated forms of β-catenin associated equally well with Pez (Fig 5.2) indicating that regions other than the catalytic cleft of Pez were involved in the association. Deletion mutants of

Figure 6.3

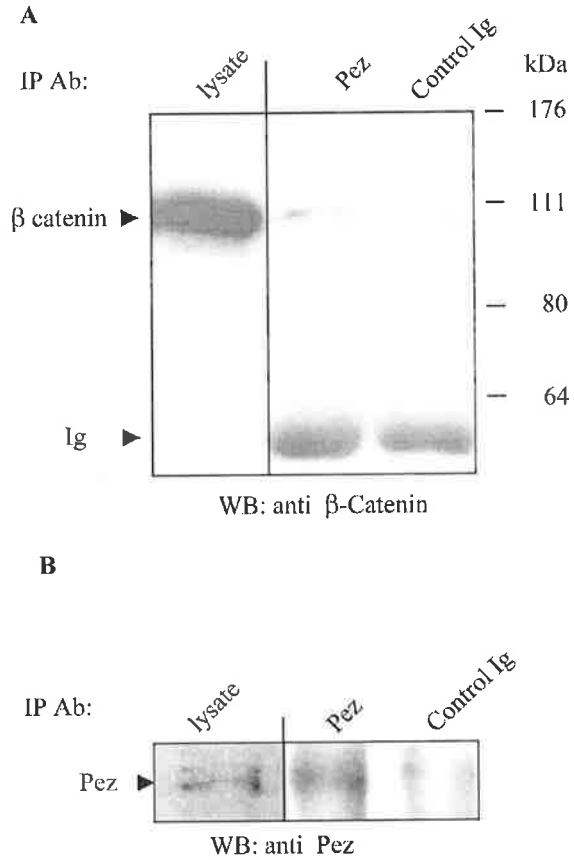


Figure 6.3 β-Catenin co-immunoprecipitates with Pez from HUVEC lysates Confluent P2 HUVEC were lysed in situ in ST buffer and equal volumes of lysate were immunoprecipitated with either anti-PezR1 antiserum or preimmune serum. The figures are representative of at least three experiments. **A.** Western blot of the IPs and total cell lysate blotted with β Catenin Ab **B.** The membrane was subsequently re-blotted with anti-Pez R1 antiserum. These data are representative of at least three separate experiments.

Pez (Fig 6.4A) were constructed in order to investigate which of the regions are involved in binding to β-catenin. WT Pez and ΔPTP-Pez both co-immunoprecipitated with equally well with β-catenin from transiently transfected HEK293 cell lysates (Fig. 6.4 B). There were no proteins co-immunoprecipitating from the empty vector control. β-Catenin also co-immunoprecipitated with ΔFERM-Pez from stably transfected MDCK cell lysates (Fig.6.4 C); no β-catenin was immunoprecipitated with the Flag Ab from the cells expressing vector alone. Figure 6 4 C suggests that wt-Pez co-immunoprecipitates β-catenin much better than ΔFERM Pez indicating that there may be some involvement of the FERM domain sequence in binding to β-catenin. The binding of ΔPTP- and ΔFERM-Pez to β-catenin indicates that the intervening domains of Pez between the FERM and PTP domains are involved in substrate binding.

6.2.1.2 wt-Pez and deletion mutants of Pez localise to the cell-cell junctions in MDCK cells

Having demonstrated that wt and the truncation mutants of Pez are co-immunoprecipitated with β-catenin in a similar manner we investigated whether they also localised to the AJs in a similar manner. Confluent monolayers of parental MDCK cells and stable MDCK cell lines expressing, wt-Pez, ΔFERM-Pez or ΔPTP-Pez were stained with an anti-flag Ab and examined by indirect immunofluorescence using epifluorescent microscopy. The flag epitope was detected at the cell junctions in all cell lines expressing wt or mutant forms of Pez (Fig 6.5). These data indicate that neither FERM nor PTP domains on their own are absolutely essential for localisation of Pez to cell junctions. They also suggest that the intervening sequence, either alone or together with flanking sequences, may be important for targeting to the junctions. It was anticipated that the FERM domain would anchor the protein at the plasma membrane by binding to an integral protein. However, the deletion of this domain did not result in the loss of cell junction localisation. Therefore, it either acts together with other domains to tether Pez to the plasma

Figure 6.4

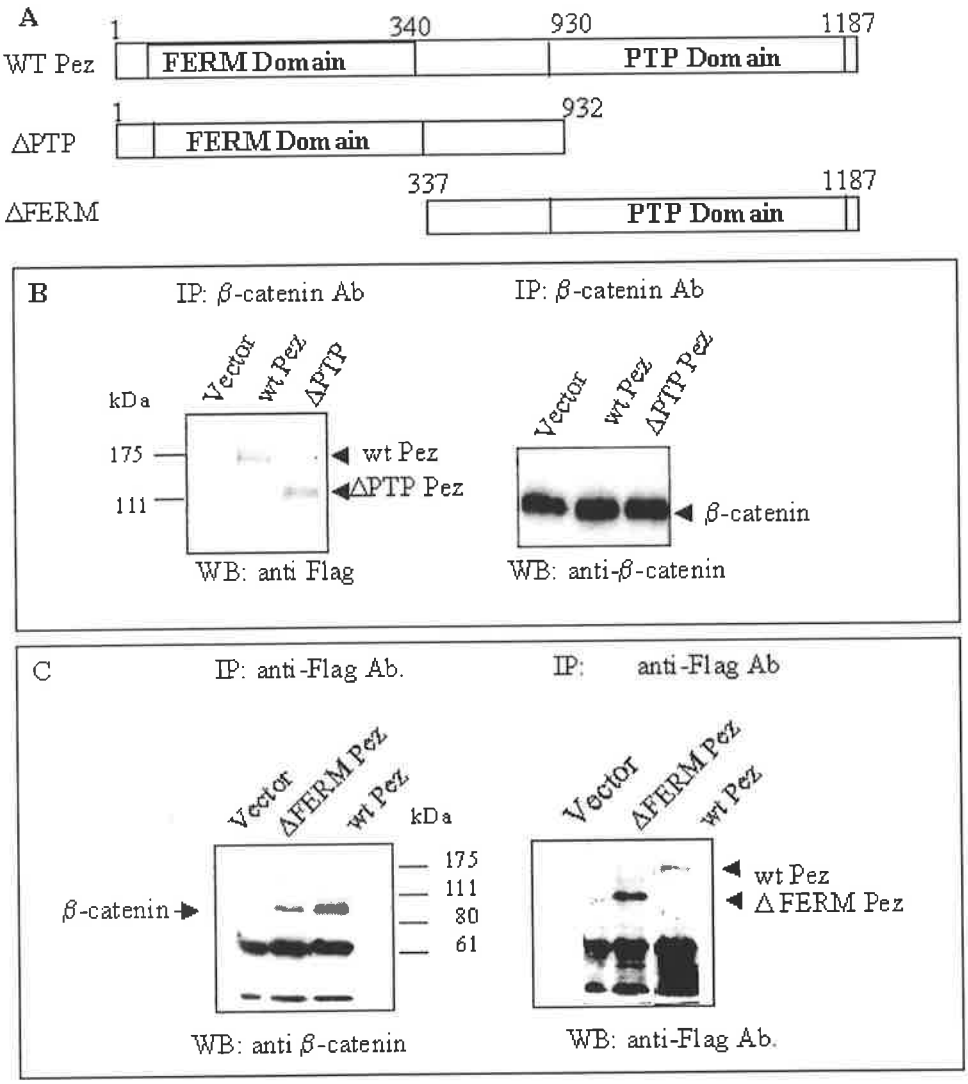


Figure 6.4 Ectopic wt-, ΔPTP- and ΔFERM-Pez coimmunoprecipitate with β-catenin. **A**. A schematic representation of wt, ΔPTP and ΔFERM Pez deletion mutants. **B**.HEK293 cells transiently transfected with either wt-Pez, ΔPTP-Pez or vector were grown to confluence then lysed *in situ* in ST buffer. Lysates were incubated with a β-catenin Ab and precipitated proteins were Western blotted using a Flag-epitope Ab to detect ectopically expressed Pez. Both wt and ΔPTP-Pez coimmunoprecipitated with β-catenin; nothing was detected in the empty vector control indicating specificity of the coimmunoprecipitation. The blot was stripped and re-blotted with a β-catenin Ab showing the presence of β-catenin in all 3 immunoprecipitations. (Transfection and IP performed by Y-Khew-Goodall) **C.** Confluent MDCK cell lines expressing either vector, wt or ΔFERM Pez were lysed *in situ* in ST buffer, immunoprecipitated with anti-flag antibody and blotted with antibodies to β-catenin. The membrane was subsequently counter-blotted with anti-flag antibody.

Figure 6.5

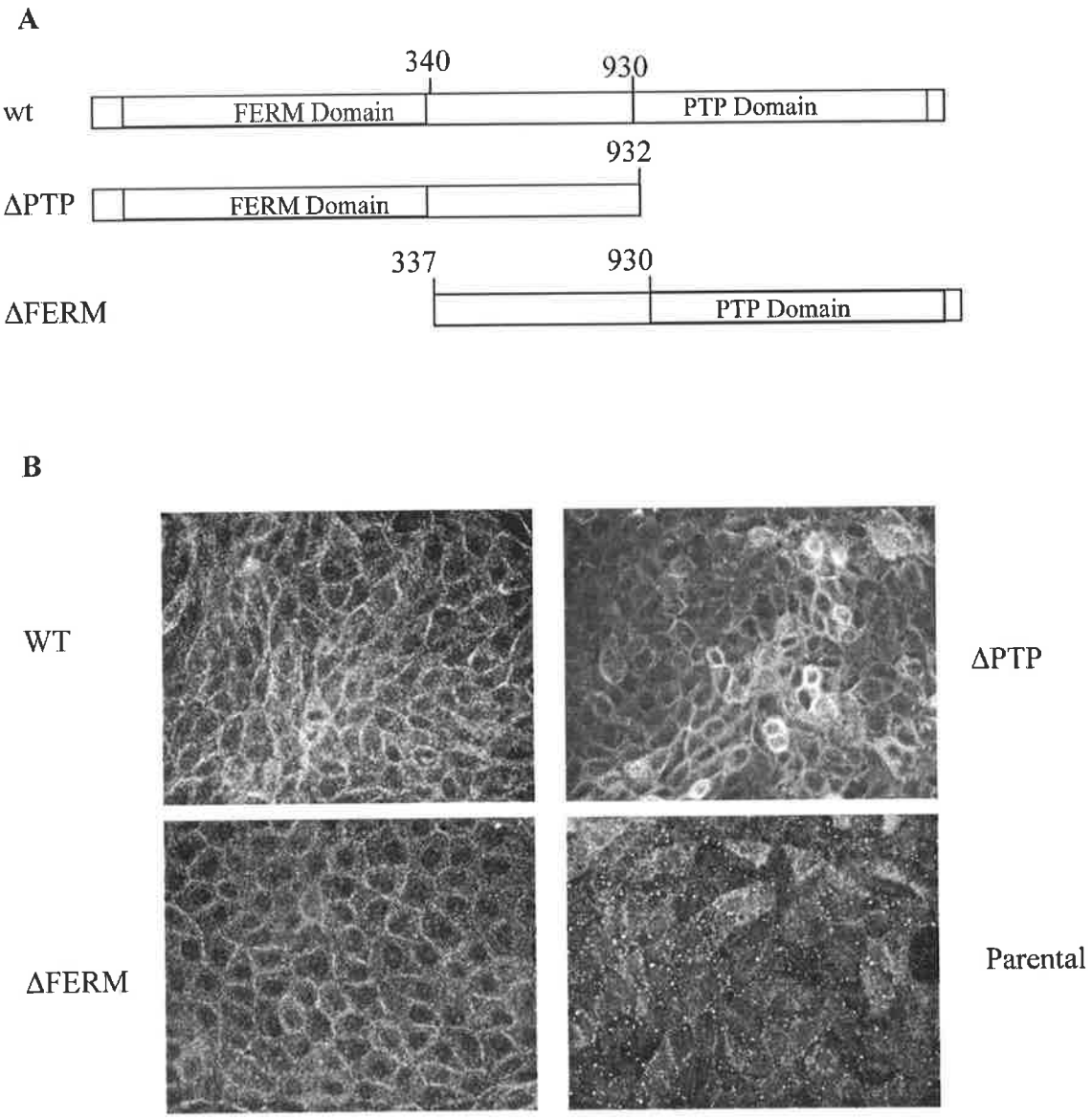


Figure 6.5 Pez deletion mutants stably expressed in MDCK cells all localise to the cell-cell junctions. **A**. A schematic representation of wt-Pez and deletion mutants. **B.** Epifluorescent micrographs of MDCK cells stably expressing wt-Pez, ΔPTP, ΔFERM or vector alone and stained with an anti-Flag Ab (M2) and detected with a FITC-conjugated anti-mouse secondary Ab.

membrane or alternatively is involved in another function such as attachment to the nuclear membrane.

6.2.2 Functions of Pez elucidated by ectopic expression of dominant negative mutants

As ΔPTP-Pez bound to β-catenin but lacked catalytic activity, the binding of this mutant could possibly inhibit the binding of endogenous PTP Pez to its substrate thereby preventing dephosphorylation of the substrate. Therefore, we were interested to determine whether this truncation mutant could act as a dominant negative (DN) of Pez. A DN mutant is one that is able to interfere with the activity of the endogenous protein. Therefore a DN mutant of Pez would be expected to prevent the dephosphorylation of Pez substrates.

6.2.2.1 ΔPTP-Pez acts as a dominant negative mutant and results in increased tyrosine phosphorylation.

An epithelial cell line (MDCK) was used to investigate the effect of ΔPTP-Pez expression on the cell junctions. These cells form morphologically and spatially distinct cell junction complexes enabling us to analyse the phosphorylation status of the different complexes.

We therefore investigated whether expression of this mutant caused an increase in tyrosine phosphorylation of proteins localised to the junctions. The tyrosine phosphorylation status of confluent monolayers of MDCK cell lines stably expressing empty vector, wt-Pez or ΔPTP-Pez that had been serum-starved were examined by indirect immunofluorescence using an anti-PY Ab. and epifluorescence microscopy (Fig 6.6). Compared to vector control cells or wt-Pez MDCK cells, a marked increase in tyrosine phosphorylation was induced by the DN mutant specifically at the cell junctions, marked by co-staining with an antibody to the tight junction protein ZO-1. Quantitation of the fluorescence intensities of the ZO-1 and anti-phosphotyrosine Abs across several cell boundaries clearly shows the co-localisation of tyrosine phosphorylation to the junctions (Fig 6.6 right hand panels). This clearly demonstrated that the ΔPTP-Pez mutant

Figure 6.6

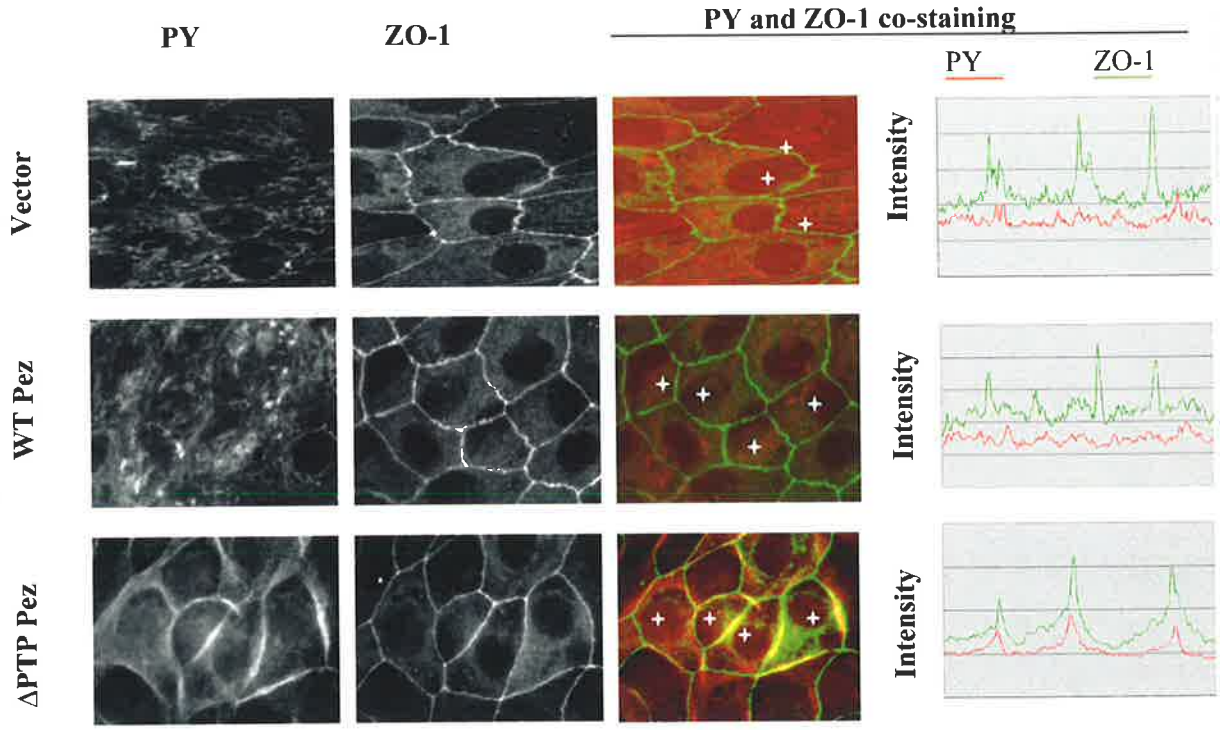


Figure 6.6 Expression of ectopic PTP-Pez increases the tyrosine phosphorylation of proteins at the cell-cell junctions

Confluent monolayers of MDCK cells stably expressing vector, wt-Pez or PTP-Pez were co-stained with anti-phosphotyrosine (mouse monoclonal Ab) and ZO-1 (rabbit polyclonal Ab) Abs. and detected by immunofluorescence using biotinylated anti-mouse Ab, followed by Texas –Red conjugated streptavidin and Alexa fluor 350-conjugated anti rabbit Ab respectively. *Columns from left to right*: phosphotyrosine Ab staining, ZO-1 staining to show the positions of cell-cell contacts, merged PY (false coloured red) and ZO-1 (false coloured green) staining and quantitation of PY and ZO-1 fluorescent intensities taken along a line connecting the crosses shown in the merged image. Specific PY staining was only observed in the ΔPTP cells, which coincided with ZO-1staining at the intercellular junctions

was able to act as a DN mutant to increase the tyrosine phosphorylation of the intercellular junctions. Expression of another Pez mutant, Pez $_{R1127M,}$ also resulted in increased tyrosine phosphorylation of junctional proteins (Wadham et al., 2003). This mutant contains a single point mutation and was predicted to act as a dominant negative mutant of Pez based on the mutational analysis of PTP1B (Flint et al., 1997).Because the analysis was carried out by epifluorescence it was not possible to determine whether the increased tyrosine phosphorylation was at the AJs only, TJs only or induced in both. Confocal microscopy is required to investigate the specificity of the phosphorylation at intercellular junctions. Since the completion of my PhD co-localisation of the phosphotyrosine staining with E-cadherin at the adherens junctions has been confirmed by Y. Khew-Goodall using confocal microscopy (Wadham et al., 2003).

6.2.2.2 Western analysis shows an increase in tyrosine phosphorylation of specific proteins by ΔPTP-Pez in MDCK cells

Western analysis was carried out to analyse the number and characteristics of proteins that were being tyrosine-phosphorylated in the presence of ΔPTP-Pez. Confluent monolayers of MDCK cells, stably expressing either empty vector or Flag epitope tagged wt-Pez or ΔPTP-Pez were serum-starved for 48-hours and then stimulated with 10% serum for 10 minutes to induce tyrosine phosphorylation. Lysates from these cells were Western blotted using an anti-phosphotyrosine Ab (Fig.6.7). The total cellular phosphorylation was low as would be expected in the absence of PTP inhibitors such as vanadate or overexpressed tyrosine kinases. There was a specific increase in the tyrosine phosphorylation of at least two proteins in the lysate from cells expressing ΔPTP-Pez but not in the lysate from empty vector control or wt expressing cell lysates. One of these tyrosine-phosphorylated proteins co-migrated with β-catenin; the other, a higher molecular weight protein, has not yet been identified. Since the completion of my PhD the

Figure 6.7

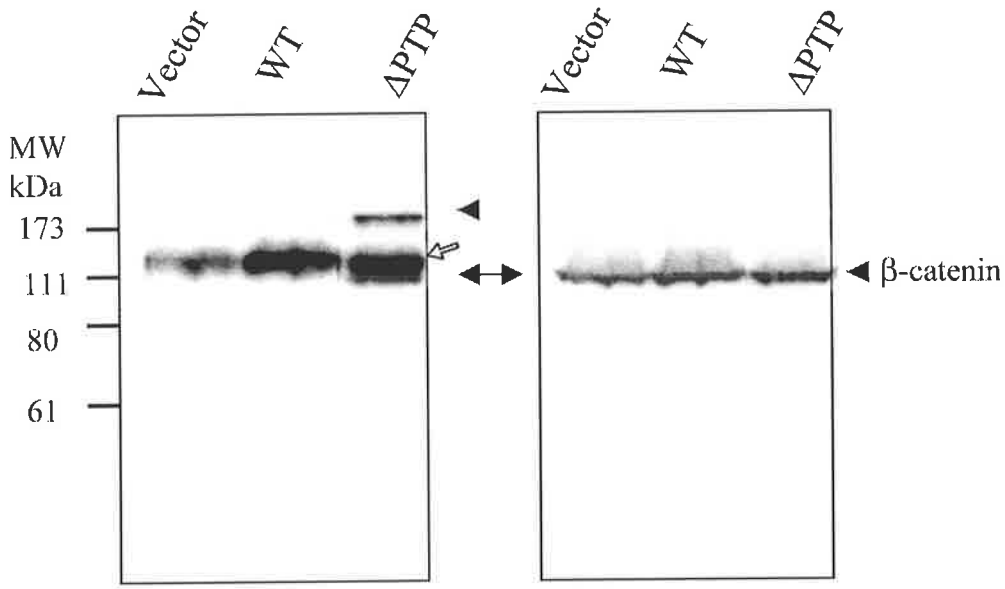


Figure 6.7 ΔPTP-Pez expression results in increased tyrosine Phosphorylation of proteins in MDCK cells MDCK cells stably expressing vector, wt or ΔPTP-Pez were serum starved for 48-hours followed by 10 minutes stimulation with 10 % serum to induce tyrosine phosphorylation. Lysates were western blotted with a phosphotyrosine Ab (left panel). In this cell line two proteins were specifically phosphorylated in the ΔPTP-Pez cells (indicated by arrowheads, one of which co-migrates with β-catenin (double-arrowhead) when the blot was stripped and re-blotted with a β-catenin Ab.A non-specifically phosphorylated protein, present in all lysates, is indicated by an open arrow. An additional unidentified protein is also tyrosine phosphorylated in the presence of ΔPTP-Pez (single arrowhead). This blot is representative of two experiments.

increased tyrosine phosphorylation by β-catenin in the presence of ΔPTP-Pez has been confirmed by Y. Khew-Goodall by immunoprecipitation with a β-catenin Ab. followed by Western blotting with an anti phosphotyrosine Ab (Wadham et al., 2003). These data provide further evidence that ΔPTP-Pez is acting as a dominant negative mutant.

6.2.2.3 Expression of ΔPTP-Pez in A431 cells

A431 cells were used to verify the dominant negative role of ΔPTP-Pez in a human cell line. This is a human epidermoid carcinoma cell line that over-expresses the EGF receptor, potentially giving rise to a higher level of β-catenin tyrosine phosphorylation. (Ref: section 6.2.1.3). When ΔPTP-Pez was transiently expressed in A431 cells it was found to act as a dominant negative increasing the level of tyrosine phosphorylation of proteins that co-migrated with both β-catenin and the tight junctions protein occludin (see chapter 7) compared to cells expressing the vector control (Fig 6.8). As in figure 6.7, there were other bands that also showed increased tyrosine phosphorylation in the presence of ΔPTP-Pez suggesting that there are other Pez substrates that were not identified by "substrate-trapping". It is probable that these are physiological substrates of Pez, as the action of a dominant negative requires competitive binding of the substrate to prevent the action of the endogenous phosphatase. The level of overexpression is low decreasing the likelihood that there is mislocalisation and forced association with non-physiological binding partners.

Figure 6.8

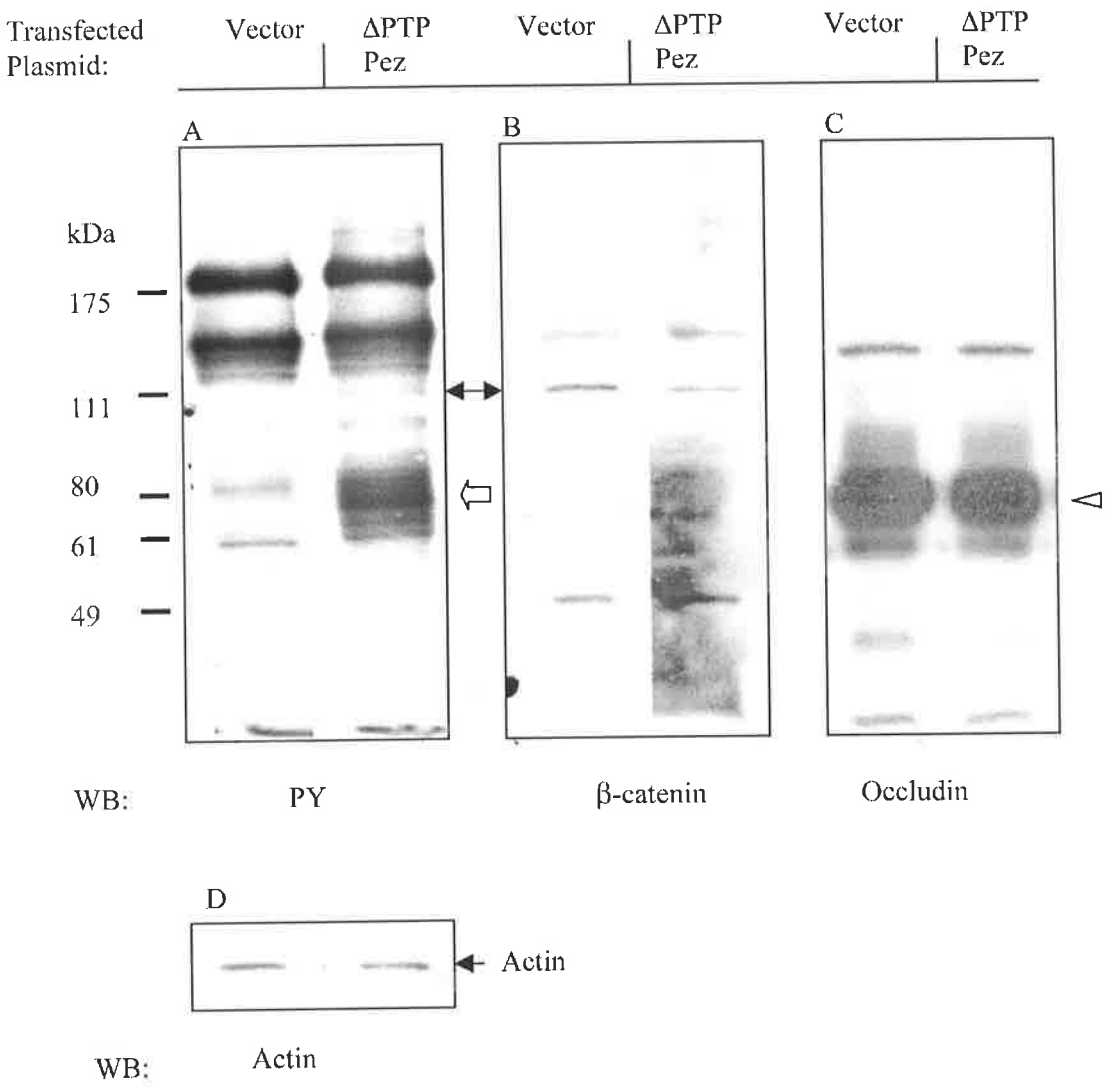


Figure 6.8 ΔPTP-Pez acts as a dominant negative mutant resulting in increased tyrosine phosphorylation of endogenous proteins including two, which migrate with β-catenin and Occludin. Lysates from A431 cells transiently transfected with empty vector (V) or ΔPTP-Pez, were Western blotted with **A.** a PY Ab. The blot was stripped and re-blotted with **B.** β-catenin. **C.** A duplicate membrane was blotted with occludin Ab. **D.** The membrane was also counterblotted with an actin Ab to show equal amount of protein loaded in both lanes. The double-headed arrow indicates the position of β-catenin. The open arrow indicates the position of the tyrosine phosphorylated band that co-migrates with occludin and the open arrowhead indicates the position of occludin.

6.2.3 Overexpression of the dominant negative mutant (ΔPTP-Pez) enhances cell migration

Tyrosine phosphorylation of β-catenin has been shown to result in an increase in cell motility. This was investigated using an *in vitro* 'wound' assay whereby a linear scratch was made on a confluent monolayer of MDCK cells to generate a linear denuded area following which cells from the edge of the 'wound' migrated into the denuded area to repopulate it. Cells overexpressing ΔPTP-Pez migrated further into the 'wound' after 24h than cells overexpressing either empty vector or wt-Pez (Fig 6.9). Measurements of the distances migrated after 24h (Fig 6.9.B) showed that the average distance migrated by the ΔPTP-Pez transfectants were significantly greater ($p = 0.02$) than the distance migrated by either wt-Pez or vector control cells. There was no significant difference between the distances migrated by the wt-Pez cells and empty vector transfected cells ($p = 0.08$). The data suggest that ΔPTP-Pez expression increases cell motility, possibly through increasing tyrosine phosphorylation of AJs leading to a decrease in adhesiveness. Y Khew-Goodall performed these experiments.

6.3 Discussion

The data provided in this chapter and in chapter 5 provide strong evidence that β-catenin is a physiological substrate of PTP-Pez. β-Catenin binds to endogenous Pez in HUVEC and also to ectopically expressed Pez in HEK293 and MDCK cells. The co-precipitation of the ΔPTP-Pez mutant with β-catenin confirms that non-catalytic domains are involved in substrate binding. There are several protein-protein interaction motifs within Pez, which are possibly involved in substrate binding. These include the C-terminal FERM domain, a poly-proline sequence and an acidic sequence both located within the intervening sequence between the FERM domain and the PTP domain (Fig 3.1). It is possible that the FERM domain plays a role in the binding of β-catenin as co-immunoprecipitation with the ΔFERM-Pez mutant was decreased compared with

Figure 6.9

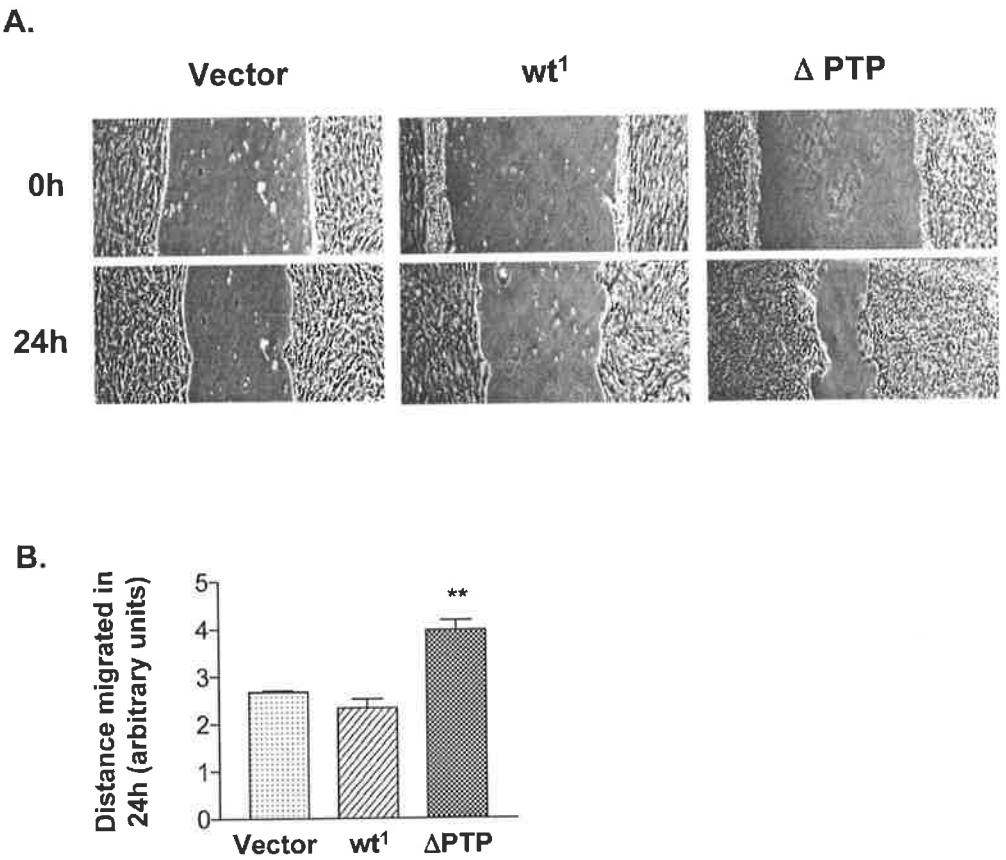


Figure 6.9. ΔPTP-Pez enhances cell motility. A. Confluent monolayers of MDCK cells stably expressing empty vector, wt[1] or ΔPTP-Pez were wounded using the edge of a cell scraper and photographed immediately (0h). Each wound was photographed again at the same spot 24h later. **B**. The distance between the wound edges was measured at the same point on each wound at 0h and at 24h. The difference in distance between the 2 edges at 24h and 0h was taken to be the distance migrated in 24h. Three wounds were measured for each cell line. $^{**}p = 0.02$ when distance migrated by ΔPTP expressing cells was compared with cells expressing empty vector. (Experiments and analysis performed by Y Khew-Goodall)

[1] The wt-Pez expressing MDCK clone was one, which retained an epithelial phenotype (Chapter 9)

wt-Pez. However more accurate quantitative analysis will be necessary to confirm this. The reduction in binding of ΔFERM-Pez to β-catenin may be the result of misfolding due to the deletion of the FERM domain, or some of the sequences in the C-terminal portion of the FERM domain flanking the intervening sequence may participate in β-catenin binding.

Many proteins are known to be binding partners of the FERM family members (see review Hoover and Bryant, 2000). From these binding partners, conserved consensus binding sequences have been proposed. These are S/TX $(K/R)_4$ (MAGUK, Ras and small GTPases) and RXK $(X)_{0-4}GXY9X)_{3E}$ (glycophorin c and syndecan 2) however, neither of these sequences is in the β-catenin amino acid sequence, supporting the notion that the β-catenin binding site of Pez is unlikely to reside solely in the FERM domain.

It was anticipated that deletion of the FERM domain would result in a failure of the mutant protein to localise at the junctions. However, immunofluorescent analysis of the subcellular localisation of wt-Pez and the two deletion mutants ΔPTP-Pez and ΔFERM-Pez showed that they all localised to the cell junctions. The junctional localisation of the two deletion mutants indicates that neither the FERM domain nor the catalytic domain is essential for localising the protein to the plasma membrane.

Another possible β-catenin binding sequence is the poly-proline stretch found in the region between the FERM and the PTP domains (see Fig 3.1). Proline rich sequences are recognised by Src homology 3 (SH3) and WW binding domains, however these domains are not present in β-catenin and there is no record in the literature of β-catenin binding to proline rich sequences.

β-Catenin associates with many proteins including α-catenin, which binds to the N-terminus of β-catenin, APC (adenomatous polyposis coli), TCF/LEF and the cadherin family, which all bind to the armadillo repeats of β-catenin. The structure of the armadillo repeat region

of β-catenin has been resolved (Huber et al., 1997). The twelve repeats generate a groove enriched in basic amino acids to which highly acidic regions in the binding domains of E-cadherin, APC or TCF/LEF bind. There is an acidic-sequence in the variable region of Pez (see Fig.3.1), which, while it does not have sequence homology with the cadherin-binding domain, is a possible β-catenin binding sequence. Detailed investigation of Pez and β-catenin binding, involving perhaps a two-hybrid assay and deletion analysis, is required to elucidate the regions within the two proteins that are involved in this interaction.

The finding that ΔPTP-Pez bound to β-catenin (Figs 6.2 to 6.4) and localised to the cell junctions (Fig 6.5) suggested that this mutant form of Pez could compete with the endogenous phosphatase for substrate, thus acting as a dominant negative, which would be reflected in increased tyrosine phosphorylation of its substrates. This inference is supported by a marked increase in tyrosine phosphorylation of proteins at the cell junctions observed by immunofluorescence. Western blots of cell lysates showed tyrosine phosphorylation of several proteins including one that co-migrates with β-catenin, following expression of ΔPTP-Pez in MDCK cells (Fig 6.7). This finding was confirmed in the human cell line A431. Overexpression of ΔPTP-Pez resulted in an increase in the motility of MDCK cells compared to wt or vector expressing cells. This finding is consistent with an increased level of tyrosine phosphorylation of junctional proteins resulting in a loosening of the junctions leading to increased motility. However this finding is only correlative and further analysis is required to determine the functional outcome of inhibiting Pez expression. The data collectively indicate that PTP-Pez is co-localising with β-catenin *in vivo* and that β-catenin is most likely a substrate of PTP-Pez. These findings support the substrate trapping data (chapter 5) showing that β-catenin is tyrosine phosphorylated in the presence of the catalytically inactive ST mutant of Pez.

Since the completion of my PhD, a GST-Pez fusion protein has been shown to dephosphorylate β-catenin in vitro whereas a GST-ST-Pez_{D1079A} fusion protein does not. Furthermore, immunoprecipitation of β-catenin followed by Western blotting with an anti-phosphotyrosine Ab confirmed that β-catenin was phosphorylated in ΔPTP-Pez-transfected but not empty vector-transfected cells, this work was performed by Y Khew-Goodall (Wadham et al., 2003). These data confirm that β-catenin is indeed a substrate of Pez.

The presence of β-catenin in the adherens junction is controlled by tyrosine phosphorylation (Balsamo et al., 1996; Hoschuetzky et al., 1994; Kinch et al., 1995; Rosato et al., 1998; Muller et al., 1999). Tyrosine phosphorylation of β-catenin has been shown to cause its dissociation from E-cadherin *in vitro* (Roura et al., 1999) resulting in weakening of the tight junctions and facilitating such processes as cell migration. Other PTPs have also been found to interact with β-catenin. PTP LAR co-localises with the cadherin-catenin complex in epithelial cells and associates with β-catenin and plakoglobin. PTP LAR inhibits phosphorylation of β-catenin and epithelial cell migration decreases. An increase in the free pool of cytoplasmic β-catenin was also observed (Muller et al., 1999). PTPμ is associated with E-cadherin and α and β-catenin in E-cadherin-positive cell lines (Brady-Kalnay et al., 1995; Hiscox and Jiang, 1998). PTP1B binds to the cytoplasmic domain of N cadherin (Balsamo et al., 1996) and β-catenin shows increased phosphorylation on tyrosine residues in the presence of catalytically inactive PTP1B (Balsamo et al., 1998).

The involvement of multiple PTPs in the regulation of the adherens junctions may be due to redundancy, underscoring the critical function of the adherens junctions. Then again there may be tissue or developmental specificity involved. Additionally each PTP may be acting on different tyrosine residues within the β-catenin sequence.

Deletion analysis of β-catenin revealed two sequences, aa1–106 and aa 575–693 that are capable of being phosphorylated by pp60c-*src* (Roura et al., 1999). The six tyrosine-residues in these fragments were compared with the sequences of known Src substrates with the result that several were discarded as not fitting well with the optimal phosphorylation sequence of this kinase. Consequently only two of these tyrosine residues were investigated for their effect on E-cadherin binding. The results indicated that, although Tyr-86 was a better substrate for pp60c-*src*, only tyrosine phosphorylation of Tyr-654 correlated with disruption of the adherens junctions. Analysis of the other tyrosine residues present in β-catenin that may also influence its binding to either E-cadherin or α-catenin has not yet been undertaken.

Tyrosine kinases other than Src are known to modulate the adherens junctions, such as the receptor tyrosine kinases for EGF (Hoschuetzky et al., 1994; Shiozaki et al., 1995; Hazan and Norton, 1998; Moon et al., 2001) hepatocyte growth factor (Birchmeier et al., 1993; Monga et al., 2002) and insulin-like growth factor 1(IGF1 (Playford et al., 2000) and other members of the Src family of tyrosine kinases such as Fyn (Calautti et al., 2002) and cytosolic tyrosine kinases such as Fer (Rosato et al., 1998). These tyrosine kinases possibly phosphorylate different tyrosine residues in β-catenin, which in turn are substrates for the different PTPs.

In support of this proposition, there is evidence to show that there are differential effects of tyrosine phosphorylation of β-catenin mediated by different tyrosine kinases. Activation of ectopically expressed TrkA, the tyrosine kinase receptor for nerve growth factor (NGF), induces tyrosine phosphorylation of β-catenin but contrary to other findings this results in increased cell-cell adhesion and promotes the assembly of cells into closely packed clusters (Cozzolino et al., 2000). Moreover, tyrosine phosphorylation of junctional proteins including β-catenin by the Fyn tyrosine kinase increases the strength of adhesion in differentiating keratinocytes (Calautti et al., 1998).

Detailed analysis of the different tyrosine residues of β-catenin is required to establish which PTKs and PTPs are involved in their modification and to determine the functional outcome, which may be a difference in adhesion, morphogenesis or cell signalling.

The ΔPTP-Pez mutant provides a valuable tool for further investigation to determine how the interaction between Pez and β-catenin is regulated and the physiological functions of this interaction. The catalytic activity of Pez $_{R1127M}$ mutant has not yet been assayed to determine whether it is indeed inactive. This could be verified by an *in vitro* phosphatase assay as shown in chapter 5 or by incubating the overexpressed Pez $_{R1127M}$ mutant with tyrosine phosphorylated β-catenin. If this is confirmed then Pez $_{R1127M}$ could be used as a dominant negative in preference to ΔPTP-Pez. As Pez $_{R1127M}$ contains only a single point mutation it is more likely to interact with all of the Pez binding proteins, whereas the deletion mutant will be unable to bind to proteins that interact with the PTP domain.

The central role of β-catenin in both cell adhesion and cell signalling (see section 1.10.1 and chapter 9) suggest that Pez is an important regulator of these functions. Analysis of Pez expression in development and cancer, in which processes the regulation of β-catenin is critical, are areas of possible future research.

The next chapter presents preliminary evidence that the tight junction protein occludin is also a Pez substrate.

CHAPTER SEVEN

PTP-Pez and the Tight Junction Complexes

Chapter Seven: PTP-Pez and the tight junction complexes

7.1 Introduction

Epithelia and endothelia serve to separate compartments within the body with different molecular, ionic and cellular composition. The tight junctions maintain the integrity of the epithelial and endothelial barriers. The junctional complexes are located towards the apical surface of the lateral plasma membrane and are comprised of three components: tight junctions, adherens junctions and desmosomes. Whereas desmosomes and adherens junctions serve to link adjacent cells together, the tight junctions are responsible for sealing the barrier between the cellular compartments.

Plasma membranes of polarised cells are functionally divided into apical and basolateral membrane domains. Integral lipids and proteins are able to diffuse freely within the plane of the plasma membrane. However, the composition of integral membrane proteins and lipids in the apical and basolateral domains is distinct. Tight junctions are composed of integral membrane proteins, occludins, claudins and JAMs which surround the top of individual cells separating the apical and basolateral membrane domains (Fig 6.1)(reviewed in Balda and Matter, 1998; Mitic et al., 2000; Tsukita et al., 2001). It is thought that the tight junction acts as a fence, limiting the lateral diffusion of lipids and integral membrane proteins between the apical and basolateral domains (Dragsten et al., 1981; van Meer et al., 1986). The TJ also functions as a gate, limiting diffusion of molecules between body compartments (reviewed in Tsukita et al., 2001; Tsukita et al., 1999).

7.1.2 Composition of the tight junctions

Freeze-fracture replica microscopy has been used to analyse the morphology of tight junctions (Staehelin et al., 1969; reviewed in Tsukita et al., 2001). These studies have revealed the tight junctions as a set of continuous, cross-linked intramembranous strands on the

protoplasmic (P) face or inner leaflet of the freeze fractured membrane lipid bilayer. On the outer leaflet of the membrane, the extracytoplasmic (E) face, are unoccupied grooves in complementary orientation to the tight junction strands. The number of the tight junction strands varies widely according to cell type. Two models were proposed to explain the structure of the tight junction strands. A lipid model in which the junctions were proposed to be comprised of lipidic cylinders (Kachar and Reese, 1982; Meyer, 1983) and a protein model in which the strands were represented as integral membrane proteins (Gumbiner, 1987; Gumbiner, 1993). The identification first of occludin (McCarthy et al., 1996) followed by the claudins (Furuse et al., 1998) and JAMs (Martin-Padura et al., 1998), all integral membrane proteins localising to the tight junctions, supports the protein model.

7.1.3 The MAGUK family

Three members of the membrane-associated guanylate kinase (MAGUK) homologue protein family, ZO-1 (Anderson et al., 1988), ZO-2 (Jesaitis and Goodenough, 1994) and ZO-3 (Haskins et al., 1998) are also incorporated in the tight junction complex. MAGUK proteins are composed of several domains including the PDZ (PSD95, DLG, ZO-1) domain that appears to bind to neighbouring proteins to promote clustering. They also contain an SH3 domain that mediates protein-protein binding to proline rich regions a catalytically inactive guanylate kinase domain that is also involved in specific protein-protein interactions and a proline-rich sequence. In addition to their role in tight junction structure the MAGUK proteins are involved in other processes such as tumour suppression, regulation of gene expression and protein clustering of ion channels (Anderson and Van Itallie, 1995).

ZO-1, the first of the tight junction proteins to be identified, is a peripheral membrane protein of 210-225 kDa that is essential to the structure and function of tight junctions. Expression of N-terminal deletion mutants of ZO-1 results in corneal epithelial and MDCK cells

undergoing an epithelial to mesenchymal transition possibly due to a modulation of the β-catenin signalling pathway (Reichert et al., 2000; Ryeom et al., 2000).

7.1.4 Occludin

The first of the integral membrane proteins to be characterised in the tight junctions was occludin (Furuse et al., 1993). Sequence analysis predicts that the protein has four transmembrane domains, with both the amino and the carboxy termini located intracellularly. The first of the two extracellular loops is rich in glycine and tyrosine residues and is thought to be involved in the tight junction formation.

Several studies using overexpressed occludin demonstrated a direct role of occludin in the formation of tight junctions and in the generation of a transepithelial barrier (Balda et al., 1996; Hirase et al., 1997; McCarthy et al., 1996). Overexpression of the carboxy-terminally truncated mutants of occludin resulted in the displacement of endogenous occludin from the tight junctions (Balda et al., 1996; Chen et al., 1997). Expression of both wild type and the truncated mutant occludin resulted in an increase in the transepithelial resistance (TER), a determination of the tightness of the junctions, which measures the electrical resistance across apical-basal axis of epithelial sheets. Expression of an N-terminal deletion, dominant negative mutant of occludin caused disruption of the tight junctions and loss of the permeability barrier (Bamforth et al., 1999). Furthermore the addition of a synthetic peptide based on the second extracellular domain of occludin to the growth medium, reversibly increased paracellular flux of macromolecules by up to 40 fold and decreased TER up to 10 fold. The amount of occludin present at the tight junction and total cellular content of occludin decreased (Wong and Gumbiner, 1997).

However, the tight junctions of mice carrying a null mutation in the occludin gene do not appear to be affected morphologically, and the transepithelial resistance of the intestinal epithelium is normal (Saitou et al., 2000). No occludin-like genes have been identified so far; accordingly this phenotype cannot be explained by functional redundancy (Tsukita and Furuse,

1999). Therefore, occludin is not required for the formation of functional tight junctions and an understanding of its function is proving elusive. It is suggested that the primary role of occludin is in signal transduction (reviewed in Tsukita et al., 1999). In support of a signalling role is the finding that the coiled-coil domain of occludin specifically interacts with the signalling molecules PKC-ζ, the non-receptor tyrosine kinase c-Yes and the p85 regulatory subunit of PI 3-kinase (Nusrat et al., 2000). Furthermore, the Raf-1 induced transformation of epithelial cells is suppressed by the overexpression of occludin (Li and Mrsny, 2000).

7.1.5 Claudins

A large family of transmembrane proteins, the claudins, has been identified as integral components of the tight junction strands. Like occludin these proteins have four transmembrane domains but do not have sequence homology to occludin. Exogenously expressed claudins 1 and 2 were sufficient to form well-developed tight junction strand networks in L-fibroblasts. Occludin expression, in contrast, resulted in short, sparsely distributed tight junction strands (Furuse et al., 1998). The claudins can interact with each other in heterogeneous combinations in a restricted manner. For example claudin 3 can interact with claudin 1 or 2 to form strands but claudin 1 cannot interact with claudin 2 (Furuse et al., 1999). While some of the claudins are expressed ubiquitously across tissue types, others are tissue specific, eg claudin 5 is specific to the tight junctions of endothelial cells (Morita et al., 1999). Claudin 11 forms tight junctions in the central nervous system within myelin sheaths, in the choroid plexus and in the Sertoli cells of the testis (Morita et al., 1999; Wolburg et al., 2001). Claudin-11 null mice do not have tight junction strands in the myelin sheath or in the Sertoli cells of the testis resulting in severe neurological defects and sterility in the male mice (Gow et al., 1999). This emphasises the importance of claudins in tight junctions and indicates that there is only limited redundancy between the claudin family members in junction formation. The implication of the defects seen in claudin knockout mice is that tight junctions are heterogeneous between tissues and therefore will

have different functional properties. The permeability of the barrier and the types of molecule that are able to cross the barrier will differ according to the molecular composition of the tight junction.

7.1.6 Junctional adhesion molecule (JAM) proteins

Another transmembrane component of the tight junctions is JAM There are three JAM proteins (Liang et al., 2000; Martin-Padura et al., 1998;Aurrand-Lions et al., 2001). They have a single transmembrane domain and two immunoglobulin domains on their extracellular regions, which form trans-interactions with the extracellular domains of JAM molecules on neighbouring cells (Kostrewa et al., 2001). The adherens junction protein nectin may be involved in recruiting JAM to the tight junctions (Fukuhara et al., 2002). The carboxy-terminus of JAM is bound to the PDZ-3 domain of ZO-1 (Itoh et al., 2001) and to the PDZ domain of polarity related protein (PAR)-3 (Itoh et al., 2001).

Recent research indicates that the JAM proteins are localised to the tight junctions in order to facilitate the transmigration of leukocytes. JAM-2 expression increases leukocyte transmigration and relocalisation of JAM-2 away from the tight junctions decreases transmigration (Del Maschio et al., 1999; Ozaki et al., 1999;Johnson-Leger et al., 2002). However, another study failed to see any change in leukocyte transmigration following reduction in JAM at the TJs (Shaw et al., 2001).

JAM-1 is a ligand of the β(2) integrin lymphocyte function-associated antigen 1 (LFA-1), assisting the LFA-1-dependent transendothelial migration of leukocytes. (Ostermann et al., 2002). JAM-2 on endothelial cells mediates adhesion to T-cells through heterotypic interactions with JAM-3 (Arrate et al., 2001; Liang et al., 2002). This interaction enables JAM2 to engage $\alpha_4\beta_1$ integrin in T-cells (Cunningham et al., 2002).

7.1.7 Other tight junction proteins

Several other peripheral membrane proteins have been localised to the tight junctions including cingulin (Citi et al., 1988), Rab3b (Weber et al., 1994), Rab 13 (Zahraoui et al., 1994), AF-6 (Prasad et al., 1993) 7H6 (Zhong et al., 1993), and symplekin (Keon et al., 1996; Ueki et al., 1997). Little is known about their respective roles.

It is thought that the tight junctions of endothelial cells function differently from epithelial cell tight junctions. Symplekin and 7H6 are not present in endothelial tight junctions and the TER appears to be dependent on interactions with other tissues. In addition endothelial tight junctions lose their barrier function in culture, whereas epithelial cell tight junctions retain their barrier properties in culture.

7.1.8 Tyrosine phosphorylation and tight junction permeability

Tyrosine phosphorylation is implicated in the increase in paracellular permeability in epithelial and endothelial cells (Esser et al., 1998; Staddon et al., 1995). Occludin, ZO-1, ZO-2 and ZO-3 can all be phosphorylated on tyrosine residues suggesting that these proteins could be involved in the regulation of permeability (Kurihara et al., 1995; Staddon et al., 1995; Takeda and Tsukita, 1995; Tsukamoto and Nigam, 1999; Van Itallie et al., 1995). ZO-1, ZO-2, ZO-3, and occludin are all intensely tyrosine-phosphorylated in the presence of pervanadate (Tsukamoto and Nigam, 1999), indicating that a PTP is involved in regulation of tight junction tyrosine phosphorylation. Acetaldehyde-induced disruption of the tight junctions in Caco-2 cell monolayers correlates with an increase in tyrosine phosphorylation of ZO-1and a loss of PTP activity. Inhibition of tyrosine phosphorylation with genistein prevented acetaldehyde-induced permeability (Atkinson and Rao, 2001).

Conversely tyrosine kinase activity has been found to be essential for the reassembly of tight junctions following disruption induced by oxidative stress (Meyer et al., 2001) or ATP depletion (Tsukamoto and Nigam, 1999). Inhibition of the MAPK pathway in Ras-transformed

MDCK cells, which lack tight junctions, resulted in a significant increase in tyrosine phosphorylation of occludin and ZO-1 associated with their recruitment to the tight junctions. Although claudin 1 also localised to the junctions this was not correlated with tyrosine phosphorylation and to date there are no reports of tyrosine phosphorylation of either the claudins or the JAMs.

Tyrosine phosphorylation is therefore essential to the dynamic restructuring of the tight junction complexes although the molecular mechanisms involved remain ill defined. It is possible that tyrosine phosphorylation results in the recruitment of scaffolding and signalling molecules involved in assembly or disassembly of the tight junction strands.

Inhibition of PTPs with vanadate results in a high level of tyrosine phosphorylation of the TJ proteins, associated with a decrease in TER and reorganisation of the junctional complexes (Staddon et al., 1995; Collares-Buzato et al., 1998; Wachtel et al., 1999). However, so far none of the PTP family members has been shown to play a specific role in the tight junctions.

7.2 Results

7.2.1 Association of Pez and occludin *in vivo*

MDCK cells were used as a source of tyrosine-phosphorylated substrates in substrate-trapping experiments (detailed in section 5.2.3) to ascertain whether there were tight junction proteins associating with Pez. These cells form impermeable tight junctions in contrast to HUVEC, which form comparatively permeable junctions. As shown in figure 5.3 two tyrosine-phosphorylated bands were pulled down from MDCK lysates by ST-Pez$_{D1079A}$ that were not phosphorylated when pulled down by wt-Pez. The two bands matched the molecular weight of unphosphorylated (65kDa) and hyperphosphorylated (~80kDa) occludin , an integral membrane component of the tight junctions. Western blots using an anti-occludin Ab on samples run in parallel showed that the higher MW band co-migrated with occludin.

To establish whether occludin is an *in vivo* substrate of Pez, several attempts were made to co-immunoprecipitate Pez and occludin from epithelial and endothelial cell lines. Occludin co-immunoprecipitated with an anti-Flag Ab from lysates of the human colon carcinoma cell line SW480 expressing wt- or ST-Pez$_{D1079A}$ but not cells expressing vector alone (Fig 7.1 A). High molecular weight forms of occludin co-immunoprecipitated from lysates of confluent HUVEC with the Pez Ab but not with the pre-immune serum control (Fig 7.1b).

7.2.2 ZO-1 Co-immunoprecipitates with Pez

The substrate-trapping experiments in chapter 5 provided evidence that ZO-1 is able to associate with Pez, although there was no evidence that ZO-1 is a substrate of Pez. To verify whether there is a physiological association between Pez and ZO-1, co-immunoprecipitation experiments were performed from lysates of confluent HUVEC. ZO-1 was found to co-immunoprecipitate with Pez antiserum but not with the pre-immune serum (Fig 7.2.A). Pez was also found to co-immunoprecipitate with a ZO-1 Ab from HUVEC lysates (Fig.7.2.B).

7.3 Discussion

The data presented in this chapter extend the findings of the substrate-trapping experiments (Chapter 5) in which occludin was identified as a potential substrate of Pez. Occludin co-immunoprecipitates with wt and ST-Pez$_{D1079A}$ from transfected SW480 cells and with endogenous Pez from HUVEC. Pez was also found to co-immunoprecipitate with the tight junction scaffolding protein ZO-1 from HUVEC cell lysates.

Increased levels of tyrosine phosphorylation of tight junctions have been repeatedly correlated with an increase in paracellular permeability and a decrease in TER (Mullin et al., 1992; Staddon et al., 1995; Bolton et al., 1998; Collares-Buzato et al., 1998; Tsukamoto and Nigam, 1999; Wachtel et al., 1999; Dye et al., 2001). Many of these findings have been based on the use of the PTP inhibitors vanadate and phenyl arsine oxide. The use of these inhibitors culminates in the tyrosine phosphorylation of many proteins, which makes interpretation of the

Figure 7.1

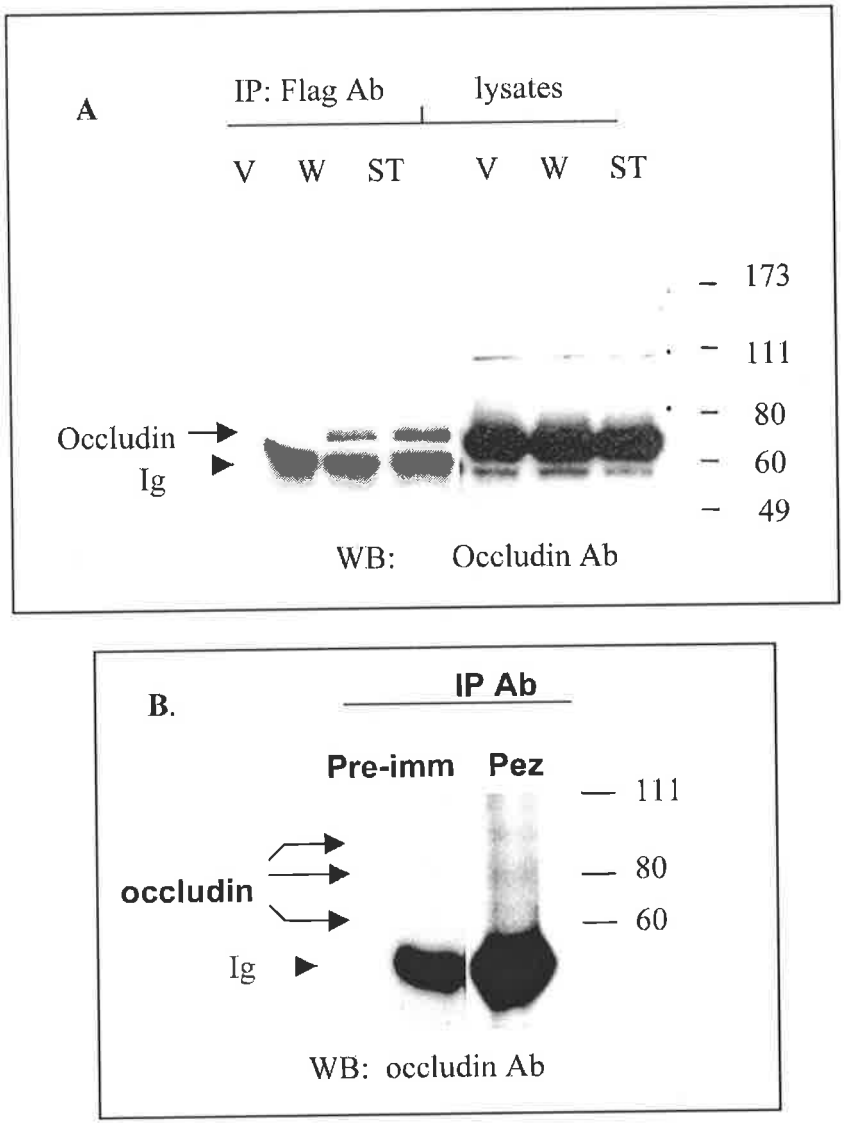


Figure 7.1 Occludin coimmunoprecipitates with ectopically expressed Pez A. Lysates from SW480 cells transiently transfected with either vector, wt-Pez or ST-Pez_{D1079A} were immunoprecipitated with an anti-Flag Ab. Precipitated proteins and lysates were Western blotted using an occludin Ab. Occludin was co-immunoprecipitated from wt- and ST-Pez_{D1079A}expressing cell lysates but not vector transfected lysates. **B.** Preimmune or Pez Abs were incubated with HUVEC lysate and the immunoprecipitated proteins were Western blotted with occludin Ab showing specific co-immunoprecipitation of occludin with Pez

Figure 7.2

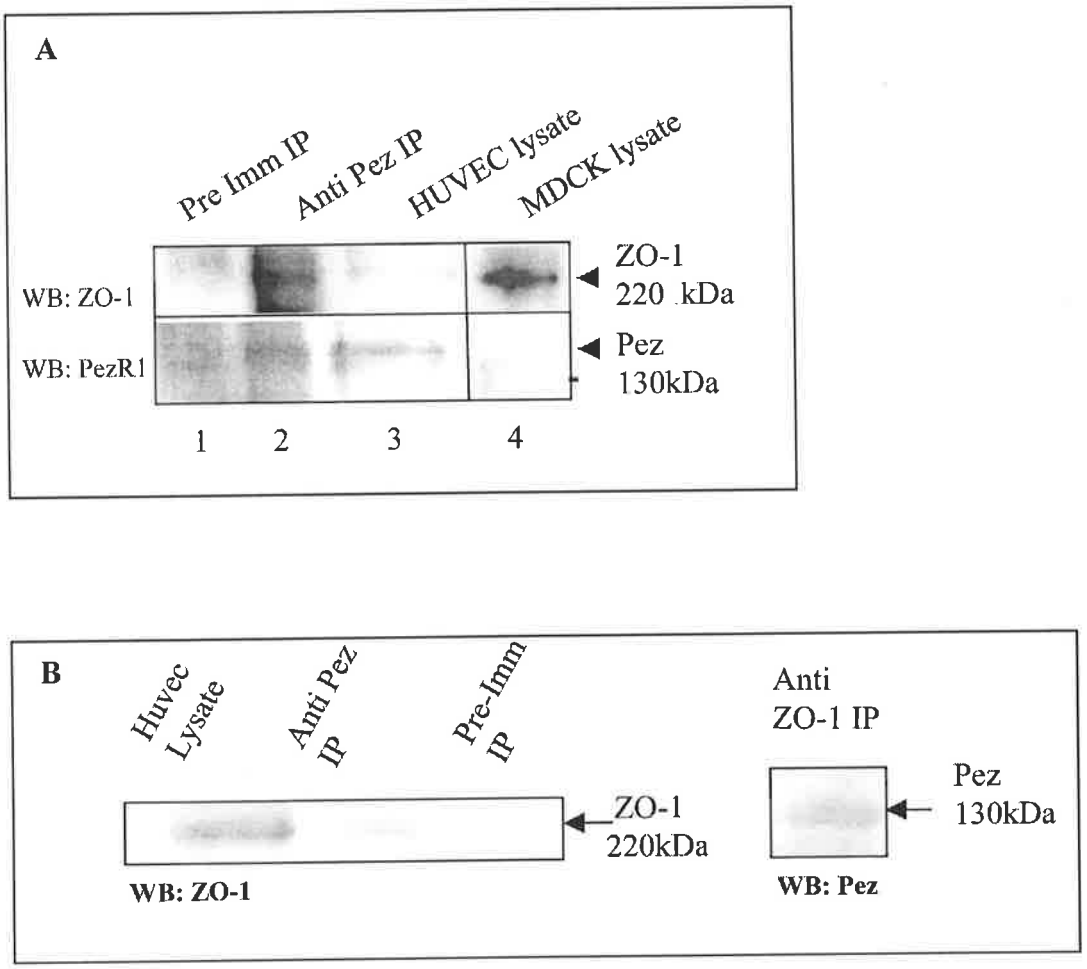


Figure 7.2. The tight junction protein ZO-1 co-immunoprecipitates with Pez. A. Confluent P2 HUVEC lysate was immunoprecipitated with either PezR1 antiserum or pre-immune serum (upper panel). Western blot of IPs and whole cell lysate blotted with an anti ZO-1 Ab (lower panel). An MDCK cell lysate is included in lane 4 as a positive control for ZO-1. The membrane was counter-blotted with PezR1 antiserum. **B.** Confluent P2 HUVEC Lysates were immunoprecipitated with pre-immune or Pez Ab (left panel) or ZO-1 Ab (right panel). Western blot ZO-1 Ab (left panel) and Pez Ab. (right panel) showing co-immunoprecipitation of ZO-1 and Pez. Data are representative of at least three experiments.

results difficult. It is possible that the disruption of the tight junctions is due to decreased adherens junction adhesion rather than tyrosine phosphorylation of the tight junction complex. Conversely it has also been demonstrated that the reassembly of the tight junctions is dependent on tyrosine phosphorylation. Following ATP repletion, which results in the reformation of tight junctions, the Triton-X insoluble occludin, localised at the tight junctions, was seen to be tyrosine-phosphorylated (Tsukamoto and Nigam, 1999). This finding was supported in a study investigating the reversible disruption of tight junctions following oxidative stress in MDCK cells. Tyrosine kinase activity was found to be critical for tight junction reassembly (Meyer et al., 2001). In another study of TJ restructuring, localisation of occludin and ZO-1 to the TJs was lost in Ras-transformed MDCK cells. Treatment with a MAPK inhibitor resulted in recruitment of occludin and ZO-1 and reassembly of the TJ, which correlated with a significant increase in tyrosine phosphorylation of both proteins (Chen et al., 2000).

Evidently serine/threonine phosphorylation and tyrosine phosphorylation are involved in the regulation of tight junctions. However, the formation and disassembly of the tight junctions is a complex process. It is probable that phosphorylation of the component proteins, and indeed of the different phosphorylation sites within the individual proteins, affects the junctions in diverse ways.

Occludin from MDCK cells has been shown to exist in multiple forms of varying MW, migrating as more than ten bands, of between 62 and 82 kDa, from MDCK lysates due to serine/threonine phosphorylation (Sakakibara et al., 1997). Formation of the tight junctions and localisation of occludin at the tight junctions correlates with the appearance of the high MW forms (Wong, 1997; Sakakibara et al., 1997). The occludin that co-immunoprecipitated with Pez from SW480 cells was the low molecular weight form that has been shown in MDCK cells to

predominate in the absence of tight junction formation (Wong, 1997) and to localise to the basolateral membranes (Sakakibara et al., 1997). This finding may mean that the association between Pez and occludin is not occurring at the tight junctions. However, the low molecular weight form of occludin is the predominant species in SW480 cells which might explain why this is the only form associating with Pez. In HUVEC Pez bound to occludin in several of its phosphorylated forms (Fig 7.1B) suggesting that the form of occludin binding to Pez may be cell type specific.

The substrate-trapping data shown in chapter 5 (Fig 5.3) suggest that occludin may be a physiological substrate of PTP-Pez. However, attempts to demonstrate *in vivo* dephosphorylation of occludin in the presence of ectopically expressed Pez have so far been unsuccessful, in part this was due to low ectopic expression of Pez. Additionally, expression of ΔPTP-Pez in MDCK cells did not result in tyrosine phosphorylation of a band with a MW corresponding to occludin (Fig 6.8). However, stimulation with serum may not have activated the kinase that tyrosine phosphorylates occludin. Possibly occludin is not a physiological substrate of Pez and is co-immunoprecipitating with Pez as part of a multi-protein complex. Further investigation is required to clarify whether occludin is a physiological substrate of Pez.

Pez may interact with occludin while in a complex with ZO-1, which possibly recruits both proteins to the tight junctions. ZO-1 is a scaffolding protein that has been found to be important in the recruitment of occludin to the tight junction fibrils (Mitic et al., 1999) via the ZO-1 guanylate kinase domain interacting with the cytosolic carboxy- terminal sequence of occludin (Schmidt et al., 2001). Another member of the FERM family of proteins 4.1R has also been found to specifically interact with the MAGUK family member ZO-2 via the amino terminus of 4.1R and to co-precipitate with ZO-1 and occludin (Mattagajasingh et al., 2000).

The functional consequences of these interactions are so far unclear. Permeability assays did not show a change in permeability in cells overexpressing either wt or ΔPTP-Pez. However, the permeability assays were preliminary and should be repeated under optimised assay conditions to verify these data. If confirmed this finding is consistent with reports that occludin-deficient embryonic stem cells can differentiate into polarised epithelial cells bearing tight junctions (Saitou et al., 1998) and that the epithelium of an occludin knockout mouse has normal barrier function (Saitou et al., 2000). Possibly, the dephosphorylation of occludin by Pez regulates a signalling pathway involved in the restructuring of the tight junctions. Future analysis of the Pez expressing MDCK cells will address these questions.

CHAPTER EIGHT

Overexpression of PTP-Pez Induces an Apparent Epithelial to Mesenchymal Transition

Chapter 8: Overexpression of PTP-Pez induces an apparent epithelial to mesenchymal transition

8.1: Introduction

To further investigate the role of Pez at the cell junctions stable MDCK cell lines were generated expressing either wt or mutant forms of Pez. In light of the data linking tyrosine phosphorylation of the junctions with decreased adhesion (Daniel and Reynolds, 1997; Kinch et al., 1995; Muller et al., 1999; Piedra et al., 2001; Roura et al., 1999; Staddon et al., 1995) it was anticipated that overexpressing wt-Pez in an epithelial cell line would decrease tyrosine phosphorylation and thus increase the strength of the cell junctions. We predicted that the expression of inactivating mutant forms of Pez in MDCK cell lines would disrupt the junctions due to an increase in tyrosine phosphorylation of cell-cell adhesion proteins.

The wt-Pez construct was tagged at either the N- or the C-terminus in order to exclude the possibility that any observed phenotype was due to mislocalisation of the ectopic protein caused by the Flag epitope. The conserved Arg_{1127} in Pez corresponds to Arg221 in PTP1B (see Table 5.1) whereby the point mutation $PTP1B_{R221M}$ was found to abrogate catalytic activity and affinity for substrate and hence act as a dominant negative mutant (Flint et al., 1997). It was therefore predicted that the Pez_{R1127M} would act as a dominant negative mutant. The Pez ST_{D1079A} mutant in section 5.1 is less desirable as a dominant negative mutant as it forms a stable complex with the substrate, which may exert an effect, that is equivalent in function to dephosphorylation of the substrate. Two ΔPTP, dominant negative mutants, ΔPTP Pez_{1-932} and ΔPTP Pez $_{1-878,}$ were generated. A putative bipartite-nuclear localisation signal ($_{aa901-920}$), adjacent to the PTP domain (see Figure 3.1) was included in ΔPTP-Pez_{1-932} and deleted in ΔPTP-Pez $_{1-878.}$ Although it is unknown whether this putative NLS is in fact involved in nuclear localisation, both mutants were included in this study to preclude a potential problem of mislocalisation of the mutant protein

confusing the interpretation of any resulting phenotype. The ΔFERM mutant had the entire FERM domain deleted. It was anticipated that deleting this domain would alter the targeting or binding properties of Pez.

It was predicted that the expression of the inactive mutant forms of Pez that could appropriately localise and bind substrate would lead to disruption of the AJ and TJ complexes due to the increased level of tyrosine phosphorylation of Pez substrates. The predicted phenotypic changes to the cells arising from expression of these mutants included decreased strength of cell-cell adhesion, increased paracellular permeability and increased cell-motility. Conversely the expression of wt Pez was predicted to increase the strength of AJs and decrease permeability due to a reduction in tyrosine phosphorylation of Pez substrates.

8.2 Results

8.2.1 Pez induces an EMT in MDCK cells

WT and mutant Pez constructs (as listed in Table 8.1) were stably transfected into MDCK cells. Following selection the resulting phenotypes of the various Pez transfectants were examined. Unexpectedly the expression of wt-Pez induced morphological alteration resembling a dramatic epithelial to mesenchymal transition (EMT) (Fig 8.1) in MDCK cells. The cells had lost their characteristic regular "cobblestone" appearance and taken on a flattened, spindle shaped fibroblastoid morphology lacking cell-cell adhesions. Although we have not directly measured invasiveness, cells sould be seen burrowing beneath neighbouring cells suggesting the acquisition of an invasive phenotype. Most of the wt-Pez expressing clonal lines underwent this phenotypic change. The transition was not immediately apparent, requiring 2-4 weeks in some cases to occur. The morphological alteration was not observed in the pooled vector transfected cells or in any of the clonal lines expressing the ΔPTP Pez mutants. This suggests that the morphological transition

Figure 8.1

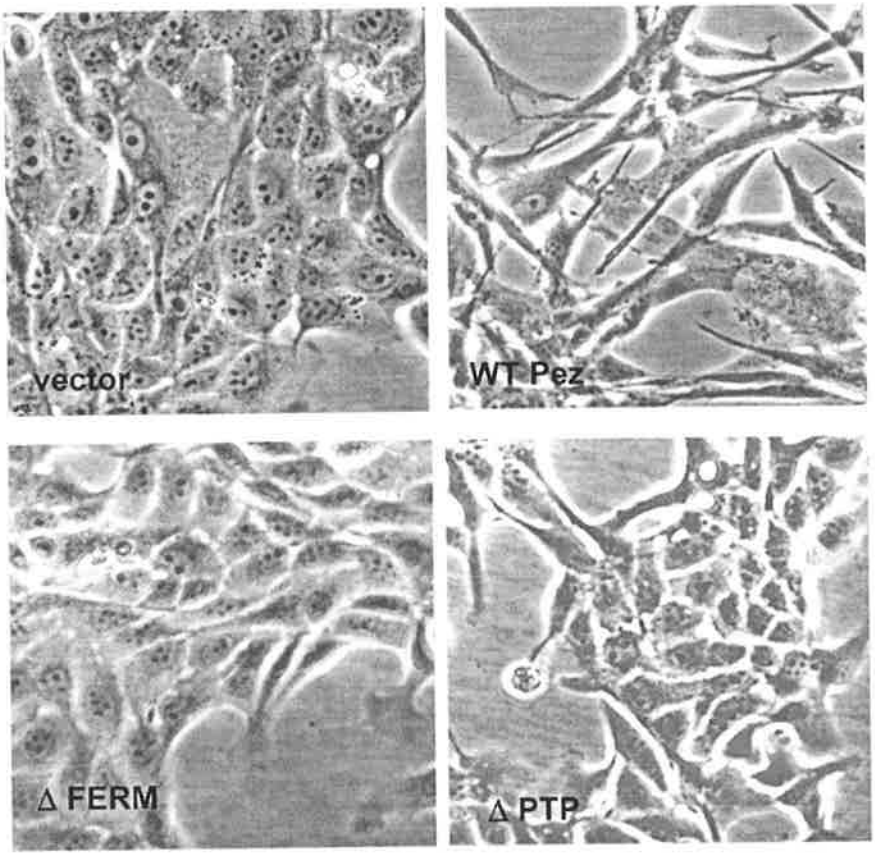


Figure 8.1 The stable expression of Pez in MDCK cells results in an Epithelial to Mesenchymal (EMT) transition. Pooled MDCK cells stably expressing vector alone and representative clonal MDCK cell lines stably expressing wt-Pez, ΔPTP-Pez and ΔFERM-Pez were plated at the same cell densities. Cells were imaged by phase contrast microscopy and photographed 48h after plating.

is specifically due to ectopic expression of the full-length Pez rather than an artifact caused by transfection or the selection procedure.

Mutant forms of Pez were also analysed for their ability to bring about the apparent EMT. Several of the mutant forms of Pez also induced an EMT but at a much lower frequency than wt-Pez. All of the ΔPTP Pez clones retained a normal epithelial phenotype whether or not the putative NLS was present, indicating that the PTP domain is important for inducing the transition. Among the clones expressing either Pez_{R1127M} or ΔFERM less than 30% underwent the apparent EMT indicating that these mutants are able to induce the transition but at a very low efficiency. Approximately half of the clones expressing the inactivating mutant ST Pez_{D1079A} also underwent an EMT.

Table 8.1 MDCK stable cell line phenotypes

Pez wt/mutants	Number of Clones			
	EMT	Epithelial	Mixed	Total
WT (N and C tagged)	9	2*	-	11
Pez_{R1127M}	2	15	2	19
ST-Pez_{D1079A}	4	3	3	10
ΔPTP-$Pez_{1-932(+NLS)}$	-	6	-	6
ΔPTP$Pez_{1-878(-NLS)}$	-	7	-	7
ΔFERM	3	8	-	11
Total	18	41	5	64

Table 8.1 MDCK cells transfected with Pez constructs were selected for stable expression. Following three to six weeks in continuous culture many clonal lines underwent an epithelial to mesenchymal transition. The "mixed" cell lines contained both epithelial and mesenchymal phenotypes and probably originated from more than one clonal line. This transition occurred in all cell lines except those expressing the Pez constructs lacking the catalytic domain.
* Pez expression could not be detected in one of the epithelial wt cell lines

Figure 8.2

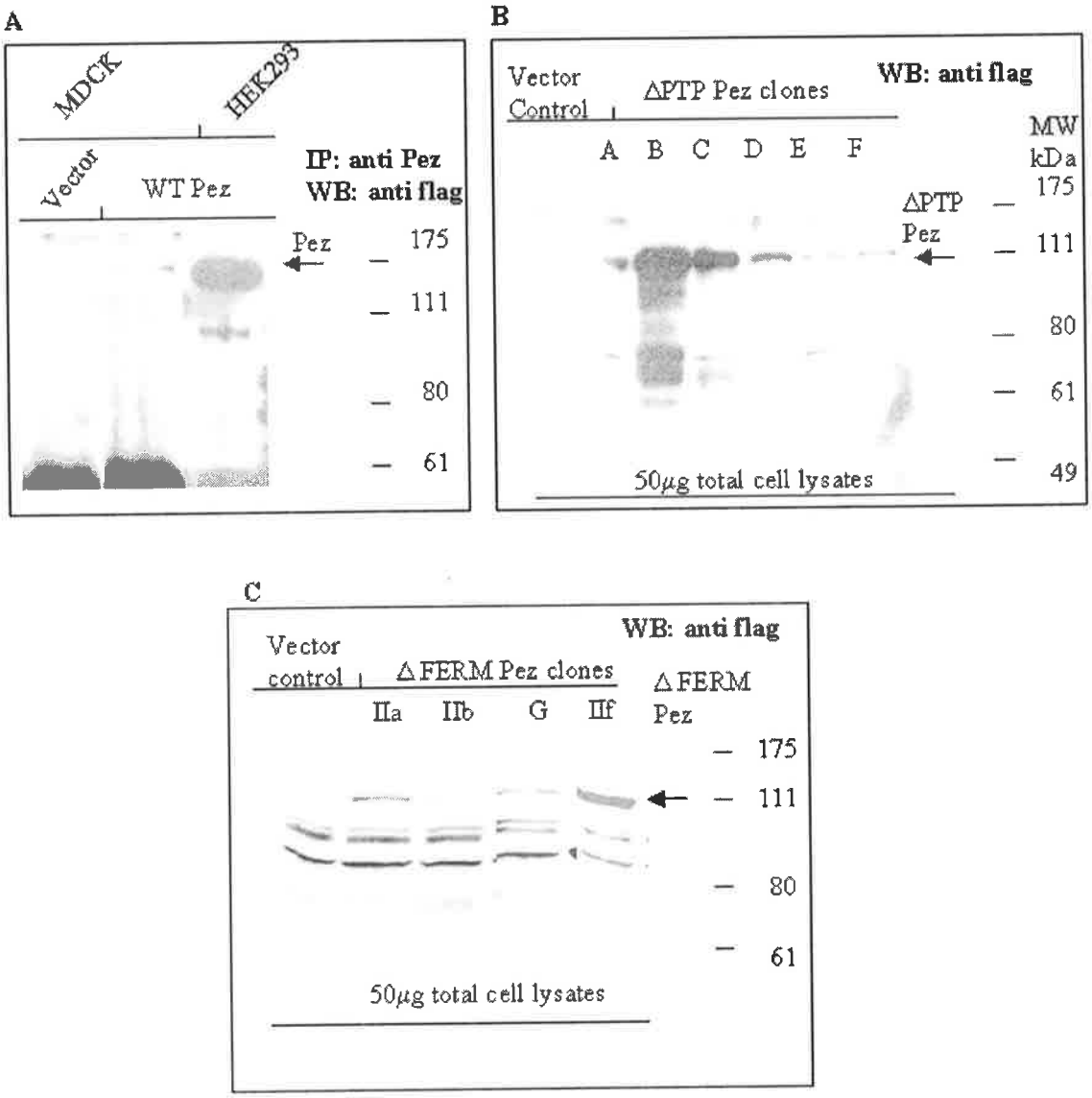


Figure 8.2 Expression of wt-Pez, ΔPTP-Pez and ΔFERM Pez protein in MDCK stable cell lines. A Lysates from pooled MDCK cells expressing vector alone and a representative clonal MDCK cell line expressing wt-Pez immunoprecipitated with anti- PezR1 antiserum. **B** Lysates from pooled MDCK cells expressing vector alone and representative clonal MDCK cell lines stably expressing ΔPTP-Pez. **C**. Lysates from pooled MDCK cells expressing vector alone and representative clonal MDCK cell lines stably expressing ΔFERM Pez. A, B and C Western blotted with an anti-Flag Ab.

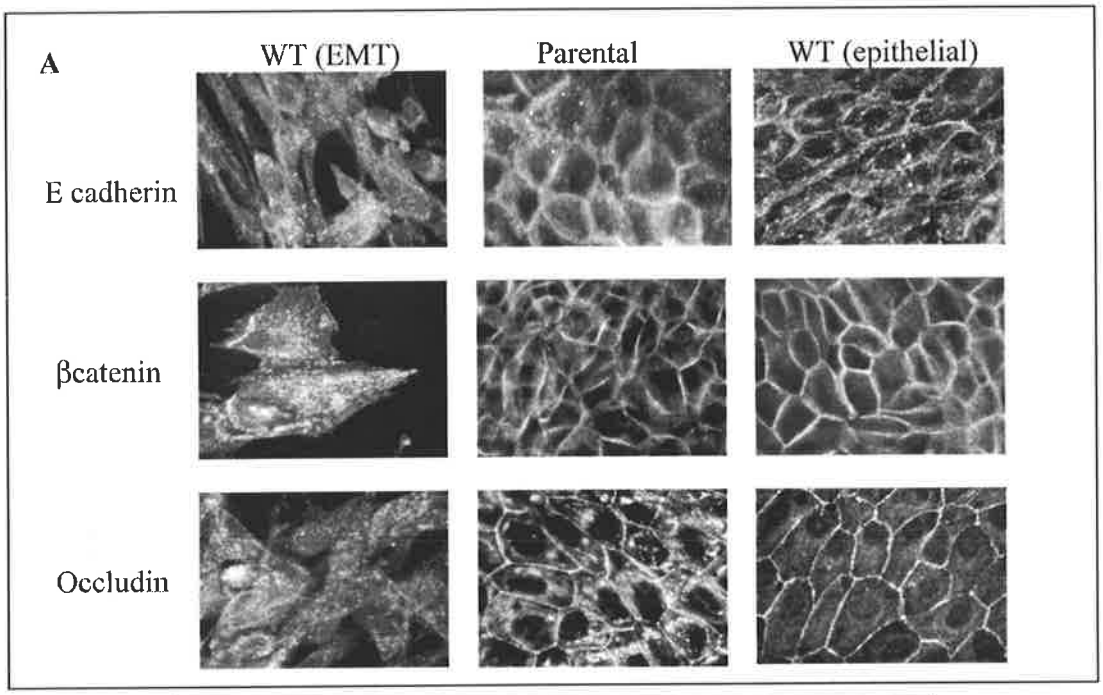
8.2.2 Expression of Pez in MDCK cells

The level of Pez expression in all of the cells expressing wt-Pez and the full length mutant Pez proteins was undetectable by Western blotting. However, expression could be detected following immunoprecipitation with PezR1 antiserum (Fig 8.2.A) and also by immunofluorescence (Fig 6.5) (note: the wt-Pez clone shown in Fig 6.6 did not undergo an EMT, therefore it was possible to see the formation of cell-cell junctions in this cell line). ST Pez_{D1079A} and Pez_{R1127M} expression levels were similar to that seen in the wt-Pez expressing cells (data not shown). ΔFERM-Pez and ΔPTP-Pez (+/- NLS) were all expressed at high levels detectable by Western analysis (Fig 8.2.B and C) and by immunofluorescence (Fig 6.5). The ΔFERM-Pez clone shown in figure 6.5 had retained an epithelial phenotype but the lysates (Fig 8.2C) were from representative clones of both epithelial and EMT phenotypes. There was considerable variation between the clonal lines in the level of expression of the Pez deletion mutants however in the case of the ΔFERM-Pez clones there was not a correlation between the level of expression and the transition to a mesenchymal phenotype. All of the Pez proteins localised to the cell-cell junctions (ΔPTP- and ΔFERM- Pez (Fig 6.5) ST Pez_{D1079A} and $Pez_{R1127\text{-}M}$ (data not shown)). Although the wt-Pez protein and the full length mutants were not detectable by Western analysis the level of protein detectable by immunofluorescence in the wt clone that did not undergo EMT was comparable to the level of protein in the cells expressing ΔPTP and ΔFERM (Fig 7.8). A possible explanation for this discrepancy is that the full-length proteins were bound more tightly to the actin cytoskeleton and were therefore insoluble under the lysis conditions used.

8.2.3 Downregulation of cell-cell adhesion proteins following EMT

The loss of cell-cell adhesion proteins is an essential step in the process of EMT (see section 8.3). A concomitant increase in the mesenchymal markers such as the intermediate filament vimentin is another hallmark of EMTs. Changes in expression levels of cell-cell adhesion proteins and mesenchymal cell markers were investigated to establish whether the

Figure 8.3



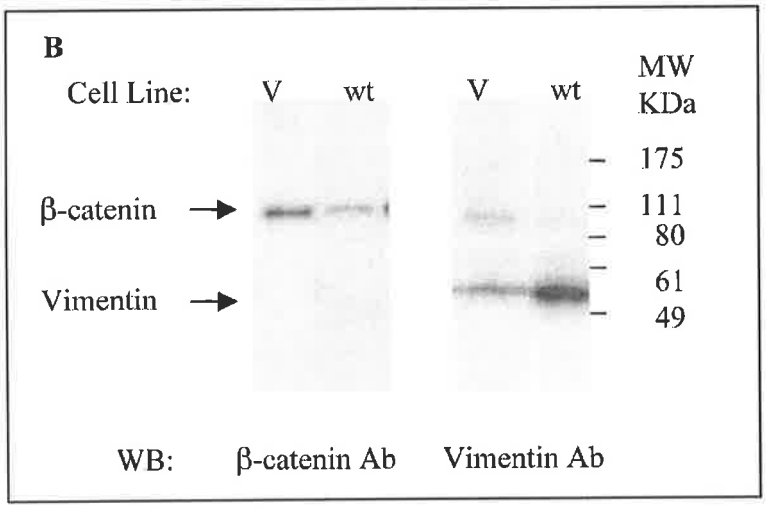


Figure 8.3 Expression of adhesion junction proteins in MDCK cells following EMT.
A. MDCK cells stably expressing empty vector and wt-Pez (both EMT and epithelial phenotype) were plated at confluent densities on 8-well chamber slides and incubated until tight junctions had formed (3 days). Following fixing and permeabilising the cells were stained for (*rows top to bottom)* (1) E-cadherin, (2) β-catenin and (3) occludin followed by anti-mouse Alexa Fluor 594 (1 & 2) or biotin conjugated anti-rabbit followed by streptavidin-FITC (3) (*columns left to right)* wt clone that has undergone an EMT, parental MDCK cells, wt clone that is still epithelial. B. EMT is correlated with a loss in β-catenin expression and increase in expression of the mesenchymal marker protein vimentin. Western blot using a β-catenin Ab of whole cell lysate (50μg) from either pooled MDCK cells expressing vector alone or a representative clonal MDCK cell line expressing wt-Pez that had undergone an apparent EMT. The membrane was subsequently reblotted with an anti-vimentin Ab.

morphological changes observed by overexpression of Pez were true EMTs. The clonal lines that had undergone an apparent EMT were immunostained with antibodies to β-catenin, E-cadherin and the TJ protein occludin. Figure 8.3.A shows that following the change in phenotype, in contrast to the junctional staining seen in the parental cells and cells expressing Pez that remained epithelial, there is diffuse cytoplasmic staining of E-cadherin, β-catenin and the TJ protein occludin. The loss of junctional localisation of β-catenin is correlated with a decrease in βcatenin protein expression (Fig.8.3 B). This is accompanied by an increase in the expression of vimentin, an intermediate filament protein characteristic of mesenchymal cells (Denk et al., 1983) (Fig 8.3 B). Together these data indicate that MDCK cells overexpressing Pez have all the characteristics of mesenchymal cells. Whether the downregulation of AJ and TJ proteins precedes the onset of EMT or is a consequence of the transitional process is unclear, although the literature indicates the former. Decreased E-cadherin at the cell-cell adhesions has been shown to be an early event in EMT. Furthermore the ectopic expression of E-cadherin can re-establish cell-cell adhesion in cells that have undergone EMT (reviewed in Hay, 1995; Savagner, 2001). It will be necessary to use an inducible gene expression system to express Pez in MDCK cells so that the onset of EMT can be analysed.

8.2.4 No evidence of the activation of β-catenin/TCF/LEF signalling by Pez expression

It has been suggested that β-catenin/TCF/LEF signalling may regulate E-cadherin expression due to the presence of TCF/LEF binding sites within its promoter region (Huber et al., 1996) (see section 8.3 for discussion). We analysed β-catenin/TCF/LEF signalling using the TOPflash and FOPflash TCF/LEF luciferase reporter constructs, a well established assay to measure transcriptional activation due to activated β-catenin signalling (Korinek et al., 1997). The pTopflash luciferase reporter construct contains three optimal TCF β-catenin binding sites upstream of a minimal promoter. The TCF sites are mutated in the pFOPflash reporter construct

preventing binding of the TCF/β-catenin complex. Cells were co-transfected with wt-Pez or vector alone and plasmids encoding multimerised wt (TOP) or mutant (FOP) LEF binding sites followed by a luciferase reporter gene (Molenaar et al., 1996). As a control for transfection efficiency, a CMV driven renilla-luciferase cDNA was also co-transfected.

Transiently transfected cells were used for these experiments as the stable cell lines that had undergone EMT were likely to have many downstream changes in cell signalling that were not necessarily directly related to the overexpression of Pez. MDCK cells did not transfect at high efficiency for transiently transfected cells to be analysed, so other cell lines were utilised. HEK293 cells, in the absence of ectopic β-catenin, did not have any detectable β-catenin/TCF/LEF transcriptional activity. Stimulation with EGF to increase the level of tyrosine phosphorylation of β-catenin (Shibamoto et al., 1994) and thus its translocation into the nucleus (Monga et al., 2002) did not affect the level of activity. Co-expression of a degradation-resistant, mutant form of *xenopus* β-catenin, resulted in β-catenin/TCF/LEF activity. However, no difference was seen between wt-Pez expressing cells and those expressing vector alone (data not shown). Expression in SW480 cells, which have accumulated cytosolic β-catenin due to a defect in the Ser/Thr-specific glycogen synthase kinase 3β (GSK3β), consistently showed a slight increase in transcriptional activity in the presence of wt-Pez (Figure 8.4). However the difference was not statistically significant ($P< 0.35$) and it is doubtful that it would result in any physiological effect.

Thus far the data do not support the involvement of the β-catenin/TCF/LEF signalling pathway in the Pez induced EMT seen in MDCK cells. However, the cell lines used in the assay were not optimal. It would be interesting to repeat these assays in hepatocytes in which β-catenin

Figure 8.4

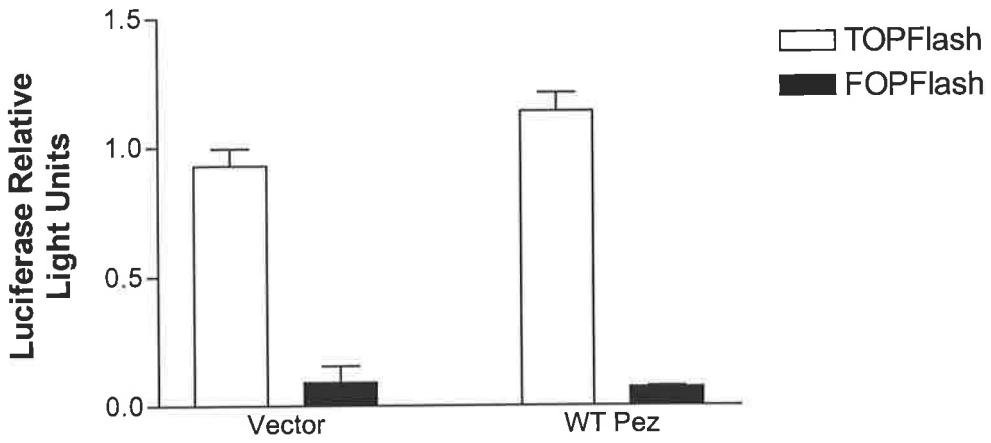


Figure 8.4 Activation of β-catenin/Tcf/Lef signalling in SW480 cells expressing wt-Pez or vector. SW480 cells were transfected with TCF/LEF reporter constructs, either pTopflash (T) (optimal motif) or pFOPflash (F) (inactive motif) together with either wt-Pez or pcDNA3. All cells were also co-transfected with a plasmid encoding pCMV Renilla luciferase to normalise transfection efficiency. The cells were harvested 48-hours after transfection in passive lysis buffer. Luciferase activities were measured using the dual luciferase assay system (Promega). The experimental LEF-luciferase reporter activity was controlled for transfection efficiency and potential toxicity of treatments using the constitutively expressed pCMV-*Renilla* luciferase. The luciferase activities obtained from the TOP or FOP reporter constructs were normalized to *Renilla* luciferase activity. Each transfection was done in triplicate. Three independent transfections were performed, and the pooled data are shown. The results represent mean ±SEM. t-Test $P < 0.35$.

has been shown to translocate into the nucleus following HGF induced tyrosine phosphorylation (Monga et al., 2002).

8.3 Discussion

8.3.1 Background

8.3.1.1 Epithelial to Mesenchymal Transition

The processes of dissociation and migration of epithelial cells are required during normal embryonic development and during pathological situations such as the dispersal of tumour cells. Epithelial cell dispersal is a complex process that requires the breakdown of cell-cell junctions as well as remodelling of the actin cytoskeleton and cell adhesion complexes. During embryogenesis subpopulations of epithelial cells down-regulate the expression of proteins involved in cell–cell adhesion and migrate to a different location in the developing embryo. This regulated alteration in phenotype is called the epithelial to mesenchymal transition (EMT) (Hay, 1995). When epithelial cells undergo EMT they lose expression of keratin filaments and cell junction proteins and acquire vimentin filaments and a flattened phenotype, which are all characteristic of a mesenchymal phenotype. They may also become migratory and secrete proteases, which break down the basement membrane to allow invasion of other tissues. Following EMT the cells differentiate into various cell types. EMT is thought to play a fundamental role during the early steps of invasion and metastasis of carcinoma cells (reviewed in Savagner, 2001).

8.3.1.2 Activators of EMT

Among the biological agents that have been identified as inducers of EMT are a number of growth factors including EGF and HGF and members of the FGF and TGFβ families as well as extracellular matrix proteins such as collagen. The effectors of the transition appear to be the same in both the developmental and the pathological processes. The coordinated changes in cell

morphology, associated with the induction of cell motility and the disruption of intercellular junctions, are the consequence of a signalling cascade originating from the plasma membrane and leading to changes in gene expression. The downstream pathways involved include activation of the GTPase Ras, the Src family of tyrosine kinases and possibly the Wnt pathway. Ras activation has been shown to be required for the relaying of EMT signals via the MAP kinase and PI3 kinase pathways (Potempa and Ridley, 1998; Bakin et al., 2000). Src kinases appear to control epithelial cell scattering by directly regulating the organisation of the cortical cytoskeleton without altering gene expression (Boyer et al., 1997).

8.3.1.3 Loss of cell-cell adhesion

The phenotypic change that occurs during EMT coincides with a loss of cell–cell contacts (reviewed in Savagner, 2001). There is a strong correlation between loss of the adherens junction protein E-cadherin and the development of metastatic tumours (Perl et al., 1998; reviewed in Christofori and Semb, 1999). A study of human carcinomas found that non-invasive, differentiated human carcinoma cell lines expressed E- cadherin, whereas invasive, dedifferentiated carcinoma lines had lost E-cadherin expression. Furthermore the invasiveness could be suppressed by the expression of E-cadherin cDNA and induced again by the addition of E-cadherin antibodies. (Behrens et al., 1991). In a transgenic mouse model of pancreatic beta-cell carcinogenesis (Rip1Tag2), loss of E-cadherin expression coincides with the transition from well-differentiated adenoma to invasive carcinoma (Perl et al., 1998). However, mutations of the E-cadherin gene are infrequently found in most types of sporadic cancers (reviewed in Strathdee, 2002). E-cadherin expression has also been found to be suppressed by epigenetic and post-translational mechanisms. Recently DNA methylation has been identified as potentially responsible for inactivation of E-cadherin during tumourigenesis (reviewed in Strathdee, 2002).

Tyrosine phosphorylation of the adherens junction complex has also been found to suppress normal E-cadherin function (reviewed in Christofori and Semb, 1999; Lilien et al., 2002). Since tyrosine phosphorylation of β-catenin correlates with EMT, it is possible that this is due to reduced E-cadherin-mediated cell adhesion (Birchmeier et al., 1996). These observations suggest that the impaired E-cadherin mediated adhesion system is a characteristic of cells with malignant transformation. The impaired expression of E-cadherin is frequently observed in tumours with aggressive histopathologic characteristics that are defined by invasiveness and metastasis (Shiozaki et al., 1996).

8.3.1.4 Transcriptional control of EMT

The altered expression profile of proteins in cells that have undergone EMT suggests the involvement of specific transcription factors.

8.3.1.4.1 The Slug/snail transcription factor family

Direct transcriptional inhibition of cell-cell adhesion molecules by the slug/snail family of zinc-finger transcription factors plays a central role in morphogenesis and in other processes that require extensive cell movements including EMT (Nieto, 2002). Different signalling molecules including FGF, TGF-β and Wnt have been implicated in the activation of the snail genes leading to EMT.

The zinc-finger protein slug and closely related members of the snail family are believed to be involved in EMT. The treatment of chick embryos with antisense oligonucleotides to slug inhibited neural crest development and mesoderm delamination. Overexpression of slug leads to an increase in neural crest production in the chick embryo ((Nieto et al., 1994; del Barrio and Nieto, 2002 reviewed in Nieto, 2002). Snail is a strong repressor of E-cadherin expression and overexpression of snail triggers an EMT through the repression of E-cadherin expression (Cano et al., 2000 reviewed in Nieto, 2002).

8.3.1.4.2 The β-catenin/TCF/LEF pathway.

In addition to its role in the adherens junctions β-catenin acts as a signalling molecule in the Wnt signalling pathway (Wodarz and Nusse, 1998). In the absence of Wnt glycoproteins, the Ser/Thr-specific glycogen synthase kinase 3β (GSK3β) phosphorylates β-catenin, APC, and axin (Behrens et al., 1998), which are present as a multi-protein complex in the cytosol. Phosphorylated β-catenin is rapidly ubiquitinated and degraded by the proteosome pathway (Aberle et al., 1997). Binding of Wnt glycoproteins to the Frizzled family of receptors leads to the inactivation of GSK3β resulting in the enhanced stability of β-catenin. Stabilised β-catenin accumulates in the cytosol and can then translocate into the nucleus. Once in the nucleus β-catenin binds to members of the TCF/LEF transcription factor family and stimulates transcription (Behrens et al., 1996), probably by recruiting the basal transcription machinery to promoter regions of Wnt target genes such as cyclin D1 (Shtutman et al., 1999; Tetsu and McCormick, 1999). Oncogenic transformation of mammalian cells is closely linked to the signalling function of β-catenin (Smits et al., 1999; Korinek et al., 1997; Harada et al., 1999).

There is evidence that EMT is associated with activation of the β-catenin/TCF/LEF pathway. Ectopically expressed LEF-1 upregulates nuclear β-catenin and promotes EMT in DLD-1 epithelial tumors that retain nuclear β-catenin. This EMT is reversible if the LEF-1 virus is removed (Kim et al., 2002). In addition, mouse mammary epithelial cells expressing a fusion protein of c-Fos and the estrogen receptor (FosER) underwent an EMT following activation of FosER by estradiol. β-Catenin significantly increased in the cytoplasm and colocalised with transcription factor LEF-1 in the nucleus (Eger et al., 2000).

Mutants of the TJ protein ZO-1, which encode the PDZ domains (ZO-1 PDZ) but no longer localise to the plasma membrane, induce an EMT in MDCK cells. The β-catenin signalling pathway is activated in the cells expressing the ZO-1 PDZ protein. The mesenchymal phenotype

is reversed by ectopic expression of the APC gene which is known to down-regulate activated β-catenin signalling.

Interestingly, it has recently been demonstrated that β-catenin also translocates into the nucleus following tyrosine phosphorylation by the intracellular kinase domain of the HGF receptor Met (Monga et al., 2002). HGF is one of the growth factors known to induce cell scattering and EMT. The Met associated pool of β-catenin is distinct from and stoichiometrically greater than the pool of E-cadherin bound β-catenin. Therefore, the correlation between EMT and tyrosine phosphorylation of β-catenin (Birchmeier et al., 1996) may be due to its role in transcriptional activation rather than, or in addition to loss of β-catenin at the AJ.

8.3.2 Summary and conclusions from this study

The expression of wt-Pez proteins containing the phosphatase domain in MDCK cells resulted in a dramatic epithelial to mesenchymal transition. This phenotypic change was accompanied by the loss of localisation of adherens and tight junction proteins to the cell junctions, a decrease in the level of β-catenin and an increase in expression of the mesenchymal marker protein vimentin. However, cells expressing Pez proteins in which the catalytic domain had been removed retained a normal epithelial phenotype despite expressing high levels of the exogenous protein (Figure 8.2B & C).

Characterisation of wt-Pez protein expression levels in individual clones indicated that the observed effect was not due to the expression of large amounts of the Pez protein as the levels of protein were undetectable by Western blot and could only be seen following immuno-precipitation, thus arguing for a physiologically relevant observation rather than an overexpression artifact. These data suggest that the phosphatase domain of Pez is required for the Pez induced EMT in MDCK cells. However, some of the clonal cell lines expressing ST-Pez_{D1079A} and $Pez_{R1127-M,}$ which are predicted to be catalytically inactive (see Figure 5.1 and Flint

et al., 1997) also underwent an EMT. This suggests that either non-catalytic sequences within the phosphatase domain are required for the EMT, or alternatively that these mutants have sufficient residual catalytic activity to promote the EMT. Another possible explanation is that if the substrate associated with the apparent EMT is inactivated following dephosphorylation by wt-Pez, then the ST-Pez_{D1079A} could also functionally inactivate it by stably binding to it. However not being able to dephosphorylate the substrate and relying only on the physical interaction would result in lower efficiency of inhibition of the function of the substrate, which would be reflected in the lower frequency of EMT. The Pez_{R1127M} inactive mutant could also bind to the substrate but with a lower affinity than ST-Pez_{D1079A} resulting in still lower efficiency of EMT. A possible explanation for the lower incidence of EMT caused by the expression of the ΔFERM-Pez deletion mutant which contains the phosphatase domain may be that it binds less efficiently to the substrate due to misfolding or the loss of the FERM protein binding domain. Further characterisation of the activity and localisation of the mutants is required to elucidate how Pez is initiating the apparent EMT.

MDCK cells originate from the renal collecting duct and consist of different cell subtypes. It has been shown that one of the clonal lines, MDCK-C7 cells, will undergo an EMT resulting from sustained alkaline stress (Oberleithner et al., 1991; Pollack et al., 1997). Following transfection there was a period of several days during which the cells were subjected to selective medium when the cell number was quite low that could possibly result in fluctuations in the pH of the growth medium. However, the growth medium was changed regularly ensuring that there was adequate buffering of the cells. The complete absence of a mesenchymal transition in cell lines expressing either of the two PTP-deleted Pez constructs or the vector-transfected control confirms that the EMT is due to the expression of Pez and not alkaline stress.

The loss of cell-cell adhesion following expression of wt-Pez was contrary to the phenotype that we expected. Tyrosine phosphorylation of AJ proteins has been widely reported to destabilise junctions. Therefore, as Pez expression causes a reduction in the tyrosine phosphorylation of the proteins at the cell junctions including β-catenin this would be expected to increase the stability of adhesive function. This unexpected finding is discussed further in chapter 9.

Truncation mutants of the tight junction protein ZO-1 induce a time dependent EMT in corneal endothelial cells (Ryeom et al., 2000) similar to that seen in Pez expressing MDCK cells. Furthermore, expression of the PDZ domains of ZO-1 in MDCK cells also resulted in an EMT, which correlated with the activation of β-catenin/TCF/LEF signalling. The co-expression of the APC tumour suppressor gene reverted the phenotype indicating that the EMT resulted from the deregulation of β-catenin signalling (Reichert et al., 2000). The association between Pez, ZO-1 and β-catenin (chapters 6 and 7) prompted the investigation of the β-catenin/TCF/LEF transactivation pathway as a possible initiator of the observed mesenchymal transition in MDCK-Pez cells.

Further investigation needs to be carried out in order to elucidate how Pez expression is initiating this transition. The time-dependent nature of the transition, which entails multiple cell divisions prior to the change in phenotype, argues against a direct affect on the cell junctions being the cause of the EMT. The presence of Pez in the nucleus hints at a role in regulating transcription. A possible mechanism that may initiate the transition is the activation of the *snail* transcription factor, which has been shown to induce EMT through the direct repression of E-cadherin expression (Cano et al., 2000).

CHAPTER NINE
General conclusions and Discussion

Chapter 9:General conclusions and discussion

9.1 Conclusions

The experimental data presented in this thesis provide evidence that PTP-Pez is an active phosphatase that interacts with and dephosphorylates the adherens junction protein β-catenin. PTP-Pez also associates with proteins that form part of the tight junction complex, the scaffolding protein ZO-1 and the transmembrane protein occludin. Preliminary evidence suggests that occludin is also a Pez substrate.

In confluent cells Pez is distributed throughout the cell but is concentrated at the cell-cell junctions, which is consistent with its role in the regulation of adhesive function. However, in subconfluent, proliferating cell cultures Pez translocates out of the cytoplasm and concentrates in the nucleus. Nuclear staining reduces as cell monolayers reach confluence and also when the cells are serum-starved to induce quiescence. Preliminary evidence suggests that nuclear/cytoplasmic translocation of Pez is regulated by serine/threonine phosphorylation.

Nuclear localisation can be induced by serum stimulation of serum-starved cells and also following the impairment of cell-cell contacts by mechanical "wounding" of the monolayer. The functional role of nuclear Pez is yet to be investigated. Possibly the relocalisation is to sequester the phosphatase away from the cell adhesions to permit phosphorylation of junctional substrates. An example of nuclear sequestration is found in the regulation of cell polarity in budding yeast in which Cdc24, the guanine-nucleotide exchange factor for the yeast GTPase Cdc42, is sequestered in the cell nucleus by Far1(Shimada et al., 2000). However, a number of junctional complex proteins including $p120^{ctn}$ (reviewed in Anastasiadis and Reynolds, 2000), β-catenin (reviewed in (Peifer and Polakis, 2000; Seidensticker and Behrens, 2000) and ZO-1 (Balda and Matter, 2000) have been found to also function as transcriptional regulators in the nucleus. Although there are no reports to date of a PTP acting as a transcriptional activator or repressor, members of the PTP

family have been shown to act as negative regulators of STATs in the nucleus. Other nuclear roles of PTP family members are discussed in section 4.4

There are differences in the reported functions and localisation of the murine homologue of Pez PTP36 compared to PTP-Pez. PTP36, ectopically expressed in HeLa cells, co-fractionated with the actin cytoskeleton and its expression was associated with a loss in cell-matrix adhesion (Ogata et al., 1999 a). When a mouse fibroblast cell line ectopically expressing PTP36 was detached from the extracellular matrix, PTP36 translocated from the cytosolic to the cytoskeletal fraction (Ogata et al., 1999 b). These data indicate that PTP36 is involved in the regulation of cell-matrix, rather than cell-cell, adhesion. Furthermore the localisation of PTP36, to the nucleus has not been reported (Sawada et al., 1994; Ogata et al, 1999 a; Ogata et al., 1999 b; Ogata et al., 1999 b). There are several possible explanations for these differences in localisation of the two PTPs. Firstly PTP36 localisation is based primarily on overexpression data. Ectopically expressed proteins do not always localise to the appropriate subcellular compartment. Furthermore, it is possible that some of the cytoskeletally attached PTP36 analysed by subcellular fractionation is localising to the adherens junctions. Unfortunately the immunofluorescence data shows only cells cultured at subconfluent levels in which the AJs have not formed. Therefore it cannot be determined whether PTP36 is at the AJs under these conditions as the fractionation data does not discriminate between the focal adhesion associated pool of actin and the adherens junction associated pool.

It is also notable that the putative bipartite nuclear-localisation signal at the C-terminal end of PTP-Pez (k_{901}kkleegmvfteyeqipkkk) is not conserved in PTP36 (k_{903}kkledgmvfteyeqip **n**kk), possibly resulting in a species-specific difference in subcellular-localisation. Species-specific differences in subcellular distribution have been reported for other proteins. For example, the putative RNA binding protein DAZL translocates between the nucleus and the cytoplasm in

human testis but is almost exclusively cytoplasmic in rat testis (Ruggiu et al., 2000). Another illustration is the retinitis pigmentosa GTPase regulator (RPGR), which is distributed differently in the photoreceptors of mice and humans (Mavlyutov et al., 2002). If there is indeed a difference in the subcellular localisation of Pez and PTP36, it will be necessary to undertake detailed mapping analysis of the two PTPs to determine which sequences are responsible.

Ectopic expression of a mutant form of Pez, in which the phosphatase domain has been removed, increases the tyrosine phosphorylation of proteins localising to the cell junction complexes. Expression of this dominant-negative mutant specifically increases the tyrosine phosphorylation of β-catenin. An extensive body of literature analysing the effects of tyrosine phosphorylation on adhesive function suggests that the ectopic expression of wt-Pez would therefore have a stabilising effect on cell-cell adhesion. However, the stable expression of wt-Pez in a canine epithelial cell line resulted in an apparent transition to a mesenchymal phenotype in which there was a complete loss of cell-cell adhesion. Solving this apparent paradox is beyond the scope of this project; however, the extensive literature emerging on the multiplicity of interactions involved in controlling the adherens junction provides some possible explanations.

β-Catenin is a critical component of both the adherens junction and the Wnt signalling pathway. The identification of β-catenin as a substrate of Pez implicates a role for Pez in both cell signalling and cell-cell adhesion. Both of these functions may potentially be involved in the apparent EMT.

Tyrosine phosphorylation of β-catenin has been repeatedly correlated with loss of cadherin function (reviewed in Lilien et al., 2002; Daniel and Reynolds, 1997) and appears to be a crucial step in the dissociation of the adherens complex from the cytoskeleton. This has been correlated with disruption of the bond between β-catenin and α-catenin (reviewed in Provost and Rimm, 1999) and also the E-cadherin-β-catenin bond (reviewed in Lilien et al., 2002). *In vitro*

analysis shows that phosphorylation of Tyr-654, a residue in the last armadillo repeat of β-catenin, decreases its binding to E-cadherin (Roura et al., 1999). Thus the prevailing view is that the function of tyrosine phosphorylation of β-catenin is the destabilisation of the adhesion complex. However, other than the *in vitro* analysis (Roura et al., 1999; Piedra et al., 2001) most of the data is correlative and relates to the effect of tyrosine phosphorylation on confluent cells that have mature junctions. Moreover the *in vitro* analysis has so far been confined to the tyrosine residues within β-catenin that can be phosphorylated by $pp60^{c\text{-}src}$ (Roura et al., 1999; Piedra et al., 2001) and of these only Tyr-654 has been demonstrated to disrupt binding to cadherin. Another β-catenin residue, Tyr-86, is also phosphorylated by $pp60^{c\text{-}src}$, but as it does not lie within the cadherin binding domain phosphorylation of this residue did not decrease binding to cadherin (Roura et al., 1999), from which it can be construed that not all tyrosine phosphorylation of β-catenin disrupts binding to cadherin. There are other tyrosine residues within the β-catenin protein that may be phosphorylated by kinases other than $pp60^{c\text{-}src}$. This is substantiated by the finding that no changes in the *in vitro* association of β- and α-catenin were observed after phosphorylation of β-catenin by $pp60^{c\text{-}}$*src* (Roura et al., 1999), indicating that another kinase must regulate this interaction.

It cannot be assumed that phosphorylation of all β-catenin tyrosine residues will necessarily disrupt adhesion. There is evidence to show that tyrosine phosphorylation is in fact required for the formation of *de novo* cell-cell adhesions. The formation of AJ complexes in differentiating keratinocytes requires the tyrosine phosphorylation of AJ proteins, including β-catenin (Calautti et al., 1998). E-cadherin bound to tyrosine-phosphorylated β-catenin in proliferating normal breast-epithelial cells that were in the process of forming new cell-cell adhesions, (Takahashi et al., 1997). In these cells, α-catenin did not associate with the complex until the cells were confluent, following dephosphorylation of β-catenin (Takahashi et al., 1997).

Remodelling of the adherens junctions in endothelial cells in response to shear stress correlated with increased tyrosine phosphorylation of β-catenin, but the amount of β-catenin bound to VE-cadherin did not alter (Ukropec JA, Hollinger MK, Woolkalis MJ 2002). In newly formed endothelial cell adhesions the tyrosine phosphorylation of VE-cadherin, $p120^{ctn}$ and β-catenin increases the efficiency of their binding to form the adherens junction complex, while the formation of mature and cytoskeletally-attached junctions is accompanied by dephosphorylation. In these cells β-catenin is only found at the adherens junction in newly formed cell contacts, in which it is tyrosine-phosphorylated. In the mature adherens junction γ-catenin takes the place of β-catenin (Lampugnani et al., 1997). A study of adhesion in bovine aortic endothelial cells showed that γ-catenin and cadherin are recruited to the junctions of cells incubated with the PTP inhibitor vanadate, which is accompanied by a dramatic increase in junctional tyrosine phosphorylation (Ayalon and Geiger, 1997). Interestingly, another study showed that short-term stimulation of tyrosine phosphorylation induced the formation of adherens junctions in NIH3T3, CEF and COS cells, whereas prolonged inhibition of PTPs caused a breakdown of the cell-cell contacts (Michalides et al., 1994). Furthermore the passage from embryonic tissue where there is dynamic remodelling of cell adhesions, to adult tissue where the cell contacts are stable, is also associated with a transition of endothelial and epithelial AJ from a phosphorylated to a dephosphorylated state (Takata and Singer, 1988).

These reports indicate that tyrosine phosphorylation is required for the assembly of the adherens junctions during the formation of cell-cell contacts followed by a decrease in tyrosine phosphorylation as the junctions mature. The picture emerging from these studies is that tyrosine phosphorylation regulates both the assembly and disassembly of the cell adhesions. It is probable that specific tyrosine residues on the constituent adherens junction proteins exert differential effects on the assembly of the AJ and the strength of adhesions.

Chapter 9

A requirement for tyrosine phosphorylation of adherens junction proteins during the formation of cell-cell contacts may provide an explanation for the apparent EMT resulting from overexpression of PTP-Pez in MDCK cells. A model is assumed in which tyrosine phosphorylation is required for the dynamic restructuring of cell-cell junctions during processes that require the disruption and reassembly of the cell-cell contacts such as cell migration during embryogenesis and wound healing. Accordingly the adherens junction proteins are transiently tyrosine-phosphorylated on specific residues thereby initiating adhesion. The tyrosine phosphorylation of β-catenin would promote attachment to E-cadherin while preventing binding to α-catenin and consequently cytoskeletal attachment. The binding of α-catenin to β-catenin following dephosphorylation (Takahashi et al., 1997) would tether the complex to the actin cytoskeleton providing the stable contacts found in quiescent epithelial and endothelial tissues. In this model tyrosine phosphorylation of specific residues of β-catenin allows the rapid formation and restructuring of cell-cell contacts by preventing the formation of the more rigid structures that result from cytoskeletal tethering.

Pez activity would therefore be required to promote the formation of strong cell-cell contacts during development and wound healing. Pez activity would also be necessary for the maintenance of cell-cell adhesion in mature adherens junction complexes. When the cells are subconfluent and forming new adherens junctions then Pez would move into the nucleus enabling the tyrosine phosphorylation of β-catenin along with the other adherens junction proteins thereby facilitating their initial binding (Lampugnani et al., 1997). Overexpression of Pez would result in reduced tyrosine phosphorylation of β-catenin. Therefore, following cell division or cell migration the reattachment of β-catenin to cadherin would be inhibited leading to the loss of cell-cell adhesion. Decreased tyrosine phosphorylation would also result in the premature binding of β-catenin to α-catenin resulting in a loss of plasticity thereby preventing normal restructuring of

the junctional complexes (Takahashi et al., 1997; Ukropec JA, Hollinger MK, Woolkalis MJ 2002).

The removal of E-cadherin from the plasma membrane is essential for the migration of cells during gastrulation and neural crest migration and is a prerequisite for EMT. During development this is mediated by transcription factors such as Snail/Slug, which block E-cadherin transcription (reviewed in Savagner, 2001). E-cadherin in adult and embryonic tissue also undergoes active endocytosis and is then either recycled to locations of new cell-cell contacts or is degraded. In cells without stable cell contacts the pool being endocytosed is markedly increased (Le et al., 1999). Therefore, in the presence of overexpressed Pez, reduced binding of β-catenin to E-cadherin may prevent aggregation, which may result in the gradual loss of E-cadherin at the junctions due to increased endocytosis. The time-dependent onset of EMT seen in the cells overexpressing Pez is compatible with such a model as it is possible that several cell cycles would take place before the amount of E- cadherin localising to the junctions reached a critically low level, precipitating EMT.

Alternatively, the presence of increased cytoplasmic β-catenin resulting from inefficient binding to E-cadherin may cause an EMT via the Wnt signalling pathway. β-Catenin becomes diffusely localised in the cytoplasm during embryonic EMT and cytoplasmic β-catenin is not tyrosine-phosphorylated. It was suggested that non-phosphorylated cytoplasmic β-catenin in mesenchyme may be related to invasive motility (Kim et al., 1998). Recently there has also been direct evidence to show that LEF-1 can induce EMT when activated by stable nuclear β-catenin (Kim et al., 2002). Thus, the decreased efficiency of binding to E-cadherin due to reduced tyrosine phosphorylation of β-catenin in the presence of overexpressed Pez may lead to an increase in stable cytoplasmic β-catenin, thereby initiating an EMT via the TCF/LEF signalling pathway.

It has been demonstrated that the transcriptionally active pool of β-catenin can be depleted by the expression of E-cadherin (Gottardi et al., 2001); therefore this pool is able to bind to either E-cadherin or to TCF/LEF. Following Pez overexpression the β-catenin that is unable to bind efficiently to E-cadherin due to loss of tyrosine phosphorylation may still bind to TCF/LEF, resulting in transcriptional activation. The lack of supporting data presented here does not exclude this possibility, as the SW480 cells used in the transactivation assays were not optimal. These cells have constitutively high levels of cytoplasmic β-catenin, which is not transcriptionally active due to a mutation in GSK3β preventing its degradation (Gottardi et al., 2001). This large pool of cytoplasmic β-catenin may interfere with the dephosphorylation of the transcriptionally active pool of β-catenin that is associated with the junctions.

While β-catenin is tyrosine-phosphorylated following growth factor stimulation, it is not necessarily the same pool of β-catenin that is associated with E-cadherin. There are several pools of β-catenin within the cell, which are associated in different protein complexes. Moreover, it is apparent that there are biochemically distinct forms of β-catenin with differential binding capacities (Stewart and Nelson, 1997). It is unclear which of these pools constitute the Pez substrate. The recent finding that β-catenin translocates into the nucleus following tyrosine phosphorylation by the hepatocyte growth factor (HGF) receptor tyrosine kinase, Met, in a manner similar to Wnt signalling (Monga et al., 2002) suggested that Pez might be involved in regulating this pathway. HGF is known to cause disassembly of the cell-cell junctions and cell scattering leading to EMT in some cell lines (Woolf et al., 1995). High levels of expression of HGF and Met are associated with invasive human breast cancer and may be causally linked to metastasis (Elliott et al., 2002). However, although there are TCF/LEF binding sites on the E-cadherin promoter (Huber et al., 1996), the TCF/LEF transcription factor is a transcriptional activator and would therefore be expected to increase E-cadherin transcription.

Alternatively, the apparent EMT may be triggered by overexpressed Pez acting on a substrate other than β-catenin. The data presented in this thesis demonstrate that occludin is also dephosphorylated in the presence of active Pez. It is probable that there are other substrates that have not been identified in the substrate-trapping assay. The use of vanadate to enrich for tyrosine-phosphorylated substrates restricts the potential pool of substrates that may be identified to those that do not require the specific activation of a kinase. The serum stimulation of quiescent MDCK cells (Fig 7.7) shows that there is another unidentified potential substrate of approximately 130kDa. Possibly the action of Pez on this protein or other substrates is precipitating the EMT.

9.2 Future Directions

There are many avenues that could be pursued in the future investigation of the role of Pez. Overexpression of ΔPTP-Pez in MDCK cells leads to an increase in the tyrosine phosphorylation at the cell-cell junctions indicating that deletion of the gene is likely to yield some significant phenotypic information. An inducible gene targeting approach would be valuable in defining its function while possibly avoiding the problems of functional redundancy resulting from conventional gene knockouts. A Cre-mediated targeting approach has been used to achieve both temporally regulated and tissue specific gene expression (reviewed in Muller, 1999). This approach could be used to disrupt Pez expression in either endothelial or epithelial tissue at different stages in development.

β-Catenin plays an important role in embryonic development, with mRNA transcripts present in the fertilized egg of *Xenopus* embryos and increasing expression throughout embryogenesis (DeMarais and Moon, 1992; Schneider et al., 1993). The critical role of β-catenin in development is demonstrated by the formation of secondary body axis in *Xenopus* embryos by the injection of antibodies to β- catenin (McCrea et al., 1993). Therefore as a phosphatase of β-

catenin, Pez is also likely to play a developmental role. Future studies investigating the distribution and patterns of expression of Pez in the developing embryo by *in situ* hybridisation is likely to yield valuable insights into the regulation of β-catenin during development.

Several phosphatases have been reported to dephosphorylate β-catenin. It will be interesting to determine to what extent Pez and the other PTPs overlap in their regulation of AJ function. Possibly they play different roles in development and/or act on different tyrosine residues or are activated by different signalling pathways. Determining the specific tyrosine that is dephosphorylated by Pez will be an important first step in dissecting its involvement in regulating β-catenin.

The role of Pez in tight junction regulation is another area deserving further investigation. The precise function of the putative Pez substrate occludin is unclear, as it is not required for the formation of tight junctions and yet is an integral part of the junction complex (see section 8.1.4). It has been suggested that occludin is an intracellular signalling molecule (reviewed in Tsukita et al., 1999). The *in vivo* binding of Pez to the tight junction protein ZO-1 together with the translocalisation of both Pez and ZO-1 into the nucleus under similar conditions is consistent with the involvement of Pez in tight junction regulation. Analysis of the effect of Pez and ΔPTP-Pez on the dynamic restructuring of the tight junctions following their disassembly may provide insight into this aspect of Pez function.

The translocation of Pez under certain conditions, shown in chapter five, raises the question of its role within the nucleus. Preliminary experiments using nuclear extracts in the "substrate-trapping" assay indicate that there is at least one nuclear-specific Pez substrate (data not shown). This finding indicates that Pez may be catalytically active in the nucleus and that the translocation of Pez into the nucleus is not merely for the purpose of sequestration away from the cell-cell junctions. Other adherens junction proteins such as ZONAB and β-catenin are involved

in transcriptional regulation. Pez may regulate the activity of transcriptional protein complexes. An example of PTP association with transcriptional regulation is SHP-2, which has recently been shown to translocate into the nucleus as a complex with STAT5a and to bind and activate the β-casein gene promoter (Chughtai et al., 2002).

Pez could be acting in the nucleus to down-regulate signalling pathways such as the JAK/STAT signalling pathway (see section 1.2.2.1). TC-PTP, SHP-2 and PTP1B have been found to dephosphorylate STAT5 (Aoki and Matsuda, 2000; Yu et al., 2000; Aoki and Matsuda, 2002) while SHP-1 is involved in the negative regulation of Jak1, Jak2, STAT1 and STAT3 activation (Bousquet et al., 1999; David et al., 1995) as well as components of the IL-4/IL-13 signalling pathway (Haque et al., 1998). Other PTPs implicated in regulation of JAK/STAT signalling include PTP epsilon C (Tanuma et al., 2000; Tanuma et al., 2001) and CD45 (Blank et al., 2001; Irie-Sasaki et al., 2001). CD45, SHP1 and TC-PTP all localise to the nucleus however only TC-PTP has been reported to dephosphorylate STAT in the nucleus (Aoki and Matsuda, 2002).

Other tyrosine-phosphorylated signalling proteins that localise to the nucleus include the MAP kinases ERK, JNK and p38. The MAP kinases are dephosphorylated in the nucleus by the dual-specificity phosphatases MKP-1, MKP-2, MKP-7 and PAC1 (Bollen and Beullens, 2002). The classical PTP Ptp2 dephosphorylates the MAP kinase Hog1 in the nucleus of yeast and also acts as a nuclear tether (Mattison and Ota, 2000).

Isoforms of the signal transduction enzyme, protein kinase C (PKC) also translocate to the nucleus in response to certain stimuli. Tyrosine phosphorylation of PKC isoforms is associated with increased activation (Konishi et al., 1997) and in the case of atypical PKC facilitates translocation into the nucleus (White et al., 2002). Clearly there are a number of tyrosine-phosphorylated proteins within the nucleus that may potentially be substrates of Pez.

Moreover, as these potential substrates are involved in essential signalling pathways, elucidating the role that Pez is playing in the nucleus will potentially lead to a better understanding of signal transduction regulation.

Future research into the role of Pez should include a detailed analysis of the regulatory mechanisms governing its activity. As would be expected of proteins involved in signalling pathways, other members of the PTP family have been shown to be subject to tight regulation (see section 1. 8). There is some evidence that nuclear translocation of Pez is subject to regulation by serine/threonine phosphorylation (see section 5.11). Pez also appears to be tyrosine-phosphorylated under some conditions. Furthermore, the murine homologue of Pez, PTP36, is expressed in several different splice isoforms, in a tissue specific manner (Aoyama et al., 1999), indicating that there is regulation of the activity of this protein by alternative splicing.

The data presented in this thesis demonstrate that PTP-Pez is an important regulator of cell-cell adhesion in endothelial and epithelial cells. We have provided strong evidence that β-catenin is a Pez substrate. The critical roles played by β-catenin at the adherens junctions and in the Wnt signalling pathway during development suggest that Pez may be involved regulating key processes such as embryogenesis, tissue repair and oncogenesis.

References

References

Aberle, H., Bauer, A., Stappert, J., Kispert, A., and Kemler, R. (1997). beta-catenin is a target for the ubiquitin-proteasome pathway. Embo J *16*, 3797-804.

Aguiar, R. C., Yakushijin, Y., Kharbanda, S., Tiwari, S., Freeman, G. J., and Shipp, M. A. (1999). PTPROt: an alternatively spliced and developmentally regulated B- lymphoid phosphatase that promotes G0/G1 arrest. Blood *94*, 2403-13.

Akimoto, S., Ochiai, A., Inomata, M., and Hirohashi, S. (1998). Expression of cadherin-catenin cell adhesion molecules, phosphorylated tyrosine residues and growth factor receptor-tyrosine kinases in gastric cancers. Jpn J Cancer Res *89*, 829-36.

Alexander, D. R. (2000). The CD45 tyrosine phosphatase: a positive and negative regulator of immune cell function. Semin Immunol *12*, 349-59.

Anastasiadis, P. Z., and Reynolds, A. B. (2000). The p120 catenin family: complex roles in adhesion, signaling and cancer. J Cell Sci *113*, 1319-34.

Anderson, J. M., Stevenson, B. R., Jesaitis, L. A., Goodenough, D. A., and Mooseker, M. S. (1988). Characterization of ZO-1, a protein component of the tight junction from mouse liver and Madin-Darby canine kidney cells. J Cell Biol *106*, 1141-9.

Anderson, J. M., and Van Itallie, C. M. (1995). Tight junctions and the molecular basis for regulation of paracellular permeability. Am J Physiol *269*, G467-75.

Angers-Loustau, A., Cote, J. F., Charest, A., Dowbenko, D., Spencer, S., Lasky, L. A., and Tremblay, M. L. (1999). Protein tyrosine phosphatase-PEST regulates focal adhesion disassembly, migration, and cytokinesis in fibroblasts. J Cell Biol *144*, 1019-31.

Angres, B., Barth, A., and Nelson, W. J. (1996). Mechanism for transition from initial to stable cell-cell adhesion: kinetic analysis of E-cadherin-mediated adhesion using a quantitative adhesion assay. J Cell Biol *134*, 549-57.

Antonelli-Orlidge, A., Saunders, K. B., Smith, S. R., and D'Amore, P. A. (1989). An activated form of transforming growth factor beta is produced by cocultures of endothelial cells and pericytes. Proc Natl Acad Sci U S A *86*, 4544-8.

Antonetti, D. A., Barber, A. J., Hollinger, L. A., Wolpert, E. B., and Gardner, T. W. (1999). Vascular endothelial growth factor induces rapid phosphorylation of tight junction proteins occludin and zonula occluden 1. A potential mechanism for vascular permeability in diabetic retinopathy and tumors. J Biol Chem *274*, 23463-7.

Aoki, N., and Matsuda, T. (2000). A cytosolic protein-tyrosine phosphatase PTP1B specifically dephosphorylates and deactivates prolactin-activated STAT5a and STAT5b. J Biol Chem *275*, 39718-26.

Aoki, N., and Matsuda, T. (2002). A Nuclear Protein Tyrosine Phosphatase TC-PTP Is a Potential Negative Regulator of the PRL-Mediated Signaling Pathway: Dephosphorylation and Deactivation of Signal Transducer and Activator of Transcription 5a and 5b by TC-PTP in Nucleus. Mol Endocrinol *16*, 58-69.

Aono, S., Nakagawa, S., Reynolds, A. B., and Takeichi, M. (1999). p120(ctn) acts as an inhibitory regulator of cadherin function in colon carcinoma cells. J Cell Biol *145*, 551-62.

Aoyama, K., Matsuda, T., and Aoki, N. (1999). Characterization of newly identified four isoforms for a putative cytosolic protein tyrosine phosphatase PTP36. Biochem Biophys Res Commun *266*, 523-31.

Arenzana-Seisdedos, F., Turpin, P., Rodriguez, M., Thomas, D., Hay, R. T., Virelizier, J. L., and Dargemont, C. (1997). Nuclear localization of I kappa B alpha promotes active transport of NF- kappa B from the nucleus to the cytoplasm. J Cell Sci *110*, 369-78.

References

Arrandale, J. M., Gore-Willse, A., Rocks, S., Ren, J. M., Zhu, J., Davis, A., Livingston, J. N., and Rabin, D. U. (1996). Insulin signaling in mice expressing reduced levels of Syp. J Biol Chem *271*, 21353-8.

Arrate, M. P., Rodriguez, J. M., Tran, T. M., Brock, T. A., and Cunningham, S. A. (2001). Cloning of human junctional adhesion molecule 3 (JAM3) and its identification as the JAM2 counter-receptor. J Biol Chem *276*, 45826-32.

Atkinson, K. J., and Rao, R. K. (2001). Role of protein tyrosine phosphorylation in acetaldehyde-induced disruption of epithelial tight junctions. Am J Physiol Gastrointest Liver Physiol *280*, G1280-8.

Aurrand-Lions, M., Duncan, L., Ballestrem, C., and Imhof, B. A. (2001). JAM-2, a novel immunoglobulin superfamily molecule, expressed by endothelial and lymphatic cells. J Biol Chem *276*, 2733-41.

Ayalon, O., and Geiger, B. (1997). Cyclic changes in the organization of cell adhesions and the associated cytoskeleton, induced by stimulation of tyrosine phosphorylation in bovine aortic endothelial cells. J Cell Sci *110*, 547-56.

Bakin, A. V., Tomlinson, A. K., Bhowmick, N. A., Moses, H. L., and Arteaga, C. L. (2000). Phosphatidylinositol 3-kinase function is required for transforming growth factor beta-mediated epithelial to mesenchymal transition and cell migration. J Biol Chem *275*, 36803-10.

Balda, M. S., and Matter, K. (2000). The tight junction protein ZO-1 and an interacting transcription factor regulate ErbB-2 expression. Embo J *19*, 2024-33.

Balda, M. S., and Matter, K. (1998). Tight junctions. J Cell Sci *111*, 541-7.

Balda, M. S., Whitney, J. A., Flores, C., Gonzalez, S., Cereijido, M., and Matter, K. (1996). Functional dissociation of paracellular permeability and transepithelial electrical resistance and disruption of the apical-basolateral intramembrane diffusion barrier by expression of a mutant tight junction membrane protein. J Cell Biol *134*, 1031-49.

Balsamo, J., Arregui, C., Leung, T., and Lilien, J. (1998). The nonreceptor protein tyrosine phosphatase PTP1B binds to the cytoplasmic domain of N-cadherin and regulates the cadherin-actin linkage. J Cell Biol *143*, 523-32.

Balsamo, J., Leung, T., Ernst, H., Zanin, M. K., Hoffman, S., and Lilien, J. (1996). Regulated binding of PTP1B-like phosphatase to N-cadherin: control of cadherin-mediated adhesion by dephosphorylation of beta-catenin. J Cell Biol *134*, 801-13.

Bamforth, S. D., Kniesel, U., Wolburg, H., Engelhardt, B., and Risau, W. (1999). A dominant mutant of occludin disrupts tight junction structure and function. J Cell Sci *112*, 1879-88.

Banville, D., Ahmad, S., Stocco, R., and Shen, S. H. (1994). A novel protein-tyrosine phosphatase with homology to both the cytoskeletal proteins of the band 4.1 family and junction-associated guanylate kinases. J Biol Chem *269*, 22320-7.

Barford, D., Flint, A. J., and Tonks, N. K. (1994). Crystal structure of human protein tyrosine phosphatase 1B. Science *263*, 1397-404.

Barford, D., and Neel, B. G. (1998). Revealing mechanisms for SH2 domain mediated regulation of the protein tyrosine phosphatase SHP-2. Structure *6*, 249-54.

Barrett, W. C., DeGnore, J. P., Keng, Y. F., Zhang, Z. Y., Yim, M. B., and Chock, P. B. (1999). Roles of superoxide radical anion in signal transduction mediated by reversible regulation of protein-tyrosine phosphatase 1B. J Biol Chem *274*, 34543-6.

Bazzoni, G., Dejana, E., and Lampugnani, M. G. (1999). Endothelial adhesion molecules in the development of the vascular tree: the garden of forking paths. Curr Opin Cell Biol *11*, 573-81.

References

Beals, C. R., Clipstone, N. A., Ho, S. N., and Crabtree, G. R. (1997b). Nuclear localization of NF-ATc by a calcineurin-dependent, cyclosporin- sensitive intramolecular interaction. Genes Dev *11*, 824-34.

Beals, C. R., Sheridan, C. M., Turck, C. W., Gardner, P., and Crabtree, G. R. (1997a). Nuclear export of NF-ATc enhanced by glycogen synthase kinase-3. Science *275*, 1930-4.

Behrens, J., Jerchow, B. A., Wurtele, M., Grimm, J., Asbrand, C., Wirtz, R., Kuhl, M., Wedlich, D., and Birchmeier, W. (1998). Functional interaction of an axin homolog, conductin, with beta- catenin, APC, and GSK3beta. Science *280*, 596-9.

Behrens, J., von Kries, J. P., Kuhl, M., Bruhn, L., Wedlich, D., Grosschedl, R., and Birchmeier, W. (1996). Functional interaction of beta-catenin with the transcription factor LEF-1. Nature *382*, 638-42.

Behrens, J., Weidner, K. M., Frixen, U. H., Schipper, J. H., Sachs, M., Arakaki, N., Daikuhara, Y., and Birchmeier, W. (1991). The role of E-cadherin and scatter factor in tumor invasion and cell motility. Exs *59*, 109-26.

Bilwes, A. M., den Hertog, J., Hunter, T., and Noel, J. P. (1996). Structural basis for inhibition of receptor protein-tyrosine phosphatase-alpha by dimerization. Nature *382*, 555-9.

Birchmeier, C., Birchmeier, W., and Brand-Saberi, B. (1996). Epithelial-mesenchymal transitions in cancer progression. Acta Anat *156*, 217-26.

Birchmeier, W., Weidner, K. M., and Behrens, J. (1993). Molecular mechanisms leading to loss of differentiation and gain of invasiveness in epithelial cells. J Cell Sci Suppl *17*, 159-64.

Blank, N., Kriegel, M., Hieronymus, T., Geiler, T., Winkler, S., Kalden, J. R., and Lorenz, H. M. (2001). CD45 tyrosine phosphatase controls common gamma-chain cytokine-mediated STAT and extracellular signal-related kinase phosphorylation in activated human lymphoblasts: inhibition of proliferation without induction of apoptosis. J Immunol *166*, 6034-40.

Blasina, A., de Weyer, I. V., Laus, M. C., Luyten, W. H., Parker, A. E., and McGowan, C. H. (1999). A human homologue of the checkpoint kinase Cds1 directly inhibits Cdc25 phosphatase. Curr Biol *9*, 1-10.

Blume-Jensen, P., and Hunter, T. (2001). Oncogenic kinase signalling. Nature *411*, 355-65.

Bockholt, S. M., and Burridge, K. (1993). Cell spreading on extracellular matrix proteins induces tyrosine phosphorylation of tensin. J Biol Chem *268*, 14565-7.

Boddy, M. N., Furnari, B., Mondesert, O., and Russell, P. (1998). Replication checkpoint enforced by kinases Cds1 and Chk1. Science *280*, 909-12.

Bollen, M., and Beullens, M. (2002). Signaling by protein phosphatases in the nucleus. Trends Cell Biol *12*, 138-45.

Bolton, S. J., Anthony, D. C., and Perry, V. H. (1998). Loss of the tight junction proteins occludin and zonula occludens-1 from cerebral vascular endothelium during neutrophil-induced blood- brain barrier breakdown in vivo. Neuroscience *86*, 1245-57.

Bousquet, C., Susini, C., and Melmed, S. (1999). Inhibitory roles for SHP-1 and SOCS-3 following pituitary proopiomelanocortin induction by leukemia inhibitory factor. J Clin Invest *104*, 1277-85.

Boyer, B., Roche, S., Denoyelle, M., and Thiery, J. P. (1997). Src and Ras are involved in separate pathways in epithelial cell scattering. Embo J *16*, 5904-13.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem *72*, 248-54.

References

Brady-Kalnay, S. M., Rimm, D. L., and Tonks, N. K. (1995). Receptor protein tyrosine phosphatase PTPmu associates with cadherins and catenins in vivo. J Cell Biol *130*, 977-86.

Brady-Kalnay, S. M., and Tonks, N. K. (1994). Identification of the homophilic binding site of the receptor protein tyrosine phosphatase PTP mu. J Biol Chem *269*, 28472-7.

Braga, V. M. (1999). Small GTPases and regulation of cadherin dependent cell-cell adhesion. Mol Pathol *52*, 197-202.

Brautigan, D. L., and Pinault, F. M. (1993). Serine phosphorylation of protein tyrosine phosphatase (PTP1B) in HeLa cells in response to analogues of cAMP or diacylglycerol plus okadaic acid. Mol Cell Biochem *127-128*, 121-9.

Brieher, W. M., Yap, A. S., and Gumbiner, B. M. (1996). Lateral dimerization is required for the homophilic binding activity of C-cadherin. J Cell Biol *135*, 487-96.

Brown, M. T., and Cooper, J. A. (1996). Regulation, substrates and functions of src. Biochim Biophys Acta *1287*, 121-49.

Buist, A., Blanchetot, C., Tertoolen, L. G., and den Hertog, J. (2000). Identification of p130cas as an in vivo substrate of receptor protein- tyrosine phosphatase alpha. J Biol Chem *275*, 20754-61.

Burns, C. M., Sakaguchi, K., Appella, E., and Ashwell, J. D. (1994). CD45 regulation of tyrosine phosphorylation and enzyme activity of src family kinases. J Biol Chem *269*, 13594-600.

Burridge, K., Turner, C. E., and Romer, L. H. (1992). Tyrosine phosphorylation of paxillin and pp125FAK accompanies cell adhesion to extracellular matrix: a role in cytoskeletal assembly. J Cell Biol *119*, 893-903.

Byth, K. F., Conroy, L. A., Howlett, S., Smith, A. J., May, J., Alexander, D. R., and Holmes, N. (1996). CD45-null transgenic mice reveal a positive regulatory role for CD45 in early thymocyte development, in the selection of CD4+CD8+ thymocytes, and B cell maturation. J Exp Med *183*, 1707-18.

Calautti, E., Cabodi, S., Stein, P. L., Hatzfeld, M., Kedersha, N., and Paolo Dotto, G. (1998). Tyrosine phosphorylation and src family kinases control keratinocyte cell-cell adhesion. J Cell Biol *141*, 1449-65.

Calautti, E., Grossi, M., Mammucari, C., Aoyama, Y., Pirro, M., Ono, Y., Li, J., and Dotto, G. P. (2002). Fyn tyrosine kinase is a downstream mediator of Rho/PRK2 function in keratinocyte cell-cell adhesion. J Cell Biol *156*, 137-48.

Cangi, M. G., Cukor, B., Soung, P., Signoretti, S., Moreira, G., Jr., Ranashinge, M., Cady, B., Pagano, M., and Loda, M. (2000). Role of the Cdc25A phosphatase in human breast cancer. J Clin Invest *106*, 753-61.

Cano, A., Perez-Moreno, M. A., Rodrigo, I., Locascio, A., Blanco, M. J., del Barrio, M. G., Portillo, F., and Nieto, M. A. (2000). The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. Nat Cell Biol *2*, 76-83. java/Propub/cellbio/ncb0200_76.fulltext java/Propub/cellbio/ncb0200_76.abstract.

Charest, A., Wagner, J., Jacob, S., McGlade, C. J., and Tremblay, M. L. (1996). Phosphotyrosine-independent binding of SHC to the NPLH sequence of murine protein-tyrosine phosphatase-PEST. Evidence for extended phosphotyrosine binding/phosphotyrosine interaction domain recognition specificity. J Biol Chem *271*, 8424-9.

Chen, H. E., Chang, S., Trub, T., and Neel, B. G. (1996). Regulation of colony-stimulating factor 1 receptor signaling by the SH2 domain-containing tyrosine phosphatase SHPTP1. Mol Cell Biol *16*, 3685-97.

Chen, Y., Lu, Q., Schneeberger, E. E., and Goodenough, D. A. (2000). Restoration of tight junction structure and barrier function by down- regulation of the mitogen-activated protein kinase pathway in ras-transformed Madin-Darby canine kidney cells. Mol Biol Cell *11*, 849-62.

References

Chen, Y., Merzdorf, C., Paul, D. L., and Goodenough, D. A. (1997). COOH terminus of occludin is required for tight junction barrier function in early Xenopus embryos. J Cell Biol *138*, 891-9.

Chen, Z., and Han, M. (2000). Building a protein interaction map: research in the post-genome era. Bioessays *22*, 503-6.

Cheng, A., Dube, N., Gu, F., and Tremblay, M. L. (2002). Coordinated action of protein tyrosine phosphatases in insulin signal transduction. Eur J Biochem *269*, 1050-9.

Cheng, J., Wu, K., Armanini, M., O'Rourke, N., Dowbenko, D., and Lasky, L. A. (1997). A novel protein-tyrosine phosphatase related to the homotypically adhering kappa and mu receptors. J Biol Chem *272*, 7264-77.

Chiarugi, P., Cirri, P., Taddei, L., Giannoni, E., Camici, G., Manao, G., Raugei, G., and Ramponi, G. (2000). The low M(r) protein-tyrosine phosphatase is involved in Rho-mediated cytoskeleton rearrangement after integrin and platelet-derived growth factor stimulation. J Biol Chem *275*, 4640-6.

Cho, H., Krishnaraj, R., Itoh, M., Kitas, E., Bannwarth, W., Saito, H., and Walsh, C. T. (1993). Substrate specificities of catalytic fragments of protein tyrosine phosphatases (HPTP beta, LAR, and CD45) toward phosphotyrosylpeptide substrates and thiophosphotyrosylated peptides as inhibitors. Protein Sci *2*, 977-84.

Christofori, G., and Semb, H. (1999). The role of the cell-adhesion molecule E-cadherin as a tumour-suppressor gene. Trends Biochem Sci *24*, 73-6.

Chughtai, N., Schimchowitsch, S., Lebrun, J. J., and Ali, S. (2002). Prolactin induces SHP-2 association with Stat5, nuclear translocation, and binding to the beta-casein gene promoter in mammary cells. J Biol Chem *11*, 11.

Citi, S., and Denisenko, N. (1995). Phosphorylation of the tight junction protein cingulin and the effects of protein kinase inhibitors and activators in MDCK epithelial cells. J Cell Sci *108*, 2917-26.

Citi, S., Sabanay, H., Jakes, R., Geiger, B., and Kendrick-Jones, J. (1988). Cingulin, a new peripheral component of tight junctions. Nature *333*, 272-6.

Collares-Buzato, C. B., Jepson, M. A., Simmons, N. L., and Hirst, B. H. (1998). Increased tyrosine phosphorylation causes redistribution of adherens junction and tight junction proteins and perturbs paracellular barrier function in MDCK epithelia. Eur J Cell Biol *76*, 85-92.

Cong, F., Spencer, S., Cote, J. F., Wu, Y., Tremblay, M. L., Lasky, L. A., and Goff, S. P. (2000). Cytoskeletal protein PSTPIP1 directs the PEST-type protein tyrosine phosphatase to the c-Abl kinase to mediate Abl dephosphorylation. Mol Cell *6*, 1413-23.

Cote, J. F., Charest, A., Wagner, J., and Tremblay, M. L. (1998). Combination of gene targeting and substrate trapping to identify substrates of protein tyrosine phosphatases using PTP-PEST as a model. Biochemistry *37*, 13128-37.

Cozzolino, M., Giovannone, B., Serafino, A., Knudsen, K., Levi, A., Alema, S., and Salvatore, A. (2000). Activation of TrkA tyrosine kinase in embryonal carcinoma cells promotes cell compaction, independently of tyrosine phosphorylation of catenins. J Cell Sci *113*, 1601-10.

Cunningham, S. A., Rodriguez, J. M., Arrate, M. P., Tran, T. M., and Brock, T. A. (2002). JAM2 interacts with alpha4beta1. Facilitation by JAM3. J Biol Chem *277*, 27589-92.

Daniel, J. M., and Reynolds, A. B. (1997). Tyrosine phosphorylation and cadherin/catenin function. Bioessays *19*, 883-91.

References

David, M., Chen, H. E., Goelz, S., Larner, A. C., and Neel, B. G. (1995). Differential regulation of the alpha/beta interferon-stimulated Jak/Stat pathway by the SH2 domain-containing tyrosine phosphatase SHPTP1. Mol Cell Biol *15*, 7050-8.

del Barrio, M. G., and Nieto, M. A. (2002). Overexpression of Snail family members highlights their ability to promote chick neural crest formation. Development *129*, 1583-93.

Del Maschio, A., De Luigi, A., Martin-Padura, I., Brockhaus, M., Bartfai, T., Fruscella, P., Adorini, L., Martino, G., Furlan, R., De Simoni, M. G., and Dejana, E. (1999). Leukocyte recruitment in the cerebrospinal fluid of mice with experimental meningitis is inhibited by an antibody to junctional adhesion molecule (JAM). J Exp Med *190*, 1351-6.

DeMarais, A. A., and Moon, R. T. (1992). The armadillo homologs beta-catenin and plakoglobin are differentially expressed during early development of Xenopus laevis. Dev Biol *153*, 337-46.

Denk, H., Krepler, R., Artlieb, U., Gabbiani, G., Rungger-Brandle, E., Leoncini, P., and Franke, W. W. (1983). Proteins of intermediate filaments. An immunohistochemical and biochemical approach to the classification of soft tissue tumors. Am J Pathol *110*, 193-208.

Denu, J. M., Lohse, D. L., Vijayalakshmi, J., Saper, M. A., and Dixon, J. E. (1996b). Visualization of intermediate and transition-state structures in protein-tyrosine phosphatase catalysis. Proc Natl Acad Sci U S A *93*, 2493-8.

Denu, J. M., Stuckey, J. A., Saper, M. A., and Dixon, J. E. (1996a). Form and function in protein dephosphorylation. Cell *87*, 361-4.

Denu, J. M., and Tanner, K. G. (1998). Specific and reversible inactivation of protein tyrosine phosphatases by hydrogen peroxide: evidence for a sulfenic acid intermediate and implications for redox regulation. Biochemistry *37*, 5633-42.

Di Cristofano, A., and Pandolfi, P. P. (2000). The multiple roles of PTEN in tumor suppression. Cell *100*, 387-90.

Dingwall, C., Robbins, J., and Dilworth, S. M. (1989). Characterisation of the nuclear location sequence of Xenopus nucleoplasmin. J Cell Sci Suppl *11*, 243-8.

Dixon, D., Moyana, T., and King, M. J. (1998). Elevated expression of the cdc25A protein phosphatase in colon cancer. Exp Cell Res *240*, 236-43.

Downing, J. R., and Reynolds, A. B. (1991). PDGF, CSF-1, and EGF induce tyrosine phosphorylation of p120, a pp60src transformation-associated substrate. Oncogene *6*, 607-13.

Dragsten, P. R., Blumenthal, R., and Handler, J. S. (1981). Membrane asymmetry in epithelia: is the tight junction a barrier to diffusion in the plasma membrane? Nature *294*, 718-22.

Dye, J. F., Leach, L., Clark, P., and Firth, J. A. (2001). Cyclic AMP and acidic fibroblast growth factor have opposing effects on tight and adherens junctions in microvascular endothelial cells in vitro. Microvasc Res *62*, 94-113.

Edens, H. A., and Parkos, C. A. (2000). Modulation of epithelial and endothelial paracellular permeability by leukocytes. Adv Drug Deliv Rev *41*, 315-28.

Eger, A., Stockinger, A., Schaffhauser, B., Beug, H., and Foisner, R. (2000). Epithelial mesenchymal transition by c-Fos estrogen receptor activation involves nuclear translocation of beta-catenin and upregulation of beta- catenin/lymphoid enhancer binding factor-1 transcriptional activity. J Cell Biol *148*, 173-88.

Elchebly, M., Payette, P., Michaliszyn, E., Cromlish, W., Collins, S., Loy, A. L., Normandin, D., Cheng, A., Himms-Hagen, J., Chan, C. C., Ramachandran, C., Gresser, M. J., Tremblay, M. L., and Kennedy, B. P. (1999).

References

Increased insulin sensitivity and obesity resistance in mice lacking the protein tyrosine phosphatase-1B gene. Science *283*, 1544-8.

Elliott, B. E., Hung, W. L., Boag, A. H., and Tuck, A. B. (2002). The role of hepatocyte growth factor (scatter factor) in epithelial- mesenchymal transition and breast cancer. Can J Physiol Pharmacol *80*, 91-102.

Endo, N., Rutledge, S. J., Opas, E. E., Vogel, R., Rodan, G. A., and Schmidt, A. (1996). Human protein tyrosine phosphatase-sigma: alternative splicing and inhibition by bisphosphonates. J Bone Miner Res *11*, 535-43.

Enenkel, C., Blobel, G., and Rexach, M. (1995). Identification of a yeast karyopherin heterodimer that targets import substrate to mammalian nuclear pore complexes. J Biol Chem *270*, 16499-502.

Erdmann, K. S., Kuhlmann, J., Lessmann, V., Herrmann, L., Eulenburg, V., Muller, O., and Heumann, R. (2000). The Adenomatous Polyposis Coli-protein (APC) interacts with the protein tyrosine phosphatase PTP-BL via an alternatively spliced PDZ domain. Oncogene *19*, 3894-901.

Esser, S., Lampugnani, M. G., Corada, M., Dejana, E., and Risau, W. (1998). Vascular endothelial growth factor induces VE-cadherin tyrosine phosphorylation in endothelial cells. J Cell Sci *111*, 1853-65.

Fachinger, G., Deutsch, U., and Risau, W. (1999). Functional interaction of vascular endothelial-protein-tyrosine phosphatase with the angiopoietin receptor Tie-2. Oncogene *18*, 5948-53.

Falet, H., Pain, S., and Rendu, F. (1998). Tyrosine unphosphorylated platelet SHP-1 is a substrate for calpain. Biochem Biophys Res Commun *252*, 51-5.

Fantus, I. G., and Tsiani, E. (1998). Multifunctional actions of vanadium compounds on insulin signaling pathways: evidence for preferential enhancement of metabolic versus mitogenic effects. Mol Cell Biochem *182*, 109-19.

Fauman (1996). Structure and Function of protein tyrosine phosphatases. TIBS, 413 -417.

Fauman, E. B., Yuvaniyama, C., Schubert, H. L., Stuckey, J. A., and Saper, M. A. (1996). The X-ray crystal structures of Yersinia tyrosine phosphatase with bound tungstate and nitrate. Mechanistic implications. J Biol Chem *271*, 18780-8.

Fitzgerald, L. R., Walton, K. M., Dixon, J. E., and Largent, B. L. (1997). PTP NE-6: a brain-enriched receptor-type protein tyrosine phosphatase with a divergent catalytic domain. J Neurochem *68*, 1820-9.

Flint, A. J., Gebbink, M. F., Franza, B. R., Jr., Hill, D. E., and Tonks, N. K. (1993). Multi-site phosphorylation of the protein tyrosine phosphatase, PTP1B: identification of cell cycle regulated and phorbol ester stimulated sites of phosphorylation. Embo J *12*, 1937-46.

Flint, A. J., Tiganis, T., Barford, D., and Tonks, N. K. (1997). Development of "substrate-trapping" mutants to identify physiological substrates of protein tyrosine phosphatases. Proc Natl Acad Sci U S A *94*, 1680-5.

Forman-Kay, J. D., and Pawson, T. (1999). Diversity in protein recognition by PTB domains. Curr Opin Struct Biol *9*, 690-5.

Fornerod, M., Ohno, M., Yoshida, M., and Mattaj, I. W. (1997). CRM1 is an export receptor for leucine-rich nuclear export signals. Cell *90*, 1051-60.

Frangioni, J. V., Beahm, P. H., Shifrin, V., Jost, C. A., and Neel, B. G. (1992). The nontransmembrane tyrosine phosphatase PTP-1B localizes to the endoplasmic reticulum via its 35 amino acid C-terminal sequence. Cell *68*, 545-60.

Frangioni, J. V., Oda, A., Smith, M., Salzman, E. W., and Neel, B. G. (1993). Calpain-catalyzed cleavage and subcellular relocation of protein phosphotyrosine phosphatase 1B (PTP-1B) in human platelets. Embo J *12*, 4843-56.

References

Fuchs, M., Muller, T., Lerch, M. M., and Ullrich, A. (1996). Association of human protein-tyrosine phosphatase kappa with members of the armadillo family. J Biol Chem *271*, 16712-9.

Fujioka, Y., Matozaki, T., Noguchi, T., Iwamatsu, A., Yamao, T., Takahashi, N., Tsuda, M., Takada, T., and Kasuga, M. (1996). A novel membrane glycoprotein, SHPS-1, that binds the SH2-domain- containing protein tyrosine phosphatase SHP-2 in response to mitogens and cell adhesion. Mol Cell Biol *16*, 6887-99.

Fukuhara, A., Irie, K., Nakanishi, H., Takekuni, K., Kawakatsu, T., Ikeda, W., Yamada, A., Katata, T., Honda, T., Sato, T., Shimizu, K., Ozaki, H., Horiuchi, H., Kita, T., and Takai, Y. (2002). Involvement of nectin in the localization of junctional adhesion molecule at tight junctions. Oncogene *21*, 7642-55.

Furnari, B., Rhind, N., and Russell, P. (1997). Cdc25 mitotic inducer targeted by chk1 DNA damage checkpoint kinase. Science *277*, 1495-7.

Furukawa, T., Itoh, M., Krueger, N. X., Streuli, M., and Saito, H. (1994). Specific interaction of the CD45 protein-tyrosine phosphatase with tyrosine-phosphorylated CD3 zeta chain. Proc Natl Acad Sci U S A *91*, 10928-32.

Furuse, M., Fujita, K., Hiiragi, T., Fujimoto, K., and Tsukita, S. (1998). Claudin-1 and -2: novel integral membrane proteins localizing at tight junctions with no sequence similarity to occludin. J Cell Biol *141*, 1539-50.

Furuse, M., Hirase, T., Itoh, M., Nagafuchi, A., Yonemura, S., and Tsukita, S. (1993). Occludin: a novel integral membrane protein localizing at tight junctions. J Cell Biol *123*, 1777-88.

Furuse, M., Sasaki, H., and Tsukita, S. (1999). Manner of interaction of heterogeneous claudin species within and between tight junction strands. J Cell Biol *147*, 891-903.

Ganapati, U., Gupta, S., Radha, V., Sudhakar, C., Manogaran, P. S., and Swarup, G. (2001). A nuclear protein tyrosine phosphatase induces shortening of G1 phase and increase in c-Myc protein level. Exp Cell Res *265*, 1-10.

Garton, A. J., Burnham, M. R., Bouton, A. H., and Tonks, N. K. (1997). Association of PTP-PEST with the SH3 domain of p130cas; a novel mechanism of protein tyrosine phosphatase substrate recognition. Oncogene *15*, 877-85.

Garton, A. J., Flint, A. J., and Tonks, N. K. (1996). Identification of p130(cas) as a substrate for the cytosolic protein tyrosine phosphatase PTP-PEST. Mol Cell Biol *16*, 6408-18.

Gil-Henn, H., Volohonsky, G., and Elson, A. (2001). Regulation of protein tyrosine phosphatases {alpha} and {epsilon} by calpain-mediated proteolytic processing. J Biol Chem *27*, 27.

Gorlich, D., Prehn, S., Laskey, R. A., and Hartmann, E. (1994). Isolation of a protein that is essential for the first step of nuclear protein import. Cell *79*, 767-78.

Gottardi, C. J., Wong, E., and Gumbiner, B. M. (2001). E-Cadherin Suppresses Cellular Transformation by Inhibiting beta- Catenin Signaling in an Adhesion-independent Manner. J Cell Biol *153*, 1049-60.

Gow, A., Southwood, C. M., Li, J. S., Pariali, M., Riordan, G. P., Brodie, S. E., Danias, J., Bronstein, J. M., Kachar, B., and Lazzarini, R. A. (1999). CNS myelin and sertoli cell tight junction strands are absent in Osp/claudin-11 null mice. Cell *99*, 649-59.

Green, M. C., and Shultz, L. D. (1975). Motheaten, an immunodeficient mutant of the mouse. I. Genetics and pathology. J Hered *66*, 250-8.

Greer, S. F., Wang, Y., Raman, C., and Justement, L. B. (2001). CD45 function is regulated by an acidic 19-amino acid insert in domain II that serves as a binding and phosphoacceptor site for casein kinase 2. J Immunol *166*, 7208-18.

References

Grosheva, I., Shtutman, M., Elbaum, M., and Bershadsky, A. D. (2001). p120 catenin affects cell motility via modulation of activity of Rho- family GTPases: a link between cell-cell contact formation and regulation of cell locomotion. J Cell Sci *114*, 695-707.

Gross, C., Heumann, R., and Erdmann, K. S. (2001). The protein kinase C-related kinase PRK2 interacts with the protein tyrosine phosphatase PTP-BL via a novel PDZ domain binding motif. FEBS Lett *496*, 101-4.

Gu, H., Pratt, J. C., Burakoff, S. J., and Neel, B. G. (1998). Cloning of p97/Gab2, the major SHP2-binding protein in hematopoietic cells, reveals a novel pathway for cytokine-induced gene activation. Mol Cell *2*, 729-40.

Gu, H., Saito, K., Klaman, L. D., Shen, J., Fleming, T., Wang, Y., Pratt, J. C., Lin, G., Lim, B., Kinet, J. P., and Neel, B. G. (2001). Essential role for Gab2 in the allergic response. Nature *412*, 186-90.

Gu, M., and Majerus, P. W. (1996). The properties of the protein tyrosine phosphatase PTPMEG. J Biol Chem *271*, 27751-9.

Gu, M., York, J., Warshawsky, I., and Majerus, P. (1991). Identification, Cloning, and Expression of a Cytosolic Megakaryocyte Protein-Tyrosine-Phosphatase with Sequence Homology to Cytoskeletal Protein 4.1. PNAS *88*, 5867-5871.

Guger, K. A., and Gumbiner, B. M. (2000). A mode of regulation of beta-catenin signaling activity in Xenopus embryos independent of its levels. Dev Biol *223*, 441-8.

Guilford, P., Hopkins, J., Harraway, J., McLeod, M., McLeod, N., Harawira, P., Taite, H., Scoular, R., Miller, A., and Reeve, A. E. (1998). E-cadherin germline mutations in familial gastric cancer. Nature *392*, 402-5.

Gumbiner, B. (1987). Structure, biochemistry, and assembly of epithelial tight junctions. Am J Physiol *253*, C749-58.

Gumbiner, B. M. (1993). Breaking through the tight junction barrier. J Cell Biol *123*, 1631-3.

Habib, T., Herrera, R., and Decker, S. J. (1994). Activators of protein kinase C stimulate association of Shc and the PEST tyrosine phosphatase. J Biol Chem *269*, 25243-6.

Hagting, A., Jackman, M., Simpson, K., and Pines, J. (1999). Translocation of cyclin B1 to the nucleus at prophase requires a phosphorylation-dependent nuclear import signal. Curr Biol *9*, 680-9.

Haj, F. G., Verveer, P. J., Squire, A., Neel, B. G., and Bastiaens, P. I. (2002). Imaging sites of receptor dephosphorylation by PTP1B on the surface of the endoplasmic reticulum. Science *295*, 1708-11.

Hanahan, D., and Weinberg, R. A. (2000). The hallmarks of cancer. Cell *100*, 57-70.

Haque, S. J., Harbor, P., Tabrizi, M., Yi, T., and Williams, B. R. (1998). Protein-tyrosine phosphatase Shp-1 is a negative regulator of IL-4- and IL-13-dependent signal transduction. J Biol Chem *273*, 33893-6.

Harada, N., Tamai, Y., Ishikawa, T., Sauer, B., Takaku, K., Oshima, M., and Taketo, M. M. (1999). Intestinal polyposis in mice with a dominant stable mutation of the beta-catenin gene. Embo J *18*, 5931-42.

Harder, K. W., Moller, N. P., Peacock, J. W., and Jirik, F. R. (1998). Protein-tyrosine phosphatase alpha regulates Src family kinases and alters cell-substratum adhesion. J Biol Chem *273*, 31890-900.

Harroch, S., Palmeri, M., Rosenbluth, J., Custer, A., Okigaki, M., Shrager, P., Blum, M., Buxbaum, J. D., and Schlessinger, J. (2000). No obvious abnormality in mice deficient in receptor protein tyrosine phosphatase beta. Mol Cell Biol *20*, 7706-15.

Hashimoto, N., Feener, E. P., Zhang, W. R., and Goldstein, B. J. (1992). Insulin receptor protein-tyrosine phosphatases. Leukocyte common antigen-related phosphatase rapidly deactivates the insulin receptor kinase by preferential dephosphorylation of the receptor regulatory domain. J Biol Chem *267*, 13811-4.

References

Haskins, J., Gu, L., Wittchen, E. S., Hibbard, J., and Stevenson, B. R. (1998). ZO-3, a novel member of the MAGUK protein family found at the tight junction, interacts with ZO-1 and occludin. J Cell Biol *141*, 199-208.

Hay, E. D. (1995). An overview of epithelio-mesenchymal transformation. Acta Anat *154*, 8-20.

Hazan, R. B., and Norton, L. (1998). The epidermal growth factor receptor modulates the interaction of E-cadherin with the actin cytoskeleton. J Biol Chem *273*, 9078-84.

Hellberg, C. B., Burden-Gulley, S. M., Pietz, G. E., and Brady-Kalnay, S. M. (2002). Expression of the receptor protein tyrosine phosphatase, PTPmu, restores E-cadherin-dependent adhesion in human prostate carcinoma cells. J Biol Chem *18*, 18.

Henkel, T., Zabel, U., van Zee, K., Muller, J. M., Fanning, E., and Baeuerle, P. A. (1992). Intramolecular masking of the nuclear location signal and dimerization domain in the precursor for the p50 NF-kappa B subunit. Cell *68*, 1121-33.

Herbst, R., Carroll, P. M., Allard, J. D., Schilling, J., Raabe, T., and Simon, M. A. (1996). Daughter of sevenless is a substrate of the phosphotyrosine phosphatase Corkscrew and functions during sevenless signaling. Cell *85*, 899-909.

Higashitsuji, H., Arii, S., Furutani, M., Imamura, M., Kaneko, Y., Takenawa, J., Nakayama, H., and Fujita, J. (1995). Enhanced expression of multiple protein tyrosine phosphatases in the regenerating mouse liver: isolation of PTP-RL10, a novel cytoplasmic- type phosphatase with sequence homology to cytoskeletal protein 4.1. Oncogene *10*, 407-14.

Hippen, K. L., Jakes, S., Richards, J., Jena, B. P., Beck, B. L., Tabatabai, L. B., and Ingebritsen, T. S. (1993). Acidic residues are involved in substrate recognition by two soluble protein tyrosine phosphatases, PTP-5 and rrbPTP-1. Biochemistry *32*, 12405-12.

Hirase, T., Furuse, M., and Tsukita, S. (1997). A 155-kDa undercoat-constitutive protein of cell-to-cell adherens junctions. Eur J Cell Biol *72*, 174-81.

Hirschberg, H., Evensen, S. A., Henriksen, T., and Thorsby, E. (1975). The human mixed lymphocyte-endothelium culture interaction. Transplantation *19*, 495-504.

Hiscox, S., and Jiang, W. G. (1998). Association of PTPmu with catenins in cancer cells: a possible role for E-cadherin. Int J Oncol *13*, 1077-80.

Hoffmann, K. M., Tonks, N. K., and Barford, D. (1997). The crystal structure of domain 1 of receptor protein-tyrosine phosphatase mu. J Biol Chem *272*, 27505-8.

Holsinger, L. J., Ward, K., Duffield, B., Zachwieja, J., and Jallal, B. (2002). The transmembrane receptor protein tyrosine phosphatase DEP1 interacts with p120(ctn). Oncogene *21*, 7067-76.

Honkaniemi, J., Zhang, J. S., Yang, T., Zhang, C., Tisi, M. A., and Longo, F. M. (1998). LAR tyrosine phosphatase receptor: proximal membrane alternative splicing is coordinated with regional expression and intraneuronal localization. Brain Res Mol Brain Res *60*, 1-12.

Hooft van Huijsduijnen, R. (1998). Protein tyrosine phosphatases: counting the trees in the forest. Gene *225*, 1-8.

Hoover, K. B., and Bryant, P. J. (2000). The genetics of the protein 4.1 family: organizers of the membrane and cytoskeleton. Curr Opin Cell Biol *12*, 229-34.

Hoschuetzky, H., Aberle, H., and Kemler, R. (1994). Beta-catenin mediates the interaction of the cadherin-catenin complex with epidermal growth factor receptor. J Cell Biol *127*, 1375-80.

References

Hu, P., O'Keefe, E. J., and Rubenstein, D. S. (2001). Tyrosine phosphorylation of human keratinocyte beta-catenin and plakoglobin reversibly regulates their binding to E-cadherin and alpha- catenin. J Invest Dermatol *117*, 1059-67.

Hubbard, S. R., and Till, J. H. (2000). Protein tyrosine kinase structure and function. Annu Rev Biochem *69*, 373-98.

Huber, A. H., Nelson, W. J., and Weis, W. I. (1997). Three-dimensional structure of the armadillo repeat region of beta- catenin. Cell *90*, 871-82.

Huber, O., Korn, R., McLaughlin, J., Ohsugi, M., Herrmann, B. G., and Kemler, R. (1996). Nuclear localization of beta-catenin by interaction with transcription factor LEF-1. Mech Dev *59*, 3-10.

Hubner, S., Xiao, C. Y., and Jans, D. A. (1997). The protein kinase CK2 site (Ser111/112) enhances recognition of the simian virus 40 large T-antigen nuclear localization sequence by importin. J Biol Chem *272*, 17191-5.

Huyer, G., Kelly, J., Moffat, J., Zamboni, R., Jia, Z., Gresser, M. J., and Ramachandran, C. (1998). Affinity selection from peptide libraries to determine substrate specificity of protein tyrosine phosphatases. Anal Biochem *258*, 19-30.

Iavarone, A., and Massague, J. (1997). Repression of the CDK activator Cdc25A and cell-cycle arrest by cytokine TGF-beta in cells lacking the CDK inhibitor p15. Nature *387*, 417-22.

Ibarra-Sanchez, M. J., Simoncic, P. D., Nestel, F. R., Duplay, P., Lapp, W. S., and Tremblay, M. L. (2000). The T-cell protein tyrosine phosphatase. Semin Immunol *12*, 379-86.

Ibarra-Sanchez, M. J., Wagner, J., Ong, M. T., Lampron, C., and Tremblay, M. L. (2001). Murine embryonic fibroblasts lacking TC-PTP display delayed G1 phase through defective NF-kappaB activation. Oncogene *20*, 4728-39.

Irie-Sasaki, J., Sasaki, T., Matsumoto, W., Opavsky, A., Cheng, M., Welstead, G., Griffiths, E., Krawczyk, C., Richardson, C. D., Aitken, K., Iscove, N., Koretzky, G., Johnson, P., Liu, P., Rothstein, D. M., and Penninger, J. M. (2001). CD45 is a JAK phosphatase and negatively regulates cytokine receptor signalling. Nature *409*, 349-54.

Itoh, M., Nagafuchi, A., Yonemura, S., Kitani-Yasuda, T., and Tsukita, S. (1993). The 220-kD protein colocalizing with cadherins in non-epithelial cells is identical to ZO-1, a tight junction-associated protein in epithelial cells: cDNA cloning and immunoelectron microscopy. J Cell Biol *121*, 491-502.

Itoh, M., Sasaki, H., Furuse, M., Ozaki, H., Kita, T., and Tsukita, S. (2001). Junctional adhesion molecule (JAM) binds to PAR-3: a possible mechanism for the recruitment of PAR-3 to tight junctions. J Cell Biol *154*, 491-7.

Jallal, B., Mossie, K., Vasiloudis, G., Knyazev, P., Zachwieja, J., Clairvoyant, F., Schilling, J., and Ullrich, A. (1997). The receptor-like protein-tyrosine phosphatase DEP-1 is constitutively associated with a 64-kDa protein serine/threonine kinase. J Biol Chem *272*, 12158-63.

Jesaitis, L. A., and Goodenough, D. A. (1994). Molecular characterization and tissue distribution of ZO-2, a tight junction protein homologous to ZO-1 and the Drosophila discs-large tumor suppressor protein. J Cell Biol *124*, 949-61.

Jia, Z., Barford, D., Flint, A. J., and Tonks, N. K. (1995). Structural basis for phosphotyrosine peptide recognition by protein tyrosine phosphatase 1B. Science *268*, 1754-8.

Jiang, G., den Hertog, J., Su, J., Noel, J., Sap, J., and Hunter, T. (1999). Dimerization inhibits the activity of receptor-like protein-tyrosine phosphatase-alpha. Nature *401*, 606-10.

References

Jiang, S., Tulloch, A. G., Kim, T. A., Fu, Y., Rogers, R., Gaskell, A., White, R. A., Avraham, H., and Avraham, S. (1998). Characterization and chromosomal localization of PTP-NP-2, a new isoform of protein tyrosine phosphatase-like receptor, expressed on synaptic boutons. Gene *215*, 345-59.

Jiao, H., Berrada, K., Yang, W., Tabrizi, M., Platanias, L. C., and Yi, T. (1996). Direct association with and dephosphorylation of Jak2 kinase by the SH2- domain-containing protein tyrosine phosphatase SHP-1. Mol Cell Biol *16*, 6985-92.

Johnson-Leger, C. A., Aurrand-Lions, M., Beltraminelli, N., Fasel, N., and Imhof, B. A. (2002). Junctional adhesion molecule-2 (JAM-2) promotes lymphocyte transendothelial migration. Blood *100*, 2479-86.

Jons, T., and Drenckhahn, D. (1992). Identification of the binding interface involved in linkage of cytoskeletal protein 4.1 to the erythrocyte anion exchanger. Embo J *11*, 2863-7.

Jou, T. S., and Nelson, W. J. (1998). Effects of regulated expression of mutant RhoA and Rac1 small GTPases on the development of epithelial (MDCK) cell polarity. J Cell Biol *142*, 85-100.

Kachar, B., and Reese, T. S. (1982). Evidence for the lipidic nature of tight junction strands. Nature *296*, 464-6.

Kaffman, A., Rank, N. M., O'Neill, E. M., Huang, L. S., and O'Shea, E. K. (1998a). The receptor Msn5 exports the phosphorylated transcription factor Pho4 out of the nucleus. Nature *396*, 482-6.

Kaffman, A., Rank, N. M., and O'Shea, E. K. (1998b). Phosphorylation regulates association of the transcription factor Pho4 with its import receptor Pse1/Kap121. Genes Dev *12*, 2673-83.

Kaibuchi, K., Kuroda, S., and Amano, M. (1999). Regulation of the cytoskeleton and cell adhesion by the Rho family GTPases in mammalian cells. Annu Rev Biochem *68*, 459-86.

Kalderon, D., Roberts, B. L., Richardson, W. D., and Smith, A. E. (1984). A short amino acid sequence able to specify nuclear location. Cell *39*, 499-509.

Kaufmann, S. H., and Hengartner, M. O. (2001). Programmed cell death: alive and well in the new millennium. Trends Cell Biol *11*, 526-34.

Keon, B. H., Schafer, S., Kuhn, C., Grund, C., and Franke, W. W. (1996). Symplekin, a novel type of tight junction plaque protein. J Cell Biol *134*, 1003-18.

Kim, H., Hawley, T. S., Hawley, R. G., and Baumann, H. (1998). Protein tyrosine phosphatase 2 (SHP-2) moderates signaling by gp130 but is not required for the induction of acute-phase plasma protein genes in hepatic cells. Mol Cell Biol *18*, 1525-33.

Kim, K., Daniels, K. J., and Hay, E. D. (1998). Tissue-specific expression of beta-catenin in normal mesenchyme and uveal melanomas and its effect on invasiveness. Exp Cell Res *245*, 79-90.

Kim, K., Lu, Z., and Hay, E. D. (2002). Direct evidence for a role of beta-catenin/LEF-1 signaling pathway in induction of EMT. Cell Biol Int *26*, 463-76.

Kinch, M. S., Clark, G. J., Der, C. J., and Burridge, K. (1995). Tyrosine phosphorylation regulates the adhesions of ras-transformed breast epithelia. J Cell Biol *130*, 461-71.

Kintner, C. (1992). Regulation of embryonic cell adhesion by the cadherin cytoplasmic domain. Cell *69*, 225-36.

Klagsbrun, M., and D'Amore, P. A. (1996). Vascular endothelial growth factor and its receptors. Cytokine Growth Factor Rev *7*, 259-70.

References

Klaman, L. D., Boss, O., Peroni, O. D., Kim, J. K., Martino, J. L., Zabolotny, J. M., Moghal, N., Lubkin, M., Kim, Y. B., Sharpe, A. H., Stricker-Krongrad, A., Shulman, G. I., Neel, B. G., and Kahn, B. B. (2000). Increased energy expenditure, decreased adiposity, and tissue-specific insulin sensitivity in protein-tyrosine phosphatase 1B-deficient mice. Mol Cell Biol *20*, 5479-89.

Klingmuller, U., Lorenz, U., Cantley, L. C., Neel, B. G., and Lodish, H. F. (1995). Specific recruitment of SH-PTP1 to the erythropoietin receptor causes inactivation of JAK2 and termination of proliferative signals. Cell *80*, 729-38.

Kolibaba, K. S., and Druker, B. J. (1997). Protein tyrosine kinases and cancer. Biochim Biophys Acta *1333*, F217-48.

Komeili, A., and O'Shea, E. K. (2000). Nuclear transport and transcription. Curr Opin Cell Biol *12*, 355-60.

Konishi, H., Tanaka, M., Takemura, Y., Matsuzaki, H., Ono, Y., Kikkawa, U., and Nishizuka, Y. (1997). Activation of protein kinase C by tyrosine phosphorylation in response to H2O2. Proc Natl Acad Sci U S A *94*, 11233-7.

Koretzky, G. A., Kohmetscher, M. A., Kadleck, T., and Weiss, A. (1992). Restoration of T cell receptor-mediated signal transduction by transfection of CD45 cDNA into a CD45-deficient variant of the Jurkat T cell line. J Immunol *149*, 1138-42.

Korinek, V., Barker, N., Morin, P. J., van Wichen, D., de Weger, R., Kinzler, K. W., Vogelstein, B., and Clevers, H. (1997). Constitutive transcriptional activation by a beta-catenin-Tcf complex in APC-/- colon carcinoma. Science *275*, 1784-7.

Kornberg, L., Earp, H. S., Parsons, J. T., Schaller, M., and Juliano, R. L. (1992). Cell adhesion or integrin clustering increases phosphorylation of a focal adhesion-associated tyrosine kinase. J Biol Chem *267*, 23439-42.

Kostrewa, D., Brockhaus, M., D'Arcy, A., Dale, G. E., Nelboeck, P., Schmid, G., Mueller, F., Bazzoni, G., Dejana, E., Bartfai, T., Winkler, F. K., and Hennig, M. (2001). X-ray structure of junctional adhesion molecule: structural basis for homophilic adhesion via a novel dimerization motif. Embo J *20*, 4391-8.

Kotani, H., Takaishi, K., Sasaki, T., and Takai, Y. (1997). Rho regulates association of both the ERM family and vinculin with the plasma membrane in MDCK cells. Oncogene *14*, 1705-13.

Krause, K., Haugwitz, U., Wasner, M., Wiedmann, M., Mossner, J., and Engeland, K. (2001). Expression of the cell cycle phosphatase cdc25C is down-regulated by the tumor suppressor protein p53 but not by p73. Biochem Biophys Res Commun *284*, 743-50.

Kurihara, H., Anderson, J. M., and Farquhar, M. G. (1995). Increased Tyr phosphorylation of ZO-1 during modification of tight junctions between glomerular foot processes. Am J Physiol *268*, F514-24.

Kypta, R. M., Su, H., and Reichardt, L. F. (1996). Association between a transmembrane protein tyrosine phosphatase and the cadherin-catenin complex. J Cell Biol *134*, 1519-29.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature *227*, 680-5.

LaMontagne, K. R., Jr., Flint, A. J., Franza, B. R., Jr., Pandergast, A. M., and Tonks, N. K. (1998). Protein tyrosine phosphatase 1B antagonizes signalling by oncoprotein tyrosine kinase p210 bcr-abl in vivo. Mol Cell Biol *18*, 2965-75.

LaMontagne, K. R., Jr., Hannon, G., and Tonks, N. K. (1998). Protein tyrosine phosphatase PTP1B suppresses p210 bcr-abl-induced transformation of rat-1 fibroblasts and promotes differentiation of K562 cells. Proc Natl Acad Sci U S A *95*, 14094-9.

References

Lampugnani, M. G., Corada, M., Andriopoulou, P., Esser, S., Risau, W., and Dejana, E. (1997). Cell confluence regulates tyrosine phosphorylation of adherens junction components in endothelial cells. J Cell Sci *110*, 2065-77.

Lander, E. S., Linton, L. M., Birren, B., Nusbaum, C., Zody, M. C., Baldwin, J., Devon, K., Dewar, K., Doyle, M., FitzHugh, W., Funke, R., Gage, D., Harris, K., Heaford, A., Howland, J., Kann, L., Lehoczky, J., LeVine, R., McEwan, P., McKernan, K., Meldrim, J., Mesirov, J. P., Miranda, C., Morris, W., Naylor, J., Raymond, C., Rosetti, M., Santos, R., Sheridan, A., Sougnez, C., Stange-Thomann, N., Stojanovic, N., Subramanian, A., Wyman, D., Rogers, J., Sulston, J., Ainscough, R., Beck, S., Bentley, D., Burton, J., Clee, C., Carter, N., Coulson, A., Deadman, R., Deloukas, P., Dunham, A., Dunham, I., Durbin, R., French, L., Grafham, D., Gregory, S., Hubbard, T., Humphray, S., Hunt, A., Jones, M., Lloyd, C., McMurray, A., Matthews, L., Mercer, S., Milne, S., Mullikin, J. C., Mungall, A., Plumb, R., Ross, M., Shownkeen, R., Sims, S., Waterston, R. H., Wilson, R. K., Hillier, L. W., McPherson, J. D., Marra, M. A., Mardis, E. R., Fulton, L. A., Chinwalla, A. T., Pepin, K. H., Gish, W. R., Chissoe, S. L., Wendl, M. C., Delehaunty, K. D., Miner, T. L., Delehaunty, A., Kramer, J. B., Cook, L. L., Fulton, R. S., Johnson, D. L., Minx, P. J., Clifton, S. W., Hawkins, T., Branscomb, E., Predki, P., Richardson, P., Wenning, S., Slezak, T., Doggett, N., Cheng, J. F., Olsen, A., Lucas, S., Elkin, C., Uberbacher, E., Frazier, M., et al. (2001). Initial sequencing and analysis of the human genome. Nature *409*, 860-921.

Le, T. L., Yap, A. S., and Stow, J. L. (1999). Recycling of E-cadherin: a potential mechanism for regulating cadherin dynamics. J Cell Biol *146*, 219-32.

Lee, S. R., Kwon, K. S., Kim, S. R., and Rhee, S. G. (1998). Reversible inactivation of protein-tyrosine phosphatase 1B in A431 cells stimulated with epidermal growth factor. J Biol Chem *273*, 15366-72.

Levea, C. M., McGary, C. T., Symons, J. R., and Mooney, R. A. (2000). PTP LAR expression compared to prognostic indices in metastatic and non- metastatic breast cancer. Breast Cancer Res Treat *64*, 221-8.

Li, D., and Mrsny, R. J. (2000). Oncogenic Raf-1 disrupts epithelial tight junctions via downregulation of occludin. J Cell Biol *148*, 791-800.

Li, P. M., Zhang, W. R., and Goldstein, B. J. (1996). Suppression of insulin receptor activation by overexpression of the protein-tyrosine phosphatase LAR in hepatoma cells. Cell Signal *8*, 467-73.

Li, W., Nishimura, R., Kashishian, A., Batzer, A. G., Kim, W. J., Cooper, J. A., and Schlessinger, J. (1994). A new function for a phosphotyrosine phosphatase: linking GRB2-Sos to a receptor tyrosine kinase. Mol Cell Biol *14*, 509-17.

Liang, T. W., Chiu, H. H., Gurney, A., Sidle, A., Tumas, D. B., Schow, P., Foster, J., Klassen, T., Dennis, K., DeMarco, R. A., Pham, T., Frantz, G., and Fong, S. (2002). Vascular endothelial-junctional adhesion molecule (VE-JAM)/JAM 2 interacts with T, NK, and dendritic cells through JAM 3. J Immunol *168*, 1618-26.

Liang, T. W., DeMarco, R. A., Mrsny, R. J., Gurney, A., Gray, A., Hooley, J., Aaron, H. L., Huang, A., Klassen, T., Tumas, D. B., and Fong, S. (2000). Characterization of huJAM: evidence for involvement in cell-cell contact and tight junction regulation. Am J Physiol Cell Physiol *279*, C1733-43.

Lilien, J., Balsamo, J., Arregui, C., and Xu, G. (2002). Turn-off, drop-out: functional state switching of cadherins. Dev Dyn *224*, 18-29.

Lipfert, L., Haimovich, B., Schaller, M. D., Cobb, B. S., Parsons, J. T., and Brugge, J. S. (1992). Integrin-dependent phosphorylation and activation of the protein tyrosine kinase pp125FAK in platelets. J Cell Biol *119*, 905-12.

Lorenzen, J. A., Dadabay, C. Y., and Fischer, E. H. (1995). COOH-terminal sequence motifs target the T cell protein tyrosine phosphatase to the ER and nucleus. J Cell Biol *131*, 631-43.

Luo, J. C., and Shibuya, M. (2001). A variant of nuclear localization signal of bipartite-type is required for the nuclear translocation of hypoxia inducible factors (1alpha, 2alpha and 3alpha). Oncogene *20*, 1435-44.

References

Lyons, P. D., Dunty, J. M., Schaefer, E. M., and Schaller, M. D. (2001). Inhibition of the Catalytic Activity of Cell Adhesion Kinase beta by Protein-tyrosine Phosphatase-PEST-mediated Dephosphorylation. J Biol Chem *276*, 24422-31.

Maekawa, K., Imagawa, N., Nagamatsu, M., and Harada, S. (1994). Molecular cloning of a novel protein-tyrosine phosphatase containing a membrane-binding domain and GLGF repeats. FEBS Lett *337*, 200-6.

Maekawa, K., Imagawa, N., Naito, A., Harada, S., Yoshie, O., and Takagi, S. (1999). Association of protein-tyrosine phosphatase PTP-BAS with the transcription-factor-inhibitory protein IkappaBalpha through interaction between the PDZ1 domain and ankyrin repeats. Biochem J *337*, 179-84.

Maher, P. A., Pasquale, E. B., Wang, J. Y., and Singer, S. J. (1985). Phosphotyrosine-containing proteins are concentrated in focal adhesions and intercellular junctions in normal cells. Proc Natl Acad Sci U S A *82*, 6576-80.

Mahmoud, M. S., Ishikawa, H., Fujii, R., and Kawano, M. M. (1998). Induction of CD45 expression and proliferation in U-266 myeloma cell line by interleukin-6. Blood *92*, 3887-97.

Mailand, N., Falck, J., Lukas, C., Syljuasen, R. G., Welcker, M., Bartek, J., and Lukas, J. (2000). Rapid destruction of human Cdc25A in response to DNA damage. Science *288*, 1425-9.

Maile, L. A., and Clemmons, D. R. (2002). Regulation of insulin-like growth factor I receptor dephosphorylation by SHPS-1 and the tyrosine phosphatase SHP-2. J Biol Chem *277*, 8955-60.

Makkerh, J. P., Dingwall, C., and Laskey, R. A. (1996). Comparative mutagenesis of nuclear localization signals reveals the importance of neutral and acidic amino acids. Curr Biol *6*, 1025-7.

Marfatia, S. M., Leu, R. A., Branton, D., and Chishti, A. H. (1995). Identification of the protein 4.1 binding interface on glycophorin C and p55, a homologue of the Drosophila discs-large tumor suppressor protein. J Biol Chem *270*, 715-9.

Martin-Padura, I., Lostaglio, S., Schneemann, M., Williams, L., Romano, M., Fruscella, P., Panzeri, C., Stoppacciaro, A., Ruco, L., Villa, A., Simmons, D., and Dejana, E. (1998). Junctional adhesion molecule, a novel member of the immunoglobulin superfamily that distributes at intercellular junctions and modulates monocyte transmigration. J Cell Biol *142*, 117-27.

Maruyama, K., Ochiai, A., Nakamura, S., Baba, S., and Hirohashi, S. (1998). [Dysfunction of E-cadherin-catenin system in invasion and metastasis of colorectal cancer]. Nippon Geka Gakkai Zasshi *99*, 402-8.

Mattagajasingh, S. N., Huang, S. C., Hartenstein, J. S., and Benz, E. J., Jr. (2000). Characterization of the interaction between protein 4.1R and ZO-2. A possible link between the tight junction and the actin cytoskeleton. J Biol Chem *275*, 30573-85.

Mattison, C. P., and Ota, I. M. (2000). Two protein tyrosine phosphatases, Ptp2 and Ptp3, modulate the subcellular localization of the Hog1 MAP kinase in yeast. Genes Dev *14*, 1229-35.

Mavlyutov, T. A., Zhao, H., and Ferreira, P. A. (2002). Species-specific subcellular localization of RPGR and RPGRIP isoforms: implications for the phenotypic variability of congenital retinopathies among species. Hum Mol Genet *11*, 1899-907.

McCarthy, K. M., Skare, I. B., Stankewich, M. C., Furuse, M., Tsukita, S., Rogers, R. A., Lynch, R. D., and Schneeberger, E. E. (1996). Occludin is a functional component of the tight junction. J Cell Sci *109*, 2287-98.

McCrea, P. D., Brieher, W. M., and Gumbiner, B. M. (1993). Induction of a secondary body axis in Xenopus by antibodies to beta- catenin. J Cell Biol *123*, 477-84.

References

Melchior, F., Paschal, B., Evans, J., and Gerace, L. (1993). Inhibition of nuclear protein import by nonhydrolyzable analogues of GTP and identification of the small GTPase Ran/TC4 as an essential transport factor. J Cell Biol *123*, 1649-59.

Meng, T. C., Fukada, T., and Tonks, N. K. (2002). Reversible oxidation and inactivation of protein tyrosine phosphatases in vivo. Mol Cell *9*, 387-99.

Meyer, B. E., and Malim, M. H. (1994). The HIV-1 Rev trans-activator shuttles between the nucleus and the cytoplasm. Genes Dev *8*, 1538-47.

Meyer, H. W. (1983). Tight junction strands are lipidic cylinders. Naturwissenschaften *70*, 251-2.

Meyer, T. N., Schwesinger, C., Ye, J., Denker, B. M., and Nigam, S. K. (2001). Reassembly of the tight junction after oxidative stress depends on tyrosine kinase activity. J Biol Chem *276*, 22048-55.

Michalides, R., Volberg, T., and Geiger, B. (1994). Augmentation of adherens junction formation in mesenchymal cells by co- expression of N-CAM or short-term stimulation of tyrosine- phosphorylation. Cell Adhes Commun *2*, 481-90.

Migone, T. S., Cacalano, N. A., Taylor, N., Yi, T., Waldmann, T. A., and Johnston, J. A. (1998). Recruitment of SH2-containing protein tyrosine phosphatase SHP-1 to the interleukin 2 receptor; loss of SHP-1 expression in human T- lymphotropic virus type I-transformed T cells. Proc Natl Acad Sci U S A *95*, 3845-50.

Mitic, L. L., Schneeberger, E. E., Fanning, A. S., and Anderson, J. M. (1999). Connexin-occludin chimeras containing the ZO-binding domain of occludin localize at MDCK tight junctions and NRK cell contacts. J Cell Biol *146*, 683-93.

Mitic, L. L., Van Itallie, C. M., and Anderson, J. M. (2000). Molecular physiology and pathophysiology of tight junctions I. Tight junction structure and function: lessons from mutant animals and proteins. Am J Physiol Gastrointest Liver Physiol *279*, G250-4.

Moeslein, F. M., Myers, M. P., and Landreth, G. E. (1999). The CLK family kinases, CLK1 and CLK2, phosphorylate and activate the tyrosine phosphatase, PTP-1B. J Biol Chem *274*, 26697-704.

Molenaar, M., van de Wetering, M., Oosterwegel, M., Peterson-Maduro, J., Godsave, S., Korinek, V., Roose, J., Destree, O., and Clevers, H. (1996). XTcf-3 transcription factor mediates beta-catenin-induced axis formation in Xenopus embryos. Cell *86*, 391-9.

Moller, N. P., Moller, K. B., Lammers, R., Kharitonenkov, A., Sures, I., and Ullrich, A. (1994). Src kinase associates with a member of a distinct subfamily of protein- tyrosine phosphatases containing an ezrin-like domain. Proc Natl Acad Sci U S A *91*, 7477-81.

Monga, S. P., Mars, W. M., Pediaditakis, P., Bell, A., Mule, K., Bowen, W. C., Wang, X., Zarnegar, R., and Michalopoulos, G. K. (2002). Hepatocyte growth factor induces Wnt-independent nuclear translocation of beta-catenin after Met-beta-catenin dissociation in hepatocytes. Cancer Res *62*, 2064-71.

Moon, H. S., Choi, E. A., Park, H. Y., Choi, J. Y., Chung, H. W., Kim, J. I., and Park, W. I. (2001). Expression and tyrosine phosphorylation of E-cadherin, beta- and gamma- catenin, and epidermal growth factor receptor in cervical cancer cells. Gynecol Oncol *81*, 355-9.

Moore, M. S., and Blobel, G. (1993). The GTP-binding protein Ran/TC4 is required for protein import into the nucleus. Nature *365*, 661-3.

Morita, K., Sasaki, H., Fujimoto, K., Furuse, M., and Tsukita, S. (1999). Claudin-11/OSP-based tight junctions of myelin sheaths in brain and Sertoli cells in testis. J Cell Biol *145*, 579-88.

Morita, K., Sasaki, H., Furuse, M., and Tsukita, S. (1999). Endothelial claudin: claudin-5/TMVCF constitutes tight junction strands in endothelial cells. J Cell Biol *147*, 185-94.

Moroianu, J. (1999). Nuclear import and export pathways. J Cell Biochem *Suppl*, 76-83.

Moroianu, J., and Blobel, G. (1995). Protein export from the nucleus requires the GTPase Ran and GTP hydrolysis. Proc Natl Acad Sci U S A *92*, 4318-22.

Moroianu, J., Hijikata, M., Blobel, G., and Radu, A. (1995). Mammalian karyopherin alpha 1 beta and alpha 2 beta heterodimers: alpha 1 or alpha 2 subunit binds nuclear localization signal and beta subunit interacts with peptide repeat-containing nucleoporins. Proc Natl Acad Sci U S A *92*, 6532-6.

Mourton, T., Hellberg, C. B., Burden-Gulley, S. M., Hinman, J., Rhee, A., and Brady-Kalnay, S. M. (2001). The PTPmu protein-tyrosine phosphatase binds and recruits the scaffolding protein RACK1 to cell-cell contacts. J Biol Chem *276*, 14896-901.

Mui, A. L., Wakao, H., O'Farrell, A. M., Harada, N., and Miyajima, A. (1995). Interleukin-3, granulocyte-macrophage colony stimulating factor and interleukin-5 transduce signals through two STAT5 homologs. Embo J *14*, 1166-75.

Muller, T., Choidas, A., Reichmann, E., and Ullrich, A. (1999). Phosphorylation and free pool of beta-catenin are regulated by tyrosine kinases and tyrosine phosphatases during epithelial cell migration. J Biol Chem *274*, 10173-83.

Muller, U. (1999). Ten years of gene targeting: targeted mouse mutants, from vector design to phenotype analysis. Mech Dev *82*, 3-21

Mullin, J. M., Laughlin, K. V., Marano, C. W., Russo, L. M., and Soler, A. P. (1992). Modulation of tumor necrosis factor-induced increase in renal (LLC-PK1) transepithelial permeability. Am J Physiol *263*, F915-24.

Mustelin, T., Brockdorff, J., Rudbeck, L., Gjorloff-Wingren, A., Han, S., Wang, X., Tailor, P., and Saxena, M. (1999). The next wave: protein tyrosine phosphatases enter T cell antigen receptor signalling. Cell Signal *11*, 637-50.

Myers, M. P., Andersen, J. N., Cheng, A., Tremblay, M. L., Horvath, C. M., Parisien, J. P., Salmeen, A., Barford, D., and Tonks, N. K. (2001). TYK2 and JAK2 are substrates of protein-tyrosine phosphatase 1B. J Biol Chem *276*, 47771-4.

Nagafuchi, A., and Takeichi, M. (1988). Cell binding function of E-cadherin is regulated by the cytoplasmic domain. Embo J *7*, 3679-84.

Nakai, Y., Irie, S., and Sato, T. A. (2000). Identification of IkappaBalpha as a substrate of Fas-associated phosphatase-1. Eur J Biochem *267*, 7170-5.

Nakamura, K., Mizuno, Y., and Kikuchi, K. (1996). Molecular cloning of a novel cytoplasmic protein tyrosine phosphatase PTP epsilon. Biochem Biophys Res Commun *218*, 726-32.

Nambirajan, S., Radha, V., Kamatkar, S., and Swarup, G. (2000). PTP-S2, a nuclear tyrosine phosphatase, is phosphorylated and excluded from condensed chromosomes during mitosis. J Biosci *25*, 33-40.

Neel, B. G., and Tonks, N. K. (1997). Protein tyrosine phosphatases in signal transduction. Curr Opin Cell Biol *9*, 193-204.

Nelson, R. W., and Gumbiner, B. M. (1999). A cell-free assay system for beta-catenin signaling that recapitulates direct inductive events in the early xenopus laevis embryo. J Cell Biol *147*, 367-74.

Nieto, M. A. (2002). The snail superfamily of zinc-finger transcription factors. Nat Rev Mol Cell Biol *3*, 155-66.

References

Nieto, M. A., Sargent, M. G., Wilkinson, D. G., and Cooke, J. (1994). Control of cell behavior during vertebrate development by Slug, a zinc finger gene. Science *264*, 835-9.

Nishimura, M., Machida, K., Imaizumi, M., Abe, T., Umeda, T., Takeshima, E., Watanabe, T., Ohnishi, Y., Takagi, K., and Hamaguchi, M. (1996). Tyrosine phosphorylation of 100-130 kDa proteins in lung cancer correlates with poor prognosis. Br J Cancer *74*, 780-7.

Noren, N. K., Liu, B. P., Burridge, K., and Kreft, B. (2000). p120 catenin regulates the actin cytoskeleton via Rho family GTPases. J Cell Biol *150*, 567-80.

Norris, K., Norris, F., Kono, D. H., Vestergaard, H., Pedersen, O., Theofilopoulos, A. N., and Moller, N. P. (1997). Expression of protein-tyrosine phosphatases in the major insulin target tissues. FEBS Lett *415*, 243-8.

Nunomura, W., Takakuwa, Y., Tokimitsu, R., Krauss, S. W., Kawashima, M., and Mohandas, N. (1997). Regulation of CD44-protein 4.1 interaction by Ca2+ and calmodulin. Implications for modulation of CD44-ankyrin interaction. J Biol Chem *272*, 30322-8.

Nusrat, A., Chen, J. A., Foley, C. S., Liang, T. W., Tom, J., Cromwell, M., Quan, C., and Mrsny, R. J. (2000). The coiled-coil domain of occludin can act to organize structural and functional elements of the epithelial tight junction. J Biol Chem *275*, 29816-22.

Oberleithner, H., Westphale, H. J., and Gassner, B. (1991). Alkaline stress transforms Madin-Darby canine kidney cells. Pflugers Arch *419*, 418-20.

Ogata, M., Takada, T., Mori, Y., Oh-hora, M., Uchida, Y., Kosugi, A., Miyake, K., and Hamaoka, T. (1999 a). Effects of overexpression of PTP36, a putative protein tyrosine phosphatase, on cell adhesion, cell growth, and cytoskeletons in HeLa cells. J Biol Chem *274*, 12905-9.

Ogata, M., Takada, T., Mori, Y., Uchida, Y., Miki, T., Okuyama, A., Kosugi, A., Sawada, M., Oh-hora, M., and Hamaoka, T. (1999 b). Regulation of phosphorylation level and distribution of PTP36, a putative protein tyrosine phosphatase, by cell-substrate adhesion. J Biol Chem *274*, 20717-24.

Ohkubo, T., and Ozawa, M. (1999). p120(ctn) binds to the membrane-proximal region of the E-cadherin cytoplasmic domain and is involved in modulation of adhesion activity. J Biol Chem *274*, 21409-15.

O'Reilly, A. M., and Neel, B. G. (1998). Structural determinants of SHP-2 function and specificity in Xenopus mesoderm induction. Mol Cell Biol *18*, 161-77.

Ostergaard, H. L., Shackelford, D. A., Hurley, T. R., Johnson, P., Hyman, R., Sefton, B. M., and Trowbridge, I. S. (1989). Expression of CD45 alters phosphorylation of the lck-encoded tyrosine protein kinase in murine lymphoma T-cell lines. Proc Natl Acad Sci U S A *86*, 8959-63.

Ostermann, G., Weber, K. S., Zernecke, A., Schroder, A., and Weber, C. (2002). JAM-1 is a ligand of the beta(2) integrin LFA-1 involved in transendothelial migration of leukocytes. Nat Immunol *3*, 151-8.

Ostman, A., Yang, Q., and Tonks, N. K. (1994). Expression of DEP-1, a receptor-like protein-tyrosine-phosphatase, is enhanced with increasing cell density. Proc Natl Acad Sci U S A *91*, 9680-4.

Ozaki, H., Ishii, K., Horiuchi, H., Arai, H., Kawamoto, T., Okawa, K., Iwamatsu, A., and Kita, T. (1999). Cutting edge: combined treatment of TNF-alpha and IFN-gamma causes redistribution of junctional adhesion molecule in human endothelial cells. J Immunol *163*, 553-7.

Ozawa, M. (1998). Identification of the region of alpha-catenin that plays an essential role in cadherin-mediated cell adhesion. J Biol Chem *273*, 29524-9.

Ozawa, M., Ringwald, M., and Kemler, R. (1990). Uvomorulin-catenin complex formation is regulated by a specific domain in the cytoplasmic region of the cell adhesion molecule. Proc Natl Acad Sci U S A *87*, 4246-50.

References

Pani, G., Siminovitch, K. A., and Paige, C. J. (1997). The motheaten mutation rescues B cell signaling and development in CD45- deficient mice. J Exp Med *186*, 581-8.

Pannifer, A. D., Flint, A. J., Tonks, N. K., and Barford, D. (1998). Visualization of the cysteinyl-phosphate intermediate of a protein- tyrosine phosphatase by x-ray crystallography. J Biol Chem *273*, 10454-62.

Papkoff, J. (1997). Regulation of complexed and free catenin pools by distinct mechanisms. Differential effects of Wnt-1 and v-Src. J Biol Chem *272*, 4536-43.

Pathre, P., Arregui, C., Wampler, T., Kue, I., Leung, T. C., Lilien, J., and Balsamo, J. (2001). PTP1B regulates neurite extension mediated by cell-cell and cell-matrix adhesion molecules. J Neurosci Res *63*, 143-50.

Patry, V., Arnaud, E., Amalric, F., and Prats, H. (1994). Involvement of basic fibroblast growth factor NH2 terminus in nuclear accumulation. Growth Factors *11*, 163-74.

Pei, D., Lorenz, U., Klingmuller, U., Neel, B. G., and Walsh, C. T. (1994). Intramolecular regulation of protein tyrosine phosphatase SH-PTP1: a new function for Src homology 2 domains. Biochemistry *33*, 15483-93.

Pei, D., Wang, J., and Walsh, C. T. (1996). Differential functions of the two Src homology 2 domains in protein tyrosine phosphatase SH-PTP1. Proc Natl Acad Sci U S A *93*, 1141-5.

Peifer, M., and Polakis, P. (2000). Wnt signaling in oncogenesis and embryogenesis--a look outside the nucleus. Science *287*, 1606-9.

Peles, E., Nativ, M., Campbell, P. L., Sakurai, T., Martinez, R., Lev, S., Clary, D. O., Schilling, J., Barnea, G., Plowman, G. D., and et al. (1995). The carbonic anhydrase domain of receptor tyrosine phosphatase beta is a functional ligand for the axonal cell recognition molecule contactin. Cell *82*, 251-60.

Pellegrini, M. C., Liang, H., Mandiyan, S., Wang, K., Yuryev, A., Vlattas, I., Sytwu, T., Li, Y. C., and Wennogle, L. P. (1998). Mapping the subsite preferences of protein tyrosine phosphatase PTP-1B using combinatorial chemistry approaches. Biochemistry *37*, 15598-606.

Peraldi, P., Zhao, Z., Filloux, C., Fischer, E. H., and Van Obberghen, E. (1994). Protein-tyrosine-phosphatase 2C is phosphorylated and inhibited by 44- kDa mitogen-activated protein kinase. Proc Natl Acad Sci U S A *91*, 5002-6.

Peretz, A., Gil-Henn, H., Sobko, A., Shinder, V., Attali, B., and Elson, A. (2000). Hypomyelination and increased activity of voltage-gated K(+) channels in mice lacking protein tyrosine phosphatase epsilon. Embo J *19*, 4036-45.

Perl, A. K., Wilgenbus, P., Dahl, U., Semb, H., and Christofori, G. (1998). A causal role for E-cadherin in the transition from adenoma to carcinoma. Nature *392*, 190-3.

Piedra, J., Martinez, D., Castano, J., Miravet, S., Dunach, M., and Garcia De Herreros, A. (2001). Regulation of {beta}-catenin structure and activity by tyrosine phosphorylation. J Biol Chem *13*, 13.

Playford, M. P., Bicknell, D., Bodmer, W. F., and Macaulay, V. M. (2000). Insulin-like growth factor 1 regulates the location, stability, and transcriptional activity of beta-catenin. Proc Natl Acad Sci U S A *97*, 12103-8.

Pluskey, S., Wandless, T. J., Walsh, C. T., and Shoelson, S. E. (1995). Potent stimulation of SH-PTP2 phosphatase activity by simultaneous occupancy of both SH2 domains. J Biol Chem *270*, 2897-900.

Pollack, V., Scheiber, K., Pfaller, W., and Schramek, H. (1997). Loss of cytokeratin expression and formation of actin stress fibers in dedifferentiated MDCK-C7 cell lines. Biochem Biophys Res Commun *241*, 541-7.

Ponniah, S., Wang, D. Z., Lim, K. L., and Pallen, C. J. (1999). Targeted disruption of the tyrosine phosphatase PTPalpha leads to constitutive downregulation of the kinases Src and Fyn. Curr Biol *9*, 535-8.

References

Potempa, S., and Ridley, A. J. (1998). Activation of both MAP kinase and phosphatidylinositide 3-kinase by Ras is required for hepatocyte growth factor/scatter factor-induced adherens junction disassembly. Mol Biol Cell *9*, 2185-200.

Prasad, R., Gu, Y., Alder, H., Nakamura, T., Canaani, O., Saito, H., Huebner, K., Gale, R. P., Nowell, P. C., Kuriyama, K., and et al. (1993). Cloning of the ALL-1 fusion partner, the AF-6 gene, involved in acute myeloid leukemias with the t(6;11) chromosome translocation. Cancer Res *53*, 5624-8.

Pregel, M. J., Shen, S. H., and Storer, A. C. (1995). Regulation of protein tyrosine phosphatase 1C: opposing effects of the two src homology 2 domains. Protein Eng *8*, 1309-16.

Provost, E., and Rimm, D. L. (1999). Controversies at the cytoplasmic face of the cadherin-based adhesion complex. Curr Opin Cell Biol *11*, 567-72.

Qu, C. K., Shi, Z. Q., Shen, R., Tsai, F. Y., Orkin, S. H., and Feng, G. S. (1997). A deletion mutation in the SH2-N domain of Shp-2 severely suppresses hematopoietic cell development. Mol Cell Biol *17*, 5499-507.

Qu, C. K., Yu, W. M., Azzarelli, B., and Feng, G. S. (1999). Genetic evidence that Shp-2 tyrosine phosphatase is a signal enhancer of the epidermal growth factor receptor in mammals. Proc Natl Acad Sci U S A *96*, 8528-33.

Rajasekaran, A. K., Hojo, M., Huima, T., and Rodriguez-Boulan, E. (1996). Catenins and zonula occludens-1 form a complex during early stages in the assembly of tight junctions. J Cell Biol *132*, 451-63.

Ratcliffe, M. J., Rubin, L. L., and Staddon, J. M. (1997). Dephosphorylation of the cadherin-associated p100/p120 proteins in response to activation of protein kinase C in epithelial cells. J Biol Chem *272*, 31894-901.

Ratcliffe, M. J., Smales, C., and Staddon, J. M. (1999). Dephosphorylation of the catenins p120 and p100 in endothelial cells in response to inflammatory stimuli. Biochem J *338*, 471-8.

Ravetch, J. V., and Lanier, L. L. (2000). Immune inhibitory receptors. Science *290*, 84-9.

Ravichandran, L. V., Chen, H., Li, Y., and Quon, M. J. (2001). Phosphorylation of PTP1B at Ser(50) by Akt impairs its ability to dephosphorylate the insulin receptor. Mol Endocrinol *15*, 1768-80.

Reddy, R. S., and Swarup, G. (1995). Alternative splicing generates four different forms of a non-transmembrane protein tyrosine phosphatase mRNA. DNA Cell Biol *14*, 1007-15.

Reichert, M., Muller, T., and Hunziker, W. (2000). The PDZ domains of zonula occludens-1 induce an epithelial to mesenchymal transition of Madin-Darby canine kidney I cells. Evidence for a role of beta-catenin/Tcf/Lef signaling. J Biol Chem *275*, 9492-500.

Ren, J. M., Li, P. M., Zhang, W. R., Sweet, L. J., Cline, G., Shulman, G. I., Livingston, J. N., and Goldstein, B. J. (1998). Transgenic mice deficient in the LAR protein-tyrosine phosphatase exhibit profound defects in glucose homeostasis. Diabetes *47*, 493-7.

Rexach, M., and Blobel, G. (1995). Protein import into nuclei: association and dissociation reactions involving transport substrate, transport factors, and nucleoporins. Cell *83*, 683-92.

Reynolds, A. B., Herbert, L., Cleveland, J. L., Berg, S. T., and Gaut, J. R. (1992). p120, a novel substrate of protein tyrosine kinase receptors and of p60v-src, is related to cadherin-binding factors beta-catenin, plakoglobin and armadillo. Oncogene *7*, 2439-45.

Rhee, J., Lilien, J., and Balsamo, J. (2001). Essential tyrosine residues for interaction of the non-receptor protein- tyrosine phosphatase PTP1B with N-cadherin. J Biol Chem *276*, 6640-4.

References

Rihs, H. P., Jans, D. A., Fan, H., and Peters, R. (1991). The rate of nuclear cytoplasmic protein transport is determined by the casein kinase II site flanking the nuclear localization sequence of the SV40 T-antigen. Embo J *10*, 633-9.

Rihs, H. P., and Peters, R. (1989). Nuclear transport kinetics depend on phosphorylation-site-containing sequences flanking the karyophilic signal of the Simian virus 40 T- antigen. Embo J *8*, 1479-84.

Robinson DR, Wu YM, Lin SF. The protein tyrosine kinase family of the human genome. *Oncogene.* 2000;19:5548-57.

Robbins, J., Dilworth, S. M., Laskey, R. A., and Dingwall, C. (1991). Two interdependent basic domains in nucleoplasmin nuclear targeting sequence: identification of a class of bipartite nuclear targeting sequence. Cell *64*, 615-23.

Rosato, R., Veltmaat, J. M., Groffen, J., and Heisterkamp, N. (1998). Involvement of the tyrosine kinase fer in cell adhesion. Mol Cell Biol *18*, 5762-70.

Roura, S., Miravet, S., Piedra, J., Garcia de Herreros, A., and Dunach, M. (1999). Regulation of E-cadherin/Catenin association by tyrosine phosphorylation. J Biol Chem *274*, 36734-40.

Ruggiu, M., Saunders, P. T., and Cooke, H. J. (2000). Dynamic subcellular distribution of the DAZL protein is confined to primate male germ cells. J Androl *21*, 470-7.

Ruzzene, M., Donella-Deana, A., Marin, O., Perich, J. W., Ruzza, P., Borin, G., Calderan, A., and Pinna, L. A. (1993). Specificity of T-cell protein tyrosine phosphatase toward phosphorylated synthetic peptides. Eur J Biochem *211*, 289-95.

Ryeom, S. W., Paul, D., and Goodenough, D. A. (2000). Truncation mutants of the tight junction protein ZO-1 disrupt corneal epithelial cell morphology. Mol Biol Cell *11*, 1687-96.

Saitou, M., Fujimoto, K., Doi, Y., Itoh, M., Fujimoto, T., Furuse, M., Takano, H., Noda, T., and Tsukita, S. (1998). Occludin-deficient Embryonic Stem Cells Can Differentiate into Polarized Epithelial Cells Bearing Tight Junctions. J. Cell Biol. *141*, 397-408.

Saitou, M., Furuse, M., Sasaki, H., Schulzke, J. D., Fromm, M., Takano, H., Noda, T., and Tsukita, S. (2000). Complex phenotype of mice lacking occludin, a component of tight junction strands. Mol Biol Cell *11*, 4131-42.

Sakakibara, A., Furuse, M., Saitou, M., Ando-Akatsuka, Y., and Tsukita, S. (1997). Possible involvement of phosphorylation of occludin in tight junction formation. J Cell Biol *137*, 1393-401.

Salmeen, A., Andersen, J. N., Myers, M. P., Tonks, N. K., and Barford, D. (2000). Molecular basis for the dephosphorylation of the activation segment of the insulin receptor by protein tyrosine phosphatase 1B. Mol Cell *6*, 1401-12.

Sap, J., Jiang, Y. P., Friedlander, D., Grumet, M., and Schlessinger, J. (1994). Receptor tyrosine phosphatase R-PTP-kappa mediates homophilic binding. Mol Cell Biol *14*, 1-9.

Saras, J., Claesson-Welsh, L., Heldin, C. H., and Gonez, L. J. (1994). Cloning and characterization of PTPL1, a protein tyrosine phosphatase with similarities to cytoskeletal-associated proteins. J Biol Chem *269*, 24082-9.

Sato, T., Irie, S., Kitada, S., and Reed, J. C. (1995). FAP-1: a protein tyrosine phosphatase that associates with Fas. Science *268*, 411-5.

Sato, Y., and Rifkin, D. B. (1989). Inhibition of endothelial cell movement by pericytes and smooth muscle cells: activation of a latent transforming growth factor-beta 1-like molecule by plasmin during co-culture. J Cell Biol *109*, 309-15.

References

Savagner, P. (2001). Leaving the neighborhood: molecular mechanisms involved during epithelial-mesenchymal transition. Bioessays *23*, 912-23.

Sawada, M., Ogata, M., Fujino, Y., and Hamaoka, T. (1994). cDNA cloning of a novel protein tyrosine phosphatase with homology to cytoskeletal protein 4.1 and its expression in T-lineage cells. Biochem Biophys Res Commun *203*, 479-84.

Saxton, T. M., Henkemeyer, M., Gasca, S., Shen, R., Rossi, D. J., Shalaby, F., Feng, G. S., and Pawson, T. (1997). Abnormal mesoderm patterning in mouse embryos mutant for the SH2 tyrosine phosphatase Shp-2. Embo J *16*, 2352-64.

Schaper, F., Gendo, C., Eck, M., Schmitz, J., Grimm, C., Anhuf, D., Kerr, I. M., and Heinrich, P. C. (1998). Activation of the protein tyrosine phosphatase SHP2 via the interleukin- 6 signal transducing receptor protein gp130 requires tyrosine kinase Jak1 and limits acute-phase protein expression. Biochem J *335*, 557-65.

Schievella, A. R., Paige, L. A., Johnson, K. A., Hill, D. E., and Erikson, R. L. (1993). Protein tyrosine phosphatase 1B undergoes mitosis-specific phosphorylation on serine. Cell Growth Differ *4*, 239-46.

Schlessinger, J. (2000). Cell signaling by receptor tyrosine kinases. Cell *103*, 211-25.

Schmidt, A., Utepbergenov, D. I., Krause, G., and Blasig, I. E. (2001). Use of surface plasmon resonance for real-time analysis of the interaction of ZO-1 and occludin. Biochem Biophys Res Commun *288*, 1194-9.

Schneeberger, E. E., and Lynch, R. D. (1992). Structure, function, and regulation of cellular tight junctions. Am J Physiol *262*, L647-61.

Schneider, S., Herrenknecht, K., Butz, S., Kemler, R., and Hausen, P. (1993). Catenins in Xenopus embryogenesis and their relation to the cadherin- mediated cell-cell adhesion system. Development *118*, 629-40.

Schumann, G., Fiebich, B. L., Menzel, D., Hull, M., Butcher, R., Nielsen, P., and Bauer, J. (1998). Cytokine-induced transcription of protein-tyrosine-phosphatases in human astrocytoma cells. Brain Res Mol Brain Res *62*, 56-64.

Seidensticker, M. J., and Behrens, J. (2000). Biochemical interactions in the wnt pathway. Biochim Biophys Acta *1495*, 168-82.

Serra-Pages, C., Kedersha, N. L., Fazikas, L., Medley, Q., Debant, A., and Streuli, M. (1995). The LAR transmembrane protein tyrosine phosphatase and a coiled-coil LAR-interacting protein co-localize at focal adhesions. Embo J *14*, 2827-38.

Shao, J., Gao, Y., and Yuan, Z. (1998). [Free fatty acids promoting PTP1B expression in rat skeletal muscle and hepatic cells]. Zhonghua Yi Xue Za Zhi *78*, 753-5.

Shaw, S. K., Perkins, B. N., Lim, Y. C., Liu, Y., Nusrat, A., Schnell, F. J., Parkos, C. A., and Luscinskas, F. W. (2001). Reduced expression of junctional adhesion molecule and platelet/endothelial cell adhesion molecule-1 (CD31) at human vascular endothelial junctions by cytokines tumor necrosis factor-alpha plus interferon-gamma Does not reduce leukocyte transmigration under flow. Am J Pathol *159*, 2281-91.

Shen, Y., Schneider, G., Cloutier, J. F., Veillette, A., and Schaller, M. D. (1998). Direct association of protein-tyrosine phosphatase PTP-PEST with paxillin. J Biol Chem *273*, 6474-81.

Shibamoto, S., Hayakawa, M., Takeuchi, K., Hori, T., Oku, N., Miyazawa, K., Kitamura, N., Takeichi, M., and Ito, F. (1994). Tyrosine phosphorylation of beta-catenin and plakoglobin enhanced by hepatocyte growth factor and epidermal growth factor in human carcinoma cells. Cell Adhes Commun *1*, 295-305.

Shimada, Y., Gulli, M. P., and Peter, M. (2000). Nuclear sequestration of the exchange factor Cdc24 by Far1 regulates cell polarity during yeast mating. Nat Cell Biol *2*, 117-24.

References

Shiozaki, H., Kadowaki, T., Doki, Y., Inoue, M., Tamura, S., Oka, H., Iwazawa, T., Matsui, S., Shimaya, K., Takeichi, M., and et al. (1995). Effect of epidermal growth factor on cadherin-mediated adhesion in a human oesophageal cancer cell line. Br J Cancer *71*, 250-8.

Shiozaki, H., Oka, H., Inoue, M., Tamura, S., and Monden, M. (1996). E-cadherin mediated adhesion system in cancer cells. Cancer *77*, 1605-13.

Shtutman, M., Zhurinsky, J., Simcha, I., Albanese, C., D'Amico, M., Pestell, R., and Ben-Ze'ev, A. (1999). The cyclin D1 gene is a target of the beta-catenin/LEF-1 pathway. Proc Natl Acad Sci U S A *96*, 5522-7.

Shuai, K., Ziemiecki, A., Wilks, A. F., Harpur, A. G., Sadowski, H. B., Gilman, M. Z., and Darnell, J. E. (1993). Polypeptide signalling to the nucleus through tyrosine phosphorylation of Jak and Stat proteins. Nature *366*, 580-3.

Shultz, L. D., Schweitzer, P. A., Rajan, T. V., Yi, T., Ihle, J. N., Matthews, R. J., Thomas, M. L., and Beier, D. R. (1993). Mutations at the murine motheaten locus are within the hematopoietic cell protein-tyrosine phosphatase (Hcph) gene. Cell *73*, 1445-54.

Simoncic, P. D., Lee-Loy, A., Barber, D. L., Tremblay, M. L., and McGlade, C. J. (2002). The T cell protein tyrosine phosphatase is a negative regulator of janus family kinases 1 and 3. Curr Biol *12*, 446-53.

Singh, S., Darnay, B. G., and Aggarwal, B. B. (1996). Site-specific tyrosine phosphorylation of IkappaBalpha negatively regulates its inducible phosphorylation and degradation. J Biol Chem *271*, 31049-54.

Skoudy, A., Llosas, M. D., and Garcia de Herreros, A. (1996). Intestinal HT-29 cells with dysfunction of E-cadherin show increased pp60src activity and tyrosine phosphorylation of p120-catenin. Biochem J *317*, 279-84.

Smith, A. L., Mitchell, P. J., Shipley, J., Gusterson, B. A., Rogers, M. V., and Crompton, M. R. (1995). Pez: a novel human cDNA encoding protein tyrosine phosphatase- and ezrin-like domains. Biochem Biophys Res Commun *209*, 959-65.

Smits, R., Kielman, M. F., Breukel, C., Zurcher, C., Neufeld, K., Jagmohan-Changur, S., Hofland, N., van Dijk, J., White, R., Edelmann, W., Kucherlapati, R., Khan, P. M., and Fodde, R. (1999). Apc1638T: a mouse model delineating critical domains of the adenomatous polyposis coli protein involved in tumorigenesis and development. Genes Dev *13*, 1309-21.

Songyang, Z., Shoelson, S. E., Chaudhuri, M., Gish, G., Pawson, T., Haser, W. G., King, F., Roberts, T., Ratnofsky, S., Lechleider, R. J., and et al. (1993). SH2 domains recognize specific phosphopeptide sequences. Cell *72*, 767-78.

Sorenson, C. M., and Sheibani, N. (2002). Altered regulation of SHP-2 and PTP 1B tyrosine phosphatases in cystic kidneys from bcl-2 -/- mice. Am J Physiol Renal Physiol *282*, F442-50.

Spencer, S., Dowbenko, D., Cheng, J., Li, W., Brush, J., Utzig, S., Simanis, V., and Lasky, L. A. (1997). PSTPIP: a tyrosine phosphorylated cleavage furrow-associated protein that is a substrate for a PEST tyrosine phosphatase. J Cell Biol *138*, 845-60.

Staddon, J. M., Herrenknecht, K., Smales, C., and Rubin, L. L. (1995). Evidence that tyrosine phosphorylation may increase tight junction permeability. J Cell Sci *108*, 609-19.

Stade, K., Ford, C. S., Guthrie, C., and Weis, K. (1997). Exportin 1 (Crm1p) is an essential nuclear export factor. Cell *90*, 1041-50.

Staehelin, L. A., Mukherjee, T. M., and Williams, A. W. (1969). Fine structure of frozen-etched tight junctions. Naturwissenschaften *56*, 142.

References

Stevenson, B. R., and Keon, B. H. (1998). The tight junction: morphology to molecules. Annu Rev Cell Dev Biol *14*, 89-109.

Stewart, D. B., and Nelson, W. J. (1997). Identification of four distinct pools of catenins in mammalian cells and transformation-dependent changes in catenin distributions among these pools. J Biol Chem *272*, 29652-62.

Stoker, A. W. (1994). Isoforms of a novel cell adhesion molecule-like protein tyrosine phosphatase are implicated in neural development. Mech Dev *46*, 201-17.

Strack, V., Krutzfeldt, J., Kellerer, M., Ullrich, A., Lammers, R., and Haring, H. U. (2002). The Protein-tyrosine-phosphatase SHP2 is phosphorylated on serine residues 576 and 591 by protein kinase C isoforms alpha, beta 1, beta 2, and eta. Biochemistry *41*, 603-8.

Strathdee, G. (2002). Epigenetic versus genetic alterations in the inactivation of E-cadherin. Semin Cancer Biol *12*, 373-9.

Stuart, R. O., and Nigam, S. K. (1995). Regulated assembly of tight junctions by protein kinase C. Proc Natl Acad Sci U S A *92*, 6072-6.

Stuckey, J. A., Schubert, H. L., Fauman, E. B., Zhang, Z. Y., Dixon, J. E., and Saper, M. A. (1994). Crystal structure of Yersinia protein tyrosine phosphatase at 2.5 A and the complex with tungstate. Nature *370*, 571-5.

Su, M. W., Yu, C. L., Burakoff, S. J., and Jin, Y. J. (2001). Targeting Src homology 2 domain-containing tyrosine phosphatase (SHP-1) into lipid rafts inhibits CD3-induced T cell activation. J Immunol *166*, 3975-82.

Sui, X., Krantz, S. B., and Zhao, Z. (1997). Identification of increased protein tyrosine phosphatase activity in polycythemia vera erythroid progenitor cells. Blood *90*, 651-7.

Sun, H., Charles, C. H., Lau, L. F., and Tonks, N. K. (1993). MKP-1 (3CH134), an immediate early gene product, is a dual specificity phosphatase that dephosphorylates MAP kinase in vivo. Cell *75*, 487-93.

Szedlacsek, S. E., Aricescu, A. R., Fulga, T. A., Renault, L., and Scheidig, A. J. (2001). Crystal structure of PTP-SL/PTPBR7 catalytic domain: implications for MAP kinase regulation. J Mol Biol *311*, 557-68.

Takahashi, K., Suzuki, K., and Tsukatani, Y. (1997). Induction of tyrosine phosphorylation and association of beta-catenin with EGF receptor upon tryptic digestion of quiescent cells at confluence. Oncogene *15*, 71-8.

Takaishi, K., Sasaki, T., Kameyama, T., Tsukita, S., and Takai, Y. (1995). Translocation of activated Rho from the cytoplasm to membrane ruffling area, cell-cell adhesion sites and cleavage furrows. Oncogene *11*, 39-48.

Takata, K., and Singer, S. J. (1988). Localization of high concentrations of phosphotyrosine-modified proteins in mouse megakaryocytes. Blood *71*, 818-21.

Takayama, T., Shiozaki, H., Doki, Y., Oka, H., Inoue, M., Yamamoto, M., Tamura, S., Shibamoto, S., Ito, F., and Monden, M. (1998). Aberrant expression and phosphorylation of beta-catenin in human colorectal cancer. Br J Cancer *77*, 605-13.

Takayama, T., Shiozaki, H., Shibamoto, S., Oka, H., Kimura, Y., Tamura, S., Inoue, M., Monden, T., Ito, F., and Monden, M. (1996). Beta-catenin expression in human cancers. Am J Pathol *148*, 39-46.

Takeda, H., and Tsukita, S. (1995). Effects of tyrosine phosphorylation on tight junctions in temperature-sensitive v-src-transfected MDCK cells. Cell Struct Funct *20*, 387-93.

Tanuma, N., Nakamura, K., Shima, H., and Kikuchi, K. (2000). Protein-tyrosine phosphatase PTPepsilon C inhibits Jak-STAT signaling and differentiation induced by interleukin-6 and leukemia inhibitory factor in M1 leukemia cells. J Biol Chem *275*, 28216-21.

References

Tanuma, N., Shima, H., Nakamura, K., and Kikuchi, K. (2001). Protein tyrosine phosphatase epsilonC selectively inhibits interleukin- 6- and interleukin- 10-induced JAK-STAT signaling. Blood *98*, 3030-4.

Tartaglia, M., Mehler, E. L., Goldberg, R., Zampino, G., Brunner, H. G., Kremer, H., van der Burgt, I., Crosby, A. H., Ion, A., Jeffery, S., Kalidas, K., Patton, M. A., Kucherlapati, R. S., and Gelb, B. D. (2001). Mutations in PTPN11, encoding the protein tyrosine phosphatase SHP-2, cause Noonan syndrome. Nat Genet *29*, 465-8.

ten Hoeve, J., de Jesus Ibarra-Sanchez, M., Fu, Y., Zhu, W., Tremblay, M., David, M., and Shuai, K. (2002). Identification of a nuclear Stat1 protein tyrosine phosphatase. Mol Cell Biol *22*, 5662-8.

Tenev, T., Keilhack, H., Tomic, S., Stoyanov, B., Stein-Gerlach, M., Lammers, R., Krivtsov, A. V., Ullrich, A., and Bohmer, F. D. (1997). Both SH2 domains are involved in interaction of SHP-1 with the epidermal growth factor receptor but cannot confer receptor-directed activity to SHP-1/SHP-2 chimera. J Biol Chem *272*, 5966-73.

Tetsu, O., and McCormick, F. (1999). Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. Nature *398*, 422-6.

Thoreson, M. A., Anastasiadis, P. Z., Daniel, J. M., Ireton, R. C., Wheelock, M. J., Johnson, K. R., Hummingbird, D. K., and Reynolds, A. B. (2000). Selective uncoupling of p120(ctn) from E-cadherin disrupts strong adhesion. J Cell Biol *148*, 189-202.

Tiganis, T., Bennett, A. M., Ravichandran, K. S., and Tonks, N. K. (1998). Epidermal growth factor receptor and the adaptor protein p52Shc are specific substrates of T-cell protein tyrosine phosphatase. Mol Cell Biol *18*, 1622-34.

Tonks, N. K., and Neel, B. G. (2001). Combinatorial control of the specificity of protein tyrosine phosphatases. Curr Opin Cell Biol *13*, 182-95.

Tsukamoto, T., and Nigam, S. K. (1999). Role of tyrosine phosphorylation in the reassembly of occludin and other tight junction proteins. Am J Physiol *276*, F737-50.

Tsukita, S., and Furuse, M. (1999). Occludin and claudins in tight-junction strands: leading or supporting players? Trends Cell Biol *9*, 268-73.

Tsukita, S., Furuse, M., and Itoh, M. (2001). Multifunctional strands in tight junctions. Nat Rev Mol Cell Biol *2*, 285-93.

Tsukita, S., Furuse, M., and Itoh, M. (1999). Structural and signalling molecules come together at tight junctions. Curr Opin Cell Biol *11*, 628-33.

Ueki, K., Ramaswamy, S., Billings, S. J., Mohrenweiser, H. W., and Louis, D. N. (1997). Chromosomal localization to 19q13.3, partial genomic structure and 5' cDNA sequence of the human symplekin gene. Somat Cell Mol Genet *23*, 229-31.

Uetani, N., Kato, K., Ogura, H., Mizuno, K., Kawano, K., Mikoshiba, K., Yakura, H., Asano, M., and Iwakura, Y. (2000). Impaired learning with enhanced hippocampal long-term potentiation in PTPdelta-deficient mice. Embo J *19*, 2775-85.

Ukropec, J. A., Hollinger, M. K., Salva, S. M., and Woolkalis, M. J. (2000). SHP2 association with VE-cadherin complexes in human endothelial cells is regulated by thrombin. J Biol Chem *275*, 5983-6.

Ukropec, J. A., Hollinger, M. K., and Woolkalis, M. J. (2002). Regulation of VE-cadherin linkage to the cytoskeleton in endothelial cells exposed to fluid shear stress. Exp Cell Res *273*, 240-7.

Van Itallie, C. M., Balda, M. S., and Anderson, J. M. (1995). Epidermal growth factor induces tyrosine phosphorylation and reorganization of the tight junction protein ZO-1 in A431 cells. J Cell Sci *108*, 1735-42.

References

van Meer, G., Gumbiner, B., and Simons, K. (1986). The tight junction does not allow lipid molecules to diffuse from one epithelial cell to the next. Nature *322*, 639-41.

Volberg, T., Zick, Y., Dror, R., Sabanay, I., Gilon, C., Levitzki, A., and Geiger, B. (1992). The effect of tyrosine-specific protein phosphorylation on the assembly of adherens-type junctions. Embo J *11*, 1733-42.

Wachtel, M., Frei, K., Ehler, E., Fontana, A., Winterhalter, K., and Gloor, S. M. (1999). Occludin proteolysis and increased permeability in endothelial cells through tyrosine phosphatase inhibition. J Cell Sci *112*, 4347-56.

Wadham, C., Gamble, J., Vadas, M., and Khew-Goodall, Y. (2003). The Protein Tyrosine Phosphatase Pez Is A Major Phosphatase Of Adherens Junctions And Dephosphorylates beta-Catenin. MBC *In Press*.

Wadham, C., Gamble, J. R., Vadas, M. A., and Khew-Goodall, Y. (2000). Translocation of protein tyrosine phosphatase Pez/PTPD2/PTP36 to the nucleus is associated with induction of cell proliferation. J Cell Sci *113*, 3117-23.

Waksman, G., Shoelson, S. E., Pant, N., Cowburn, D., and Kuriyan, J. (1993). Binding of a high affinity phosphotyrosyl peptide to the Src SH2 domain: crystal structures of the complexed and peptide-free forms. Cell *72*, 779-90.

Walchli, S., Curchod, M. L., Gobert, R. P., Arkinstall, S., and Hooft van Huijsduijnen, R. (2000). Identification of tyrosine phosphatases that dephosphorylate the insulin receptor. A brute force approach based on "substrate-trapping" mutants. J Biol Chem *275*, 9792-6.

Wallace, M. J., Fladd, C., Batt, J., and Rotin, D. (1998). The second catalytic domain of protein tyrosine phosphatase delta (PTP delta) binds to and inhibits the first catalytic domain of PTP sigma. Mol Cell Biol *18*, 2608-16.

Wang, B., Kishihara, K., Zhang, D., Sakamoto, T., and Nomoto, K. (1999). Transcriptional regulation of a receptor protein tyrosine phosphatase gene hPTP-J by PKC-mediated signaling pathways in Jurkat and Molt-4 T lymphoma cells. Biochim Biophys Acta *1450*, 331-40.

Wang, Y., Guo, W., Liang, L., and Esselman, W. J. (1999). Phosphorylation of CD45 by casein kinase 2. Modulation of activity and mutational analysis. J Biol Chem *274*, 7454-61.

Wang, Z., Bhattacharya, N., Meyer, M. K., Seimiya, H., Tsuruo, T., Tonani, J. A., and Magnuson, N. S. (2001). Pim-1 negatively regulates the activity of PTP-U2S phosphatase and influences terminal differentiation and apoptosis of monoblastoid leukemia cells. Arch Biochem Biophys *390*, 9-18.

Weber, E., Berta, G., Tousson, A., St John, P., Green, M. W., Gopalokrishnan, U., Jilling, T., Sorscher, E. J., Elton, T. S., Abrahamson, D. R., and et al. (1994). Expression and polarized targeting of a rab3 isoform in epithelial cells. J Cell Biol *125*, 583-94.

Weng, L. P., Wang, X., and Yu, Q. (1999). Transmembrane tyrosine phosphatase LAR induces apoptosis by dephosphorylating and destabilizing p130Cas. Genes Cells *4*, 185-96.

White, W. O., Seibenhener, M. L., and Wooten, M. W. (2002). Phosphorylation of tyrosine 256 facilitates nuclear import of atypical protein kinase C. J Cell Biochem *85*, 42-53.

Wickrema, A., Chen, F., Namin, F., Yi, T., Ahmad, S., Uddin, S., Chen, Y. H., Feldman, L., Stock, W., Hoffman, R., and Platanias, L. C. (1999). Defective expression of the SHP-1 phosphatase in polycythemia vera. Exp Hematol *27*, 1124-32.

Wodarz, A., and Nusse, R. (1998). Mechanisms of Wnt signaling in development. Annu Rev Cell Dev Biol *14*, 59-88.

References

Wolburg, H., Wolburg-Buchholz, K., Liebner, S., and Engelhardt, B. (2001). Claudin-1, claudin-2 and claudin-11 are present in tight junctions of choroid plexus epithelium of the mouse. Neurosci Lett *307*, 77-80.

Wong, V. (1997). Phosphorylation of occludin correlates with occludin localization and function at the tight junction. Am J Physiol *273*, C1859-67.

Wong, V., and Gumbiner, B. M. (1997). A synthetic peptide corresponding to the extracellular domain of occludin perturbs the tight junction permeability barrier. J Cell Biol *136*, 399-409.

Woolf, A. S., Kolatsi-Joannou, M., Hardman, P., Andermarcher, E., Moorby, C., Fine, L. G., Jat, P. S., Noble, M. D., and Gherardi, E. (1995). Roles of hepatocyte growth factor/scatter factor and the met receptor in the early development of the metanephros. J Cell Biol *128*, 171-84.

Yahagi, Y., Tagawa, M., Tomoda, T., and Shirasawa, T. (1996). Binary expression of olfactory bulb-protein tyrosine phosphatase in rat central nervous system: developmental gene regulation in neonate cerebral cortex and constitutive expression in olfactory-rhinencephalon. Neurosci Lett *211*, 125-8.

Yamamoto, T., Sekine, Y., Kashima, K., Kubota, A., Sato, N., Aoki, N., and Matsuda, T. (2002). The nuclear isoform of protein-tyrosine phosphatase TC-PTP regulates interleukin-6-mediated signaling pathway through STAT3 dephosphorylation. Biochem Biophys Res Commun *297*, 811-7.

Yamauchi, K., Milarski, K. L., Saltiel, A. R., and Pessin, J. E. (1995). Protein-tyrosine-phosphatase SHPTP2 is a required positive effector for insulin downstream signaling. Proc Natl Acad Sci U S A *92*, 664-8.

Yamauchi, K., and Pessin, J. E. (1995). Epidermal growth factor-induced association of the SHPTP2 protein tyrosine phosphatase with a 115-kDa phosphotyrosine protein. J Biol Chem *270*, 14871-4.

Yamauchi, K., Ribon, V., Saltiel, A. R., and Pessin, J. E. (1995). Identification of the major SHPTP2-binding protein that is tyrosine- phosphorylated in response to insulin. J Biol Chem *270*, 17716-22.

Yang, J., Liang, X., Niu, T., Meng, W., Zhao, Z., and Zhou, G. W. (1998). Crystal structure of the catalytic domain of protein-tyrosine phosphatase SHP-1. J Biol Chem *273*, 28199-207.

Yang, Q., Co, D., Sommercorn, J., and Tonks, N. K. (1993). Cloning and expression of PTP-PEST. A novel, human, nontransmembrane protein tyrosine phosphatase. J Biol Chem *268*, 17650.

Yang, Q., and Tonks, N. (1991). Isolation of a cDNA Clone Encoding a Human Protein-Tyrosine Phosphatase with Homology to the Cytoskeletal-Associated Proteins Band 4.1, Ezrin, and Talin. PNAS *88*, 5949-5953.

Yang, T., Martignetti, J. A., Massa, S. M., and Longo, F. M. (2000). Leukocyte common-antigen-related tyrosine phosphatase receptor: altered expression of mRNA and protein in the New England Deaconess Hospital rat line exhibiting spontaneous pheochromocytoma. Carcinogenesis *21*, 125-31.

Yap, A. S., Brieher, W. M., Pruschy, M., and Gumbiner, B. M. (1997). Lateral clustering of the adhesive ectodomain: a fundamental determinant of cadherin function. Curr Biol *7*, 308-15.

Yap, A. S., Niessen, C. M., and Gumbiner, B. M. (1998). The juxtamembrane region of the cadherin cytoplasmic tail supports lateral clustering, adhesive strengthening, and interaction with p120ctn. J Cell Biol *141*, 779-89.

Yi, T., Mui, A. L., Krystal, G., and Ihle, J. N. (1993). Hematopoietic cell phosphatase associates with the interleukin-3 (IL-3) receptor beta chain and down-regulates IL-3-induced tyrosine phosphorylation and mitogenesis. Mol Cell Biol *13*, 7577-86.

Yi, T., Zhang, J., Miura, O., and Ihle, J. N. (1995). Hematopoietic cell phosphatase associates with erythropoietin (Epo) receptor after Epo-induced receptor tyrosine phosphorylation: identification of potential binding sites. Blood *85*, 87-95.

References

You, M., Yu, D. H., and Feng, G. S. (1999). Shp-2 tyrosine phosphatase functions as a negative regulator of the interferon-stimulated Jak/STAT pathway. Mol Cell Biol *19*, 2416-24.

You, M., and Zhao, Z. (1997). Positive effects of SH2 domain-containing tyrosine phosphatase SHP-1 on epidermal growth factor- and interferon-gamma-stimulated activation of STAT transcription factors in HeLa cells. J Biol Chem *272*, 23376-81.

You-Ten, K. E., Muise, E. S., Itie, A., Michaliszyn, E., Wagner, J., Jothy, S., Lapp, W. S., and Tremblay, M. L. (1997). Impaired bone marrow microenvironment and immune function in T cell protein tyrosine phosphatase-deficient mice. J Exp Med *186*, 683-93.

Yu, C. L., Jin, Y. J., and Burakoff, S. J. (2000). Cytosolic tyrosine dephosphorylation of STAT5. Potential role of SHP-2 in STAT5 regulation. J Biol Chem *275*, 599-604.

Yu, D. H., Qu, C. K., Henegariu, O., Lu, X., and Feng, G. S. (1998). Protein-tyrosine phosphatase Shp-2 regulates cell spreading, migration, and focal adhesion. J Biol Chem *273*, 21125-31.

Zabolotny, J. M., Kim, Y. B., Peroni, O. D., Kim, J. K., Pani, M. A., Boss, O., Klaman, L. D., Kamatkar, S., Shulman, G. I., Kahn, B. B., and Neel, B. G. (2001). Overexpression of the LAR (leukocyte antigen-related) protein-tyrosine phosphatase in muscle causes insulin resistance. Proc Natl Acad Sci U S A *98*, 5187-92.

Zahraoui, A., Joberty, G., Arpin, M., Fontaine, J. J., Hellio, R., Tavitian, A., and Louvard, D. (1994). A small rab GTPase is distributed in cytoplasmic vesicles in non polarized cells but colocalizes with the tight junction marker ZO-1 in polarized epithelial cells. J Cell Biol *124*, 101-15.

Zhang, S. H., Kobayashi, R., Graves, P. R., Piwnica-Worms, H., and Tonks, N. K. (1997). Serine phosphorylation-dependent association of the band 4.1-related protein-tyrosine phosphatase PTPH1 with 14-3-3beta protein. J Biol Chem *272*, 27281-7.

Zhang, S. H., Liu, J., Kobayashi, R., and Tonks, N. K. (1999). Identification of the cell cycle regulator VCP (p97/CDC48) as a substrate of the band 4.1-related protein-tyrosine phosphatase PTPH1. J Biol Chem *274*, 17806-12.

Zhang, Z. Y. (2001). Protein tyrosine phosphatases: prospects for therapeutics. Curr Opin Chem Biol *5*, 416-23.

Zhang, Z. Y., Wang, Y., and Dixon, J. E. (1994). Dissecting the catalytic mechanism of protein-tyrosine phosphatases. Proc Natl Acad Sci U S A *91*, 1624-7.

Zheng, X. M., Wang, Y., and Pallen, C. J. (1992). Cell transformation and activation of pp60c-src by overexpression of a protein tyrosine phosphatase. Nature *359*, 336-9.

Zhong, Y., Saitoh, T., Minase, T., Sawada, N., Enomoto, K., and Mori, M. (1993). Monoclonal antibody 7H6 reacts with a novel tight junction-associated protein distinct from ZO-1, cingulin and ZO-2. J Cell Biol *120*, 477-83.

Zondag, G. C., Koningstein, G. M., Jiang, Y. P., Sap, J., Moolenaar, W. H., and Gebbink, M. F. (1995). Homophilic interactions mediated by receptor tyrosine phosphatases mu and kappa. A critical role for the novel extracellular MAM domain. J Biol Chem *270*, 14247-50.

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The Protein Tyrosine Phosphatase Pez Is a Major Phosphatase of Adherens Junctions and Dephosphorylates β-Catenin

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Cell-cell adhesion regulates processes important in embryonal development, normal physiology, and cancer progression. It is regulated by various mechanisms including tyrosine phosphorylation. We have previously shown that the protein tyrosine phosphatase Pez is concentrated at intercellular junctions in confluent, quiescent monolayers but is nuclear in cells lacking cell-cell contacts. We show here with an epithelial cell model that Pez localizes to the adherens junctions in confluent monolayers. A truncation mutant lacking the catalytic domain acts as a dominant negative mutant to upregulate tyrosine phosphorylation at adherens junctions. We identified β-catenin, a component of adherens junctions, as a substrate of Pez by a "substrate trapping" approach and by in vitro dephosphorylation with recombinant Pez. Consistent with this, ectopic expression of the dominant negative mutant caused an increase in tyrosine phosphorylation of β-catenin, demonstrating that Pez regulates the level of tyrosine phosphorylation of adherens junction proteins, including β-catenin. Increased tyrosine phosphorylation of adherens junction proteins has been shown to decrease cell-cell adhesion, promoting cell migration as a result. Accordingly, the dominant negative Pez mutant enhanced cell motility in an in vitro "wound" assay. This suggests that Pez is also a regulator of cell motility, most likely through its action on cell-cell adhesion.

INTRODUCTION

Cell-cell adhesion regulates diverse cellular functions including cell proliferation, migration, and apoptosis (Vleminckx and Kemler, 1999). One important cell-cell adhesion system, the adherens junction (AJ), is mediated by a family of homophilic receptors, the cadherins (Steinberg and McNutt, 1999). The strength of cadherin-mediated adhesion is regulated by lateral clustering of cadherin molecules at the plasma membrane and also through the linkage of its intracellular cytoplasmic tail to the actin cytoskeleton. β-Catenin, a structural component of AJs and signal transducer of the wnt signaling pathway, is crucial for cross-linking cadherins to the actin cytoskeleton through another intermediate, α-catenin (Gumbiner, 1995;Cowin and Burke, 1996).

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Reversible tyrosine phosphorylation, catalyzed by the opposing actions of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs), is an important mechanism for regulating the linkage of cadherins to the cytoskeleton. A number of PTKs and PTPs have been found to be associated with AJs (Steinberg and McNutt, 1999). Inhibitors of PTPs have been shown to disrupt cell-cell adhesion, suggesting that PTPs play a critical role in maintaining the integrity of AJs (Ayalon and Geiger, 1997). The observation that phosphorylation of a critical tyrosine residue, Tyr654, on β-catenin results in its dissociation from E-cadherin (Roura *et al.*, 1999), verifies that tyrosine phosphorylation is an important mechanism for regulating the E-cadherin-catenin linkage. Tyrosine phosphorylation has also been reported to disrupt the β-catenin–α-catenin linkage (Ozawa and Kemler, 1998), although the critical tyrosine(s) in this case has not been determined. These observations suggest that multiple targets for tyrosine phosphorylation exist to regulate cell-cell adhesion.

The PTP Pez (PTPD2/PTP36) is a 130-kDa cytosolic (nontransmembrane) PTP (Smith *et al.*, 1995) expressed in a number of tissues. It is a member of the FERM (four-point-one,

ezrin, radixin, moesin) family of PTPs characterized by a conserved N-terminal FERM domain (Chishti *et al.*, 1998) and a C-terminal PTP catalytic domain separated by an intervening region. We recently showed that the subcellular localization of Pez is regulated in both HeLa and human umbilical vein endothelial cells (HUVEC); in cells grown to confluence Pez is localized to the cytosol, where it is concentrated at intercellular junctions, but it is predominantly nuclear in sparsely plated cells that have not yet formed extensive cell-cell contacts (Wadham *et al.*, 2000). Other factors also regulate the subcellular localization of Pez, including TGFβ, which inhibits translocation of Pez from the cytosol to the nucleus, and serum, which promotes the accumulation of Pez in the nucleus (Wadham *et al.*, 2000). Together these findings suggest that Pez could have multiple roles, involving the dephosphorylation of different substrates depending on whether it is in the nucleus or at intercellular junctions. Its presence at the intercellular junctions of confluent monolayers suggests that it may regulate the assembly or disassembly of adhesion complexes.

To elucidate the function of Pez, we used a "substrate trapping" approach (Flint *et al.*, 1997) in combination with the generation and overexpression of a dominant negative form of Pez to identify its substrates. We identified β-catenin as a substrate and show that the dominant negative Pez enhances both tyrosine phosphorylation of adherens junctions and cell motility.

MATERIALS AND METHODS

Tissue Culture and Cell Lines

MDCK and HEK 293 cell lines were cultured in DMEM supplemented with 10% FBS. HUVECS were obtained from discarded umbilical cords and cultured in M199 medium supplemented with 20% FBS and endothelial growth factors as previously described (Wall *et al.*, 1978). Transient transfections of HEK293 cells were carried out using Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. Stable MDCK clones were generated by transfection by standard calcium phosphate coprecipitation and transfectants selected by G418 (Promega, Madison, WI) resistance.

Antibodies

The polyclonal Pez antibody had previously been characterized (Wadham *et al.*, 2000). All other antibodies used were purchased: monoclonal Flag epitope antibody (M2) from AMRAD Biotech (Victoria, Australia), β-catenin, γ-catenin, and E-cadherin monoclonal antibodies from Transduction Laboratories, α-catenin mAb from either Transduction Laboratories (Lexington, KY) or Zymed (San Francisco, CA), monoclonal antiphosphotyrosine antibody (PY100) from New England Biolabs (Beverley, MA), and polyclonal ZO-1 and monoclonal p120catenin antibody from Zymed.

Generation of Mutant Pez Constructs

Isolation of the human Pez cDNA and generation of a Flag epitope-tagged construct in the mammalian expression vector, pcDNA3 (Invitrogen) has been described (Wadham *et al.*, 2000). The $D_{1079}A$ and R1127M mutations in Pez cDNA were made by site-directed mutagenesis using PCR. ΔFERM (amino acids 337-1187)- and ΔPTP (amino acids 1–932)-Pez were generated by PCR using the appropriate primers to remove the entire FERM or PTP domain, respectively. All constructs were tagged with the Flag epitope. The sequences of all mutated constructs were verified by sequencing from both the sense and antisense directions.

Generation of GST-Pez Fusion Proteins

wt-Pez and ST-Pez coding sequences were excised from the pcDNA3 constructs described above and cloned into the pGEX 4T-1 vector (Amersham Biosciences, Piscataway, NJ) to generate GST-fusion Pez proteins. The constructs were transformed into BL21-Codon Plus (DE3)-RIL *Escherichia coli* (Stratagene, La Jolla, CA) for protein expression. Cultures were induced with 0.15 mM IPTG for 2 h at ambient temperature, and the GST-fusion proteins were purified on glutathione sepharose. The amounts of full-length fusion proteins produced were determined by Coomassie blue staining after PAGE. Equal amounts of GST-wt-Pez and GST-ST-Pez protein were used for in vitro dephosphorylation of β-catenin.

Substrate Trapping

Newly confluent HUVEC lysates, used as a source of tyrosine-phosphorylated proteins, were prepared as described (Flint *et al.*, 1997). Briefly, the cells were incubated for 30 min with 50 μM sodium pervanadate to enrich for tyrosine-phosphorylated proteins, washed in phosphate-buffered saline (PBS), and lysed in ST buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 150 mM NaF, 10 mM sodium pyrophosphate, 5 mM EDTA, 1% Triton X-100, and protease inhibitor cocktail [P2714, Sigma, St Louis, MO]) containing 1 mM sodium orthovanadate at 4°C. The lysates were incubated on ice for 30 min in the presence of 5 mM iodoacetic acid to irreversibly inactivate endogenous PTPs. Unreacted iodoacetic acid was inactivated with 10 mM DTT. The lysates were then frozen on liquid nitrogen and stored at −70°C.

Flag-tagged wt-Pez or ST-Pez was transiently transfected into HEK293 cells and transfectants lysed in ST buffer 48 h after transfection. Equal amounts of protein from each lysate were immunoprecipitated (in the absence of orthovanadate) with an anti-Flag (M2) antibody precoupled to protein A sepharose beads. The Pez immunoprecipitates were washed three times in ST buffer, added to the phosphotyrosine enriched HUVEC lysates, and rocked at 4°C for 2 h. The beads were washed three times with ST buffer, boiled in Laemmli sample buffer, and bound proteins resolved by 8% SDS-PAGE. To detect tyrosine-phosphorylated proteins "pulled-down" by either wt or ST Pez, Western blots were performed using an antiphosphotyrosine antibody (PY100).

Immunoprecipitations and Western Blots

Immunoprecipitations were carried out after lysis of cells in ice-cold ST buffer containing 1 mM orthovanadate. Lysates were precleared with 20 μl of protein A-sepharose for 30 min at 4°C. Protein concentration was assayed using Bradford Reagent from Bio-Rad (Hercules, CA). Equal amounts (1–5 mg) of protein were incubated with 2 μg of primary antibodies supplemented with 20 μl packed protein A-sepharose for 1 h at 4°C. After washing, bound proteins were eluted by boiling in Laemmli sample buffer for 5 min separated by 8% SDS-PAGE and transferred to PVDF membrane (Hybond-P, Amersham Pharmacia Biotech) for Western blotting. Western blotting was carried out after blocking with 5% milk, 0.1% Triton X-100 in PBS using the indicated antibodies and developed using HRP-conjugated secondary antibody (Immunotech, Marseille, France) and ECL (Amersham Pharmacia Biotech). For quantitation, Western blots were developed with ECL-Plus (Amersham Pharmacia Biotech), and fluorescence intensity was imaged using a Molecular Dynamics Typhoon 9410 (Amersham Biosciences, United Kingdom) variable mode imager.

Immunofluorescence

MDCK stable cells lines expressing either wt-Pez, ΔPTP-Pez, or ΔFERM-Pez were plated at confluent density onto fibronectin

coated glass LabTek chamber slides (Nalge, Nunc International, Naperville, IL) and incubated for 2–3 d before staining. The cells were fixed in 4% paraformaldehyde/PBS for 10 min, quenched with 10 mg/ml sodium borohydride for 15 min, and then permeabilized by treatment with 0.1% Triton X-100. Primary antibodies were used at 1:100 dilution and binding detected by incubation with either fluorophore-coupled secondary antibodies or biotinylated secondary antibodies followed by fluorophore-conjugated streptavidin, as indicated (Molecular Probes, Eugene, OR).

Epifluorescence microscopy was performed on an Olympus BX-51 microscope equipped with excitation filters for Alexa Fluor 594/Texas red and fluorescein (494 nm), acquired to a Cool Snap FX, charge-coupled device (CCD) camera (Photometrics, Phoenix, AZ). Images were adjusted for brightness and contrast with V^{++} software (Digital Optics Ltd., Auckland, New Zealand). The line-profiling feature of this software was used to plot the intensity vs. position of different fluorophores along a path through the cell monolayer, in cells that had been costained for two proteins. Confocal microscopy was performed using a 60× oil-immersion objective on an Olympus IX70 inverted microscope linked to a Bio-Rad Radiance 2100 confocal microscope. Sequential scans of each fluorophore separately were carried out for two-color colocalization studies.

Wounding Assay

MDCK stable cell lines were plated onto six-well trays at densities that would give confluent monolayers after 24 h. Confluent monolayers were incubated a further 48 h to allow intercellular junctions to mature before being serum-starved for 24 h. A linear wound was generated on the monolayers by scraping with the edge of a cell scraper. Unattached cells were washed off with agitation. Cells were photographed at the same point on a grid at the time of scraping and again at 24 h later. The difference in width of the wound between the two edges at the time of scraping and 24 h later was measured and represents the distance migrated. Each line was plated and wounded in triplicate.

RESULTS

Pez Colocalizes with E-cadherin at AJs

We previously observed in confluent endothelial monolayers that endogenous Pez localizes to the intercellular junctions (Wadham *et al.*, 2000). Here, we investigate the localization of Flag-tagged-Pez stably expressed in MDCK cells, a polarized epithelial cell line in which cell-cell adhesion is well characterized. For subsequent investigations of the function of Pez, truncation mutants of Pez that lack either the catalytic (ΔPTP-Pez) or FERM (ΔFERM-Pez) domain (Figure 1A) were created and their subcellular localization when stably expressed in MDCK epithelial cell lines were also examined. wt-Pez and both truncated Pez mutants, examined by epifluorescent microscopy, were similarly localized to what appears to be the intercellular junctions (Figure 1B). To further confirm that the localization of Pez was indeed at intercellular junctions rather than at the cell surface, optical sectioning using a confocal microscope was performed on wt-Pez-MDCK cells that had been costained for the Flag-epitope (on Pez) and E-cadherin (a marker of AJs). The data showed that Pez precisely colocalized with E-cadherin at basolateral membranes both along the z-axis (Figure 1, C and D) and in the *x-y* plane (Figure 1E), confirming that it is localizing to the AJs.

ΔPTP-Pez Is a Potential Dominant Negative Mutant of Pez That Causes an Increase in Tyrosine Phosphorylation at AJs

Because ΔPTP-Pez is devoid of the catalytic domain and therefore not enzymatically active, but retains the ability to localize to intercellular junctions, it can potentially act as a dominant negative mutant. If Pez is an AJ PTP that regulates the level of tyrosine phosphorylation at AJs, then overexpression of a dominant negative mutant of Pez should result in an increase in tyrosine phosphorylation of AJ proteins. This was investigated using confluent monolayers of MDCK cells overexpressing ΔPTP-Pez. Cells were serum-starved followed by 10 min serum stimulation before staining with an antiphosphotyrosine antibody. Epifluorescence microscopy showed that there was a markedly higher level of tyrosine phosphorylation at intercellular junctions (marked by costaining with an anti–ZO-1 antibody) in ΔPTP-Pez–transfected cells compared with the empty vector control or wt-Pez transfectants (Figure 2A). Under these experimental conditions no tyrosine phosphorylation was detected at the intercellular junctions of empty vector- and wt-Pez–transfected cells. This is best demonstrated when the fluorescence intensities resulting from both the phosphotyrosine and ZO-1 antibodies were quantitated across several cell boundaries (Figure 2A, right column).

To determine the exact location of the tyrosine-phosphorylated proteins induced by overexpression of the putative dominant negative mutant ΔPTP-Pez, optical sectioning with a confocal microscope was performed on confluent ΔPTP-Pez–transfected cells costained with both the antiphosphotyrosine and anti–E-cadherin antibodies. As with Pez, the tyrosine phosphorylation induced by ΔPTP-Pez precisely colocalized with E-cadherin both along the Z-axis (Figure 2, B and C) and in the *x-y* plane, confirming that the increased tyrosine phosphorylation occurred at AJs. In addition, these data also suggest that the tyrosine-phosphorylated substrates remained in the proximity of the plasma membrane and did not translocate to other parts of the cell. Induction of tyrosine phosphorylation at intercellular junctions has also been confirmed using another potential dominant negative mutant, the R1127M point-mutant (Figure 2D; R1127 of Pez is the equivalent of R221 of PTP1B, which when mutated leads to inactivation of its PTP activity (Flint *et al.*, 1997).

The AJ Protein β-Catenin Is a Substrate of Pez and Coimmunoprecipitates with Pez

Data obtained so far suggested that Pez was an AJ PTP and in concordance with this hypothesis, a putative dominant negative mutant of Pez caused an increase in tyrosine phosphorylation of AJs. A number of components of the AJ complex can be tyrosine phosphorylated, leading to alterations in their functions (Steinberg and McNutt, 1999). We therefore used a substrate trapping approach to identify substrates of Pez at the AJ.

Asp181 of PTP1B is an essential residue for catalytic activity of PTP1B, which when mutated to Ala results in a catalytically inactive substrate trapping (ST) mutant (Flint *et al.*, 1997). Sequence alignment of the phosphatase domains of Pez and PTP1B indicate that Asp1079 of Pez corresponds to Asp181 of PTP1B. To generate a ST mutant of Pez (denot-

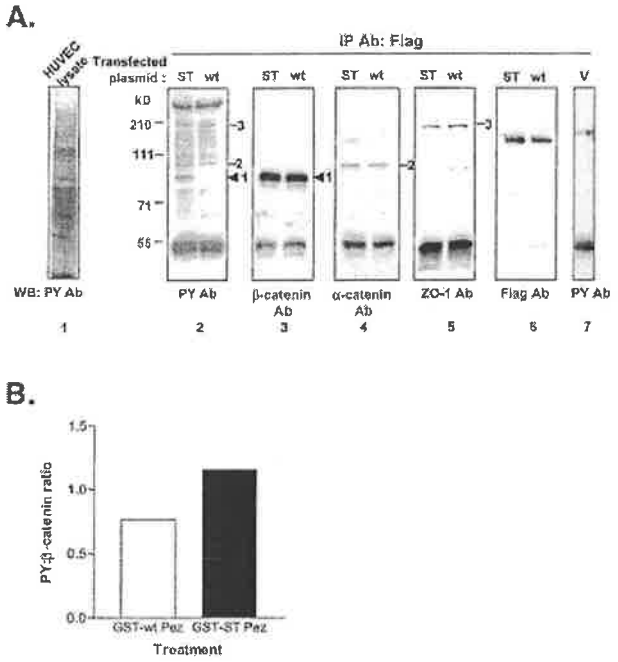

Figure 3. Identification of Pez substrates and interacting proteins by substrate trapping and by in vitro dephosphorylation. (A) Substrate trapping. HUVEC lysate enriched for tyrosine-phosphorylated proteins (panel 1) was incubated for 2 h with Sepharose-bound anti-Flag immunoprecipitates from cells transfected with wt-Pez, ST-Pez, or empty vector as shown. The beads were then washed, and bound proteins were eluted and immunoblotted with various antibodies as indicated. (B) In vitro dephosphorylation of β-catenin by GST-Pez. HUVEC lysate enriched for tyrosine-phosphorylated proteins (as in A) was incubated with either GST-wt-Pez or GST-ST-Pez bound to glutathione sepharose beads. After removal of the GST-Pez fusion proteins, β-catenin was immunoprecipitated, and the immunoprecipitates were Western blotted with an antiphosphotyrosine antibody. The amount of tyrosine-phosphorylated β-catenin was quantitated by fluorimaging. The blots were then stripped, counterblotted with anti–β-catenin antibody, and quantitated for total β-catenin. The ratio of tyrosine-phosphorylated β-catenin to total β-catenin for each GST-Pez treatment is shown (one representative of three experiments).

After stripping and reprobing the filters with antibodies to other components of junctional adhesion complexes, we identified a number of other junctional proteins, including α-catenin (band 2, Figure 3A, panel 4), ZO-1 (band 3, Figure 3A, panel 5), and plakoglobin (unpublished data), which interact with Pez but do not appear to be substrates for its PTPase activity. β-Catenin, α-catenin, and plakoglobin are all components of the AJ complex. ZO-1 is normally associated with tight junctions in polarized epithelial and endothelial monolayers but in newly confluent HUVEC, which have not formed bona fide tight junctions, it is also associated with AJs (Stevenson and Keon, 1998), suggesting that a complex of AJ proteins may be binding to Pez.

The phosphotyrosyl-enriched HUVEC lysate that was the source of phospho-β-catenin used in the substrate-trapping approach was made devoid of any active PTPs by iodoacetate treatment. However, there is a possibility that the wt-Pez immunoprecipitates incubated with the lysate may contain other active PTPs in addition to Pez. To demonstrate that phospho-β-catenin can be directly dephosphorylated by Pez, we carried out a similar experiment using recombinant Pez, prepared as a GST fusion protein from *E. coli*. As a control, we performed the same reaction with the inactive GST-ST-Pez. After incubation of recombinant Pez with the HUVEC lysate, β-catenin was immunoprecipitated with anti–β-catenin antibody and the amount of tyrosine-phosphorylated β-catenin remaining was quantitated by fluorimager analysis of phosphotyrosine immunoblots. Although the GST fusion proteins expressed poorly in *E. coli* (>90% of the products were degraded, presumably because the large fusion protein [~160 kDa] was poorly folded), the data showed that GST-wt-Pez, but not GST-ST-Pez, dephosphorylated the β-catenin in the lysate, removing about one third of the tyrosylphosphates on the β-catenin (Figure 3B). GST-Pez proteins lacking the FERM domain were also expressed and assayed, showing that the forms with wt but not ST catalytic domains dephosphorylate β-catenin to a similar extent as full-length GST-wt-Pez (unpublished data).

These experiments indicated that Pez can directly dephosphorylate β-catenin in vitro. To further assess whether Pez and β-catenin interact in vivo, we investigated whether Pez can be coimmunoprecipitated with β-catenin. Immunoprecipitation of endogenous β-catenin from cell lysates containing either endogenous or ectopically expressed Pez was carried out, followed by Western blotting to determine if Pez is coimmunoprecipitated. Endogenous Pez was detected in β-catenin immunoprecipitates when a β-catenin antibody, but not a control antibody, was used to immunoprecipitate β-catenin from confluent HUVEC monolayers (Figure 4A). Similarly, ectopically expressed Pez coimmunoprecipitated with β-catenin in cells that were transfected with an expression vector bearing Pez cDNA but not empty vector (Fig, 4B), confirming that Pez and β-catenin interact in vivo.

Although β-catenin coimmunoprecipitated with both endogenous and ectopic Pez, the coimmunoprecipitation appeared to be relatively weak. This could have a number of possible explanations. First, the coimmunoprecipitations were carried out in 1% Triton X-100 with a brief pulse of sonication to maximize recovery of Pez. These conditions may be too harsh for the proteins to remain bound. In preliminary experiments to test the strength of the interaction between Pez and β-catenin, we have observed better coimmunoprecipitations if the sonication step was omitted although more striking increases in coimmunoprecipitation were observed by reducing the concentration of detergent used in the lysis buffer from 1% to 0.5% Triton X-100 (Figure 4B, right panel). This suggests that the complex formed may be detergent labile. Such detergent lability has been well documented with p120 catenin (p120ctn)-cadherin interactions, whereby only 5–20% of the p120ctn in detergent lysates is associated with cadherin in contrast to its almost complete localization to AJs or membrane fraction under detergent-free conditions (reviewed in Anastasiadis and Reynolds, 2000). Finally, it is likely that not all the Pez within the cell is localized to the AJ. This has certainly been observed in endothelial cells where Pez expression is observed in the cytosol away from the cell junctions, even when the monolayer is confluent (Wadham *et al.*, 2000). This is also

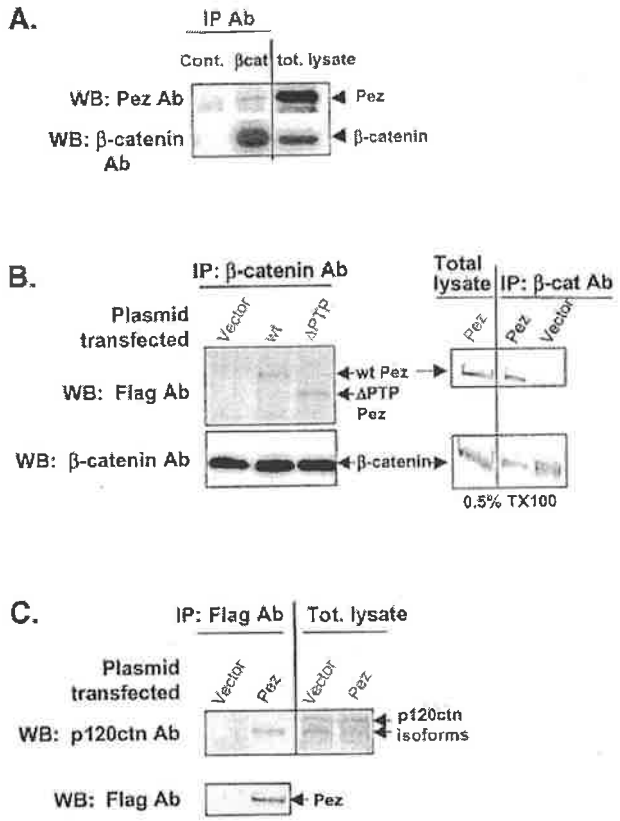

Figure 4. Coimmunoprecipitations of endogenous Pez and ectopically expressed wt- and ΔPTP-Pez with endogenous β-catenin and p120ctn. (A) HUVEC lysate was incubated with β-catenin antibody or control isotype-matched irrelevant antibody, and immunoprecipitates were Western blotted with Pez or β-catenin antibodies as indicated; right panel shows Western blots of total lysate. (B) Left panel: Empty vector, wt-Pez, or ΔPTP-Pez were transiently transfected into HEK293 cells. β-Catenin was immunoprecipitated from the lysates of transfectants and Western blotted using a Flag-epitope antibody to detect ectopically expressed Pez. The blot was stripped and reblotted with a β-catenin antibody showing the presence of β-catenin in all three immunoprecipitations. Right panel as in left panel, except that lysis was carried out in 0.5% instead of the 1% Triton X-100, which was used in all the other immunoprecipitations shown. (C) Flag epitope-tagged Pez was immunoprecipitated with an anti-Flag antibody from HEK 293 cells stably transfected with either empty vector or Flag epitope-tagged Pez. The immunoprecipitates were Western-blotted with an anti-p120ctn antibody and reblotted with the anti-Flag antibody.

particularly true when Pez is ectopically expressed in HEK 293 cells (unpublished data).

Because a number of AJ proteins were also pulled-down together with β-catenin (Figure 3A) and because of their structural relatedness, we also investigated whether p120ctn coimmunoprecipitates with Pez. Immunoprecipitates from lysates of vector- or Pez-transfected stable HEK 293 cell lines carried out using the Flag-epitope antibody were Western blotted with a p120ctn antibody. p120ctn coimmunoprecipitated with Pez that was immunoprecipitated with the Flag antibody (Figure 4C). Western blots of total lysates from both the vector- and Pez-transfected cells showed that HEK 293 cells expressed two major p120ctn isoforms of similar abundance (Figure 4C), with the larger isoform corresponding to full-length p120ctn (isoform 1). Interestingly, only the smaller MW isoform (~95 kDa) coimmunoprecipitated with Pez. Isoforms 1 and 3 are the most commonly expressed (reviewed in Anastasiadis and Reynolds, 2000), hence the smaller isoform coimmunoprecipitating with Pez is most likely isoform 3, although this remains to be confirmed. It is unclear why Pez is only associated with one isoform of p120ctn, and it is also unkown at this stage whether p120ctn interacts directly with Pez and whether it is a substrate. These will be the subject of future studies. What is clear, however, is that the cell junctional proteins that are pulled-down with Pez are likely to be specific because the coimmunoprecipitation discriminated between the two highly related isoforms of p120ctn.

ΔPTP-Pez Interacts with and Induces Tyrosine Phosphorylation of β-Catenin

If ΔPTP-Pez acts as a dominant negative mutant of Pez (as suggested by its localization to the AJ [Figure 1B] and by its ability to induce tyrosine phosphorylation of proteins at the AJ [Figure 2]), and if β-catenin is a bona fide substrate of Pez, then ΔPTP-Pez should interact with β-catenin in vivo to increase its tyrosine phosphorylation. To investigate the ability of ΔPTP-Pez to interact with β-catenin in vivo, β-catenin was immunoprecipitated from HEK293 cells stably transfected with ΔPTP-Pez. The β-catenin immunoprecipitates were then Western blotted with an anti-Flag antibody to detect ΔPTP-Pez that has coimmunoprecipitated with β-catenin. The data showed that ΔPTP-Pez was able to coimmunoprecipitate with β-catenin to the same extent as wt-Pez (Figure 4B, left panel).

To examine whether ΔPTP-Pez can induce tyrosine phophorylation of Pez substrates through a dominant negative effect, antiphosphotyrosine Western blots were performed on extracts from confluent monolayers of MDCK cells stably expressing empty vector, wt-Pez, or ΔPTP-Pez. Cells were serum-starved for 24 h before addition of serum for 10 min to induce tyrosine phosphorylation. To see specific tyrosine phosphorylation of Pez substrates, cells were not pretreated with pervanadate, which would have caused a global increase in tyrosine phosphorylation. Thus, in concordance with other studies (Ayalon and Geiger, 1997), the basal level of tyrosine-phosphorylated proteins in vector-transfected cells was very low. Two bands that were specifically phosphorylated in extracts from ΔPTP-Pez but not empty vector or wt-Pez transfected cells (Figure 5A, closed arrowheads) were observed. The lower MW band comigrated with β-catenin when the filter was counterblotted with a β-catenin antibody, suggesting that one of the proteins that is tyrosine phosphorylated through overexpression of ΔPTP-Pez is β-catenin. Immunoprecipitation of β-catenin followed by Western blotting with an antiphosphotyrosine antibody confirmed that β-catenin was indeed phosphorylated in ΔPTP-Pez–transfected but not empty vector-transfected cells (Figure 5B). These data indicate that ΔPTP-Pez acts as a dominant negative mutant of Pez leading to an increased level of β-catenin tyrosine phosphorylation. The finding that ΔPTP-Pez could interact with and increase

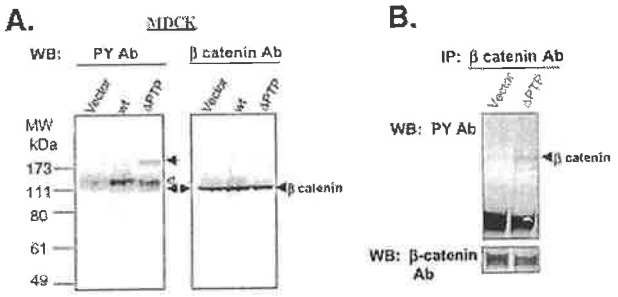

Figure 5. ΔPTP-Pez enhances tyrosine phosphorylation of β-catenin. (A) MDCK cell lines stably expressing vector, wt-Pez, or ΔPTP-Pez were serum-starved for 24 h followed by 10-min stimulation with 10% FBS to induce tyrosine phosphorylation. Lysates were Western blotted with a phosphotyrosine (PY) antibody (left). Arrowheads show two proteins specifically tyrosine phosphorylated in the ΔPTP-Pez transfected cells, one of which comigrates with β-catenin (double arrowhead) when the blot was stripped and reblotted with a β-catenin antibody (right). (B) Lysates from empty vector- or ΔPTP-Pez–transfected MDCK cells were immunoprecipitated with a β-catenin antibody followed by Western blotting of the immunoprecipitates with an antiphosphotyrosine antibody (top). The blot was stripped and counterprobed with a β-catenin antibody (bottom).

the tyrosine phosphorylation status of β-catenin further reinforces our conclusion that β-catenin is a bona fide Pez substrate. The presence of another protein with increased tyrosine phosphorylation in ΔPTP-Pez but not wt-Pez or empty vector-transfected cells (Figure 5A, open arrowhead) suggests that there are other Pez substrates that were not identified by substrate trapping. Any additional substrates are likely to also be located at the intercellular junctions because the tyrosine phosphorylation induced by ΔPTP-Pez was highly specific to intercellular junctions (Figure 2A). The presence of other substrates at intercellular junctions in addition to β-catenin would also account for the dramatic increase in tyrosine phosphorylation at intercellular junctions in ΔPTP-Pez MDCK cells. Similarly, the tyrosine-phosphorylated band comigrating with β-catenin in Figure 5A may be comprised of more than one protein of the same relative mobility. The higher degree of tyrosine phosphorylation relative to β-catenin protein in the ΔPTP-Pez lysate shown in Figure 5A compared with that in the β-catenin immunoprecipitates in Figure 5B (where the observed phosphorylation is due solely to β-catenin) suggests that this may be the case.

Overexpression of the Dominant Negative Mutant (ΔPTP-Pez) Enhances Cell Migration

Tyrosine phosphorylation of β-catenin has been correlated with increased cell migration in a number of studies (Liu *et al.*, 1997;Muller *et al.*, 1999;Hollande *et al.*, 2001). We used an in vitro "wound" assay to investigate whether the increase in tyrosine phosphorylation of β-catenin that results from overexpression of the dominant negative mutant, ΔPTP-Pez, could affect rates of cell migration. In this assay, a linear scratch was made on a confluent monolayer of MDCK cells to generate a linear denuded area, after which cells from the edge of the wound could migrate into the denuded area to repopulate it. After 24 h, cells overexpressing ΔPTP-Pez had migrated further into the wound than cells overexpressing either empty vector or wt-Pez (Figure 6A). Measurements of the distances migrated after 24 h (Figure 6B) showed that the average distance migrated by the ΔPTP transfectants was significantly greater ($p = 0.02$) than the distance migrated by either wt-Pez or vector control cells. There was no significant difference between the distances migrated by the wt-Pez cells and empty vector-transfected cells ($p = 0.08$). Higher resolution images of the "wound" shows that the cells at the edge are migrating into the wound characterized by protrusions into the wound and formation of pseudopodia (Figure 6C). Our observation that the dominant negative mutant, ΔPTP-Pez, enhanced cell motility suggests that Pez is a regulator of cell motility, most likely through its role in regulating cell-cell adhesion.

DISCUSSION

Regulation of the integrity of AJs in response to external cues is important for proper tissue and organ formation during embryonal development and for wound healing in adults. The participation of tyrosine phosphorylation in regulation of AJ function is apparent from the involvement of PTKs such as Src (Behrens *et al.*, 1993;Fujita *et al.*, 2002), EGF receptor, and HGF/scatter factor receptor (Shibamoto *et al.*, 1994) in phosphorylation of AJ proteins, leading to decreased cell-cell interaction and concomitant enhancement of cell migration. A number of PTPs have also been found to be components of the AJ complex, including the receptor PTPs μ, κ, and LAR and the cytosolic PTP, PTP1B (reviewed in (Steinberg and McNutt, 1999).

In this study, we have identified the PTP Pez as a novel PTP of AJs. A truncation mutant of Pez lacking the catalytic domain acted as a dominant negative mutant to enhance tyrosine phosphorylation of AJs and promote cell migration. Analysis of the proteins that are tyrosine phosphorylated as a result of the overexpression of the dominant negative mutant suggested that there are at least two Pez substrates in epithelial cells, one of which is the AJ protein β-catenin. Using a substrate trapping mutant to isolate potential Pez substrates followed by in vitro dephosphorylation of β-catenin by recombinant Pez, we confirmed that β-catenin is indeed a substrate of Pez. Dephosphorylation of β-catenin by recombinant Pez in the absence of any other active PTP also demonstrated that Pez could directly dephosphorylate β-catenin. Both endogenous and ectopically expressed Pez coimmunoprecipitated with endogenous β-catenin, indicating that they interact in vivo, providing further evidence that Pez is a physiological regulator of β-catenin tyrosine phosphorylation.

The highly similar complement of AJ proteins pulled-down by both wt-Pez and ST-Pez, the observation that wt-Pez and ST-Pez can pull-down β-catenin equally well, and the observation that wt-Pez can pull-down unphosphorylated β-catenin all suggest that Pez is likely to be a component of the AJ complex. This raises a number of questions. First, although we have demonstrated that Pez could directly dephosphorylate β-catenin, the question remains as to whether its association with the AJ complex is solely through binding to β-catenin or through binding to some

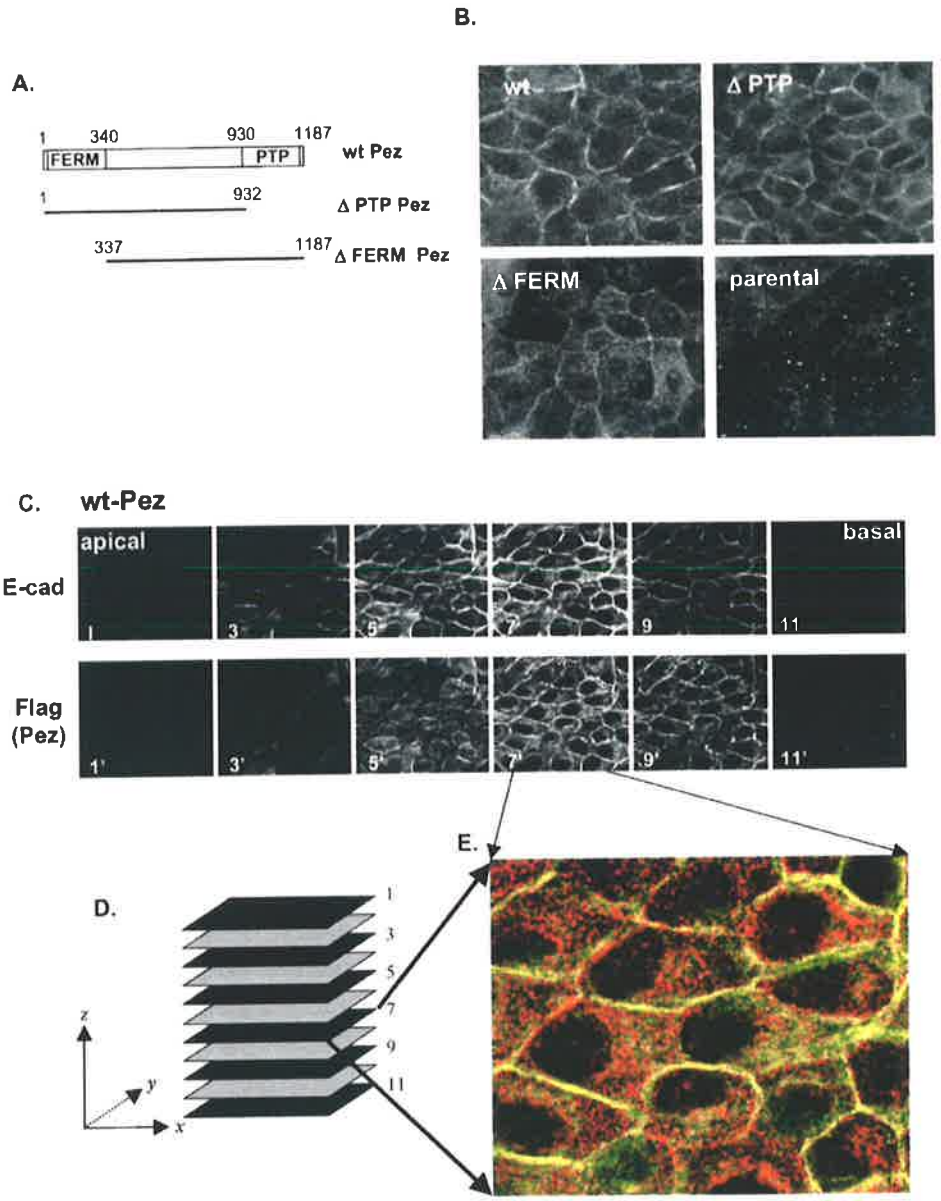

Figure 1. Flag-tagged wt-Pez and truncation mutants of Pez localize to the AJs of confluent monolayers of MDCK cells. (A) Schematic showing the structure of wt-, ΔPTP-, and ΔFERM-Pez. (B) Epifluorescence microscopy of confluent monolayers of parental MDCK cells and stable MDCK cell lines expressing wt-, ΔPTP-, and ΔFERM-Pez, labeled with an antibody against the Flag epitope and detected by indirect immunofluorescence using FITC-conjugated anti-mouse antibody. Specific labeling was observed in all Pez-expressing cell lines at the intercellular junctions. No staining was observed in the parental cells. (C) Z-series obtained by confocal laser scanning microscopy showing wt-Pez–transfected MDCK cells double-stained with the anti–E-cadherin (top row) and anti-Flag (bottom row) antibodies to indicate colocalization of Pez with E-cadherin along the z-axis. Anti–E-cadherin antibody was detected with phycoerythrin-conjugated anti-mouse IgG2a and anti-Flag antibody with biotinylated anti-mouse IgG1 antibody and FITC-conjugated streptavidin. Z-steps were carried out at 0.5-μm intervals, and alternate optical sections are shown. Top and bottom panels from each row show E-cadherin and Pez staining, respectively, from the same optical section. (D) Diagrammatic representation of the optical sections in the Z-series shown in C, indicating *x*, *y*, and z axes. (E) High-resolution merged image of one optical section (represented by panels 7 and 7′ from C) showing colocalization (yellow) in the *x*-*y* plane of Pez (green) and E-cadherin (red).

ed ST-Pez), we mutated D1079 of Pez to Ala and verified using an in vitro assay with a tyrosine-phosphorylated peptide that the catalytic activity of ST-Pez was significantly reduced compared with that of wt-Pez (between 10–20% of wt-Pez activity; unpublished data).

Without prior knowledge of the substrates of Pez, specific agonists could not be used to trigger their phosphorylation. Therefore, treatment with pervanadate, an inhibitor of PTPs, was used to upregulate tyrosine phosphorylation of proteins, including potential Pez substrates, in vivo (Figure 3A, panel 1). After lysis, endogenous PTPs in the HUVEC lysate were subsequently irreversibly inactivated by treatment with iodoacetic acid. The phosphotyrosyl-enriched HUVEC lysate devoid of endogenous PTP activities was incubated with either Flag-tagged wt- or ST-Pez (expressed in HEK 293 cells) immunoprecipitates bound to protein A-sepharose beads (Figure 3A, panel 6). Tyrosine-phosphorylated proteins that could interact with the wt- or ST-Pez immunoprecipitates, either directly or indirectly, were pulled-down and detected by Western blotting with an antiphosphotyrosine antibody. A number of tyrosine-phosphorylated proteins of similar staining intensities were pulled-down by both wt- and ST-Pez immunoprecipitates (Figure 3A, panel 2) but not immunoprecipitates from vector-transfected cells (panel 7). However, Band 1 was barely detectable in wt-Pez pull-downs but was clearly present when associated with ST-Pez. This could be because Band 1 did not

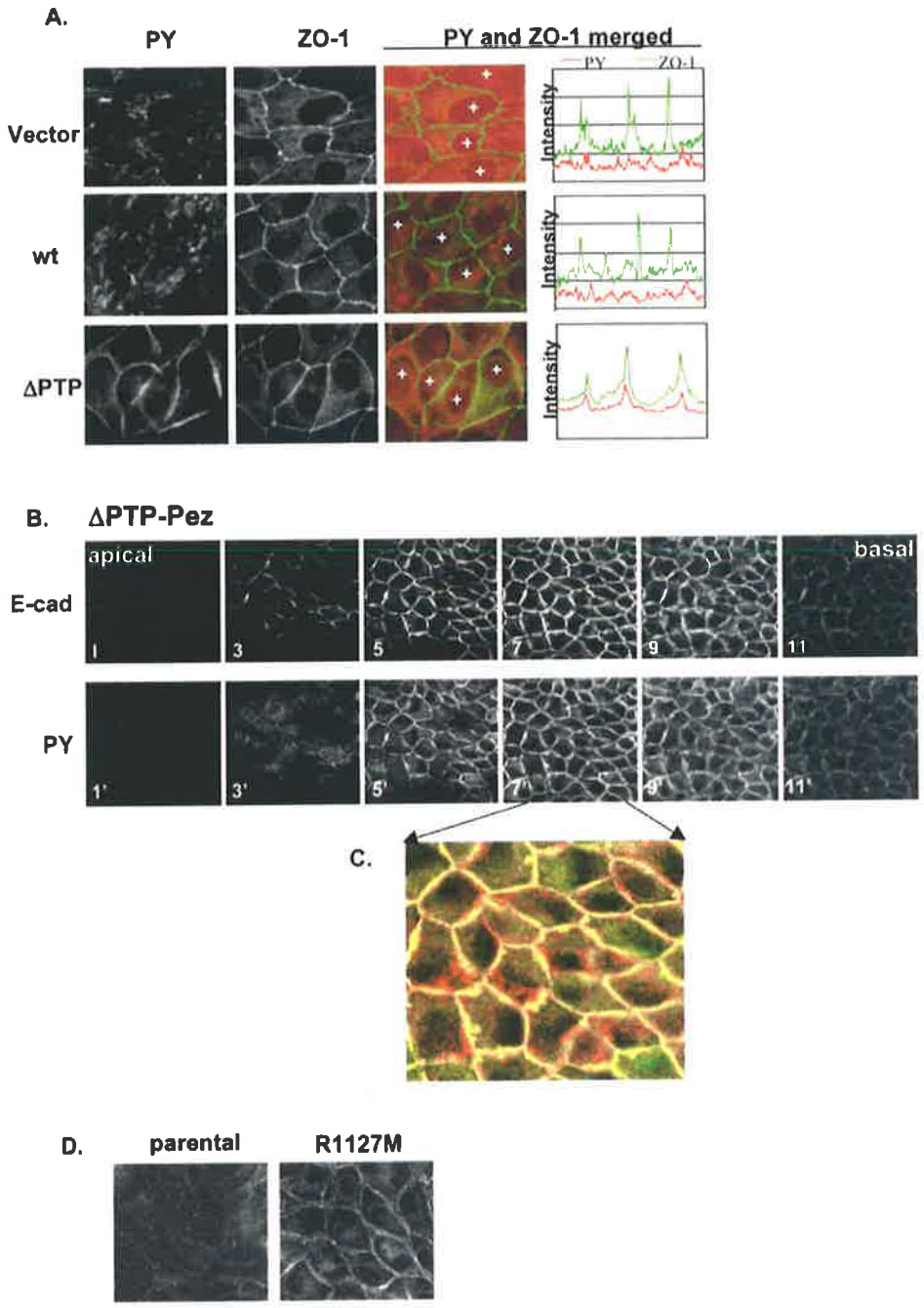

Figure 2. ΔPTP-Pez enhances tyrosine phosphorylation of AJs. (A) Epifluorescence microscopy showing confluent monolayers of MDCK cells stably expressing vector, wt- or ΔPTP-Pez, double-labeled with phosphotyrosine (PY) and ZO-1 antibodies and detected by indirect immunofluorescence using biotinylated anti-mouse antibody followed by Texas Red–conjugated streptavidin and Alexa fluor 350–conjugated anti-rabbit antibody, respectively. From left to right columns: phosphotyrosine antibody staining, ZO-1 staining to show positions of cell-cell contacts, merged image of PY staining (false colored red) and ZO-1 staining (false colored green), and quantitation of PY and ZO-1 fluorescent intensities taken along a line connecting the crosses shown in the merged image. (B) Z-series obtained by confocal laser scanning microscopy showing ΔPTP-Pez–transfected MDCK cells double-stained with the anti–E-cadherin (top row) and antiphosphotyrosine (bottom row) antibodies to show colocalization of phosphotyrosines with E-cadherin along the z-axis. Anti–E-cadherin antibody was detected with phycoerythrin-conjugated anti-mouse IgG2a, and antiphosphotyrosine antibody was detected with biotinylated anti-mouse IgG1 antibody and FITC-conjugated streptavidin. Z-steps were carried out at 0.5-μm intervals, and alternate optical sections are shown. Top and bottom panels from each row show E-cadherin and phosphotyrosine staining, respectively, from the same optical section. (C) High-resolution merged image of one optical section (represented by panels 7 and 7′ of B) showing colocalization (yellow) in the *x-y* plane of phosphotyrosine (green) and E-cadherin (red) staining. (D) Epifluorescence microscopy of confluent monolayers of parental MDCK or a stable MDCK cell-line expressing a Pez mutant, R1127M, stained with the phosphotyrosine antibody.

bind sufficiently stably to wt-Pez to be pulled-down or because the associated protein had been dephosphorylated by the catalytically active wt-Pez but not the inactive ST-Pez. Both causes for its absence in the wt-Pez pull-downs are consistent with Band 1 being a specific substrate of Pez.

Because the molecular weight of Band 1 is similar to that of the AJ protein β-catenin, we probed parallel lanes with a β-catenin antibody. β-Catenin was found to be pulled-down by both wt- and ST-Pez (Figure 3A, panel 3) and furthermore comigrated exactly with Band 1, suggesting that the substrate at this position could indeed be β-catenin. Thus, if indeed Band 1 is β-catenin, it was only tyrosine phosphorylated when in association with inactive ST-Pez, suggesting that the lack of tyrosine phosphorylation in the wt-Pez–associated protein was due to dephosphorylation by wt-Pez.

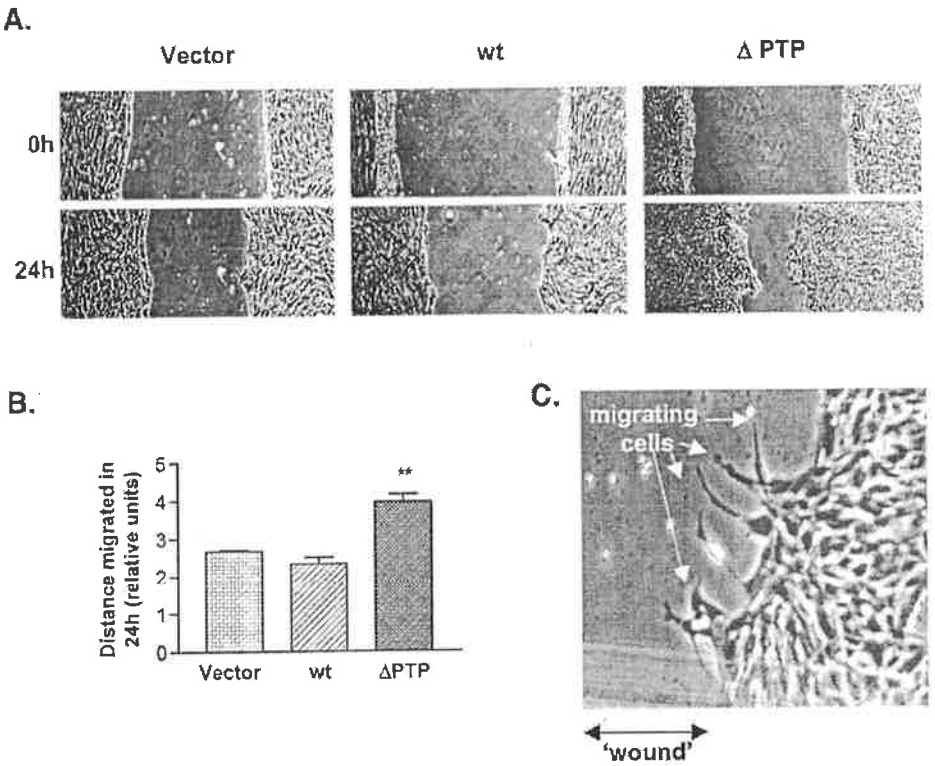

Figure 6. ΔPTP-Pez enhances cell motility. (A) Confluent monolayers of MDCK cells stably expressing empty vector, wt-Pez, or ΔPTP-Pez were wounded using the edge of a cell scraper and photographed immediately (0h). Each wound was photographed again at the same spot 24 h later. (B) The distance between the wound edges was measured at the same point on each wound at 0 h and at 24 h. The difference in distance between the two edges at 24 h and 0 h was taken to be the distance migrated in 24 h. Each cell line was plated and wounded in triplicate and the experiment performed at least twice with similar outcomes; data from one representative experiment are presented. **p = 0.02 when distance migrated by ΔPTP- expressing cells was compared with cells expressing empty vector. (C) Higher magnification image of ΔPTP-Pez MDCK cells at the wound edge 24 h after wounding.

other component of the AJ complex. Second, if Pez is a component of the AJ through direct binding to β-catenin or some other protein, than one might expect that any tyrosine phosphorylation of β-catenin at the AJ will be very transient. To achieve longer-term loosening of the AJ might then require downregulation of Pez activity.

Of the PTPs that have previously been shown to be localized to the AJ, PTP LAR and PTP 1B have been shown to dephosphorylate β-catenin, whereas the substrates of PTPμ are yet to be identified. There are potentially many reasons for multiple PTPs to be associated with AJs. These include cell type-specific expression of some PTPs, different degrees of responsiveness to external stimuli and different substrate specificities exhibited by different PTPs. In the case of β-catenin, the crystal structure indicates there are potentially up to 14 tyrosines that are accessible for phosphorylation. One of these, Tyr654, has been demonstrated to regulate the binding of β-catenin to E-cadherin (Roura *et al.*, 1999), but the phospho-tyrosine that interrupts α-catenin binding is yet to be determined. It is conceivable that dephosphorylation of different tyrosines that mediate different functions on the one molecule may be regulated by different PTPs. A comprehensive analysis of substrate specificity and responses to external stimuli for individual PTPs, which to date has not been carried out, is essential to fully elucidate the role of tyrosine phosphorylation in regulating AJ functions.

Finally, some studies have reported that after the tyrosine phosphorylation of β-catenin and its dissociation from intercellular junctions, it is translocated into the nucleus where it can, under some circumstances, interact with the LEF-1/Tcf transcription factor to alter gene expression (Adam *et al.*, 2001; Kim and Lee, 2001; Monga *et al.*, 2002). What is not clear from these studies is whether β-catenin is translocated into the nucleus in its tyrosine-phosphorylated form and if so, whether the tyrosine-phosphorylated form can interact with LEF-1/Tcf. Intriguingly, under conditions where cell-cell adhesion is disrupted, we have shown that Pez also translocates into the nucleus (Wadham *et al.*, 2000). It would be particularly important to determine whether Pez and β-catenin interact in the nucleus and if so, what the functional consequence of this interaction is.

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REFERENCES

Adam, L., Vadlamudi, R.K., McCrea, P., and Kumar, R. (2001). Tiam1 overexpression potentiates heregulin-induced lymphoid enhancer factor-1/beta-catenin nuclear signaling in breast cancer cells

by modulating the intercellular stability. J. Biol. Chem. *276*, 28443–28450.

Anastasiadis, P.Z., and Reynolds, A.B. (2000). The p120 catenin family: complex roles in adhesion, signaling and cancer. J. Cell Sci. *113* (Pt 8), 1319–1334.

Ayalon, O., and Geiger, B. (1997). Cyclic changes in the organization of cell adhesions and the associated cytoskeleton, induced by stimulation of tyrosine phosphorylation in bovine aortic endothelial cells [In Process Citation]. J. Cell Sci. *110*, 547–556.

Behrens, J., Vakaet, L., Friis, R., Winterhager, E., Van Roy, F., Mareel, M.M., and Birchmeier, W. (1993). Loss of epithelial differentiation and gain of invasiveness correlates with tyrosine phosphorylation of the E-cadherin/beta-catenin complex in cells transformed with a temperature-sensitive v-SRC gene. J. Cell Biol. *120*, 757–766.

Chishti, A.H. *et al.* (1998). The FERM domain: a unique module involved in the linkage of cytoplasmic proteins to the membrane. Trends Biochem. Sci. *23*, 281–282.

Cowin, P., and Burke, B. (1996). Cytoskeleton-membrane interactions. Curr. Opin. Cell Biol. *8*, 56–65.

Flint, A.J., Tiganis, T., Barford, D., and Tonks, N.K. (1997). Development of "substrate-trapping" mutants to identify physiological substrates of protein tyrosine phosphatases. Proc. Natl. Acad. Sci. USA *94*, 1680–1685.

Fujita, Y., Krause, G., Scheffner, M., Zechner, D., Leddy, H.E., Behrens, J., Sommer, T., and Birchmeier, W. (2002). Hakai, a c-Cbl-like protein, ubiquitinates and induces endocytosis of the E-cadherin complex. Nat. Cell Biol. *4*, 222–231.

Gumbiner, B.M. (1995). Signal transduction of beta-catenin. Curr. Opin. Cell Biol. *7*, 634–640.

Hollande, F., Choquet, A., Blanc, E.M., Lee, D.J., Bali, J.P., and Baldwin, G.S. (2001). Involvement of phosphatidylinositol 3-kinase and mitogen-activated protein kinases in glycine-extended gastrin-induced dissociation and migration of gastric epithelial cells. J. Biol. Chem. *276*, 40402–40410.

Kim, K., and Lee, K.Y. (2001). Tyrosine phosphorylation translocates beta-catenin from cell→cell interface to the cytoplasm, but does not significantly enhance the LEF-1-dependent transactivating function. Cell Biol. Int. *25*, 421–427.

Liu, D., el Hariry, I., Karayiannakis, A.J., Wilding, J., Chinery, R., Kmiot, W., McCrea, P.D., Gullick, W.J., and Pignatelli, M. (1997). Phosphorylation of beta-catenin and epidermal growth factor receptor by intestinal trefoil factor. Lab. Invest. *77*, 557–563.

Monga, S.P., Mars, W.M., Pediaditakis, P., Bell, A., Mule, K., Bowen, W.C., Wang, X., Zarnegar, R., and Michalopoulos, G.K. (2002). Hepatocyte growth factor induces Wnt-independent nuclear translocation of beta-catenin after Met-beta-catenin dissociation in hepatocytes. Cancer Res. *62*, 2064–2071.

Muller, T., Choidas, A., Reichmann, E., and Ullrich, A. (1999). Phosphorylation and free pool of beta-catenin are regulated by tyrosine kinases and tyrosine phosphatases during epithelial cell migration. J. Biol. Chem. *274*, 10173–10183.

Ozawa, M., and Kemler, R. (1998). Altered cell adhesion activity by pervanadate due to the dissociation of alpha-catenin from the E-cadherin. catenin complex. J. Biol. Chem. *273*, 6166–6170.

Roura, S., Miravet, S., Piedra, J., Garcia, d. H., Dunach, M. (1999). Regulation of E-cadherin/Catenin association by tyrosine phosphorylation. J. Biol. Chem. *274*, 36734–36740.

Shibamoto, S., Hayakawa, M., Takeuchi, K., Hori, T., Oku, N., Miyazawa, K., Kitamura, N., Takeichi, M., and Ito, F. (1994). Tyrosine phosphorylation of beta-catenin and plakoglobin enhanced by hepatocyte growth factor and epidermal growth factor in human carcinoma cells. Cell Adhes. Commun. *1*, 295–305.

Smith, A.L., Mitchell, P.J., Shipley, J., Gusterson, B.A., Rogers, M.V., and Crompton, M.R. (1995). Pez: a novel human cDNA encoding protein tyrosine phosphatase- and ezrin-like domains. Biochem. Biophys. Res. Commun. *209*, 959–965.

Steinberg, M.S., and McNutt, P.M. (1999). Cadherins and their connections: adhesion junctions have broader functions. Curr. Opin. Cell Biol. *11*, 554–560.

Stevenson, B.R., and Keon, B.H. (1998). The tight junction: morphology to molecules. Annu. Rev. Cell Dev. Biol. *14*, 89–109.

Vleminckx, K., and Kemler, R. (1999). Cadherins and tissue formation: integrating adhesion and signaling. Bioessays *21*, 211–220.

Wadham, C., Gamble, J.R., Vadas, M.A., and Khew-Goodall, Y. (2000). Translocation of protein tyrosine phosphatase Pez/PTPD2/PTP36 to the nucleus is associated with induction of cell proliferation. J. Cell Sci. *113* (Pt 17), 3117–3123.

Wall, R.T., Harker, L.A., Quadracci, L.J., and Striker, G.E. (1978). Factors influencing endothelial cell proliferation in vitro. J. Cell Physiol. *96*, 203–213.

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Translocation of protein tyrosine phosphatase Pez/PTPD2/PTP36 to the nucleus is associated with induction of cell proliferation

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SUMMARY

Pez is a non-transmembrane tyrosine phosphatase with homology to the FERM (4.1, ezrin, radixin, moesin) family of proteins. The subcellular localisation of Pez in endothelial cells was found to be regulated by cell density and serum concentration. In confluent monolayers Pez was cytoplasmic, but in cells cultured at low density Pez was nuclear, suggesting that it is a nuclear protein in proliferating cells. This notion is supported by the loss of nuclear Pez when cells are serum-starved to induce quiescence, and the rapid return of Pez to the nucleus upon refeeding with serum to induce proliferation. Vascular endothelial cells normally exist as a quiescent confluent monolayer but become proliferative during angiogenesis or upon vascular injury. Using a 'wound' assay to mimic these events in vitro, Pez was found to be nuclear in the cells that had migrated and were proliferative at the 'wound' edge. TGFβ, which inhibits cell proliferation but not migration, inhibited the translocation of Pez to the nucleus in the cells at the 'wound' edge, further strengthening the argument that Pez plays a role in the nucleus during cell proliferation. Together, the data presented indicate that Pez is a nuclear tyrosine phosphatase that may play a role in cell proliferation.

Key words: Tyrosine phosphatase, Pez, Nuclear localisation, HUVEC, Proliferation, Quiescence

INTRODUCTION

Reversible tyrosine phosphorylation catalysed by the opposing actions of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs) is a major regulatory mechanism for a diverse number of cellular functions (for a review, see Neel and Tonks, 1997). Although there has been a surge in the numbers of PTPs identified in the last 5 years, relatively little is known about the specific functions and modes of regulation of the individual PTPs.

The PTP Pez (PTPD2/PTP36) is a member of the subfamily of PTPs with homology to the band 4.1 proteins, characterised by the presence of N-terminal FERM (4.1, ezrin, radixin, moesin) domains and C-terminal PTP catalytic domains. Other members of the FERM subfamily of PTPs include PTPD1, PTPH1 and PTPMeg1. Pez was first cloned in a screen for PTPs expressed in normal breast tissue (Smith et al., 1995). It is also expressed in varying amounts in other tissues including kidney, skeletal muscle, lung and placenta. In human umbilical vein endothelial cells (HUVEC), the Pez mRNA is one of the most highly expressed PTP mRNAs (C. Wadham and Y. Khew-Goodall, unpublished observation), suggesting it may be a critical PTP in regulating endothelial cell function.

The FERM superfamily of proteins include the ERM (ezrin, radixin and moesin) proteins and talin. A feature common to all these proteins is that they interact through their N termini with integral plasma membrane proteins and through their C termini with the cytoskeleton, thus forming a bridge between the plasma membrane and the cytoskeleton (Tsukita and Yonemura, 1997, 1999). It has been postulated that the family of FERM proteins, particularly the ERM proteins, play a role in organising the cytoskeleton, perhaps in response to external cues.

By analogy to the other members of the FERM family of proteins, it is reasonable to predict that the FERM subfamily of PTPs is similarly localised and perform similar functions. Some evidence exists to support this. Ogata et al. (1999a,b) recently demonstrated that ectopic overexpression of PTP36, the murine homologue of Pez, led to decreases in actin stress fibre and focal adhesions, resulting in loss of cell-matrix adhesion and cell proliferation. They showed that ectopically expressed PTP36 was associated with the plasma membrane and the cytoskeletal fraction, suggesting that it may play a role in the regulation of the cellular cytoskeleton. Studies on PTPH1 have also shown that the N-terminal portion can mediate associations of PTPH1 with plasma membrane structures (Zhang et al., 1999). In addition, the FERM domain of PTPH1 can inhibit the catalytic activity of PTPH1 in vitro (Zhang et al., 1995), suggesting that it has functions other than in tethering PTPH1 to the plasma membrane. Furthermore, PTPH1 has been shown to be complexed to the adaptor molecule 14-3-3 when it is phosphorylated (Zhang et al., 1997), and the major substrate of PTPH1 has been identified, using a substrate-trapping mutant of PTPH1, to be the valosin-

containing protein (VCP, also known as p97/CDC48) (Zhang et al., 1999), a protein with various potential functions including cell-cycle regulation (Madeo et al., 1998). The studies with PTPH1, a representative of the FERM subfamily of PTPs, demonstrate that members of this subfamily may have functions other than those related to cytoskeletal architecture.

In this report, we show that the subcellular localisation of Pez is regulated. Pez is cytoplasmic in confluent monolayers where the cells are quiescent, a state similar to the resting endothelium lining blood vessels. However, in actively proliferating cells growing at low cell density and in cells induced to proliferate from the resting state by serum addition or by the 'wounding' of a confluent monolayer, Pez is nuclear. Collectively, our data demonstrate that the presence of Pez in the nucleus correlates with cell proliferation and suggests that it has a nuclear role during cell proliferation. In blood vessels, the normally quiescent endothelium is induced to proliferate at sites of vascular injury and during angiogenesis. Therefore, Pez may play a role in regulating endothelial cell proliferation during vascular injury and angiogenesis.

MATERIALS AND METHODS

Antibodies

Anti-Flag epitope (M2) was obtained from AMRAD Biotech (Victoria, Australia) and anti-ERK1/ERK2 from Zymed Laboratories Inc. (San Francisco, CA, USA).

Generation of Pez-specific antibodies

Two peptides predicted to be immunogenic by the algorithm 'Antigenic' (GCG, University of Wisconsin, USA), located in the variable region of Pez, were synthesised. The peptides, PYTVPYGPQGVYSNKLVSPS corresponding to amino acids (aa) 495-510 ($Pez_{495-510}$) and SHEVPQLPQYHHKK corresponding to aa 683-696 ($Pez_{683-696}$), do not exhibit significant homology to other proteins in the protein databases, including other members of the FERM PTPs, suggesting that antibodies (Abs) raised against them would be specific to Pez. The peptides were conjugated to the carrier protein, ovalbumin (Sigma, St Louis, MO, USA) and used to immunise rabbits. Antisera obtained against each of the peptides were used in an ELISA to screen for binding to unconjugated peptide. Antisera generated to $Pez_{495-510}$ and $Pez_{683-696}$ were called PezR1 and PezR2, respectively. Whole antiserum to PezR1 and PezR2 were purified using peptide affinity columns generated by coupling the peptides $Pez_{495-510}$ and $Pez_{683-696}$ cross-linked to BSA to Affigel-15 (Bio-Rad Labs, Hercules, CA, USA) matrix, according to the manufacturer's instructions.

Western blotting, subcellular fractionation and immunoprecipitation

For western blotting, the lysates were electrophoresed on an 8% SDS-polyacrylamide gel (Laemmli, 1970), transferred onto Hybond-P PVDF membranes (Amersham, Buckinghamshire, England) and blocked with 5% milk, 0.1% Triton X-100 in phosphate-buffered saline (PBS). Crude antisera against Pez were used at 1:500 dilution, affinity-purified Ab were used at 1:50 or 1:100 dilution and the blots developed using an HRP-conjugated secondary Ab (Immunotech, Marseille, France) and enhanced chemiluminescence (Amersham). For quantitative western blotting analysis, the blots were developed using an alkaline phosphatase-conjugated secondary Ab, fluorescent Vistra ECF substrates (Amersham) and the resulting band intensities quantitated using a Fluorimager and the Imagequant software (Molecular Dynamics).

In studies where the Ab was preincubated with peptides prior to use in western blots, preincubation was carried out in 1 ml PBS containing 100 μg/ml peptide for 1 hour at room temperature or overnight at 4°C.

Total cell lysates were prepared by harvesting cells with trypsin, followed by washing in PBS prior to lysing in Laemmli sample buffer (Laemmli, 1970) containing 300 mM NaCl. Subcellular fractionation into nuclear and cytoplasmic fractions was carried out according to published protocols (Mui et al., 1995). Briefly, cells were harvested by trypsinisation, washed in PBS and resuspended in Buffer A (50 mM Hepes, pH 7.5, 100 mM NaF, 10 mM sodium pyrophosphate, 10 mM MgCl, 2 mM sodium orthovanadate, 2 mM sodium molybdate, 2 mM EDTA, 1× protease inhibitor cocktail (Sigma)) containing 0.2% Nonidet P-40 (Sigma) for 1 minute on ice. The nuclear pellet was collected by a brief centrifugation for 15 seconds and washed twice with Buffer A. The nuclear pellet was then either lysed in Laemmli sample buffer to obtain a total nuclear fraction or resuspended in Buffer B (50 mM Hepes, pH 7.5, 100 mM NaF, 300 mM NaCl, 10% glycerol, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 2 mM sodium molybdate, 2 mM EDTA, 1× protease inhibitor cocktail) and rocked vigorously to obtain nuclear extract (supernatant after centrifugation at 13000 rpm, 5 minutes at 4°C) or nuclear pellet fraction. Immunoprecipitations were carried out by lysing cells in RIPA buffer (50 mM Tris-HCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1× protease inhibitor cocktail) followed by preclearing with protein-A sepharose beads. Precleared lysate was incubated with antiserum for 2 hours at 4°C, then protein-A sepharose beads were added and incubated a further 1 hour at 4°C. The protein-A sepharose complex was pelleted by centrifugation, washed twice with RIPA buffer and boiled in Laemmli sample buffer.

Confocal microscopy

HUVEC were plated onto fibronectin (50 μg/ml) coated glass Lab-Tek chamber slides (Nalge Nunc International, Naperville, IL, USA) at the indicated densities. Prior to staining, cells were fixed in 4% paraformaldehyde/PBS for 10 minutes, quenched with 10 mg/ml sodium borohydride for 15 minutes and then permeabilised by treatment with 0.1% Triton X-100. Affinity-purified Ab to Pez was used at 1:10 dilution (whole sera was used at 1:250) and indirect immunofluorescence was detected by incubation with biotinylated anti-rabbit secondary Ab (Rockland, Gilbertsville, PA, USA) followed by FITC-conjugated streptavidin (Dako A/S, Denmark). Confocal microscopy was carried out using a BioRad MRC-600 confocal microscope. For comparison of different treatments, the laser power, confocal aperture and contrast settings were kept constant.

Culture of primary HUVEC

HUVEC were extracted by collagenase treatment according to a modified version of Wall et al. (1978). Cells were grown in 25 cm^2 gelatin-coated tissue culture flasks (Costar, Cambridge, MA, USA) in endotoxin-free M 199 medium (Cytosystems, Sydney, Australia) supplemented with 20% FCS (PA Biological, Sydney, Australia), 20 mM Hepes, sodium pyruvate and non-essential amino acids at 37°C in a 5% CO_2 atmosphere. Cells were replated 2-5 days after establishment of culture by harvesting with 0.05% trypsin-0.02% EDTA. Endothelial cell growth supplement (Multicel, Trace Biosystems, Australia) at 25 mg/ml and heparin were added to cells that were passaged twice or more. In general, cells were used at first or second passage. All reagents used in the growth and passaging of HUVEC were made up under endotoxin-free conditions and contained between 10-100 pg/ml endotoxin determined by the *Limulus* amoebocyte assay.

Plasmids and transfection

Full-length Pez cDNA was tagged at the N terminus with the Flag-epitope and cloned into the eukaryotic expression vector pcDNA3

(Invitrogen, Groningen, The Netherlands). The Flag-tagged mutant, Δ4.1, was generated by deletion of amino acids 2-336, which essentially removes the entire FERM domain. HUVEC and 293 cells were transfected using Lipofectamine 2000 (Life Technologies Inc., Grand Island, NY). Cells were harvested or analysed 48 hours after transfection.

RESULTS

Endogenous Pez expression detected by Pez-specific antisera

Two specific antisera generated to two separate regions of Pez were used to determine the subcellular localisation of endogenous Pez in primary HUVEC. Each of the antisera detected the peptide that was used for immunisation but not the reciprocal peptide, indicating that they are each specific for the peptide used for immunisation (data not shown). The single open reading frame in the Pez cDNA sequence (Smith et al., 1995) predicted a protein of 1187 amino acids or approximately 130 kDa. By western blotting analysis of HUVEC lysate, each Ab (named PezR1 and PezR2) detected two major bands, but only one major band of approx. 130 kDa is common between the Abs and this was not detected by the preimmune sera (Fig. 1A,C). A Flag epitope-tagged Pez ectopically expressed in 293 cells comigrated with the 130 kDa band from HUVEC lysate and was recognised by PezR1, in both western blotting and immunoprecipitation (Fig. 1B). This suggests that the 130 kDa band from HUVEC detected by both Abs is most likely endogenous Pez. Further evidence that the 130 kDa band is indeed Pez is demonstrated in Fig. 1C, where preincubating the Abs with the peptides used for immunisation resulted in removal of the 130 kDa band but not the nonspecific bands. Preincubation with nonspecific peptides had no effect. Together these data suggest that we have generated two Pez-specific Abs that recognise endogenous Pez.

Both the antisera were affinity-purified using the appropriate peptide affinity columns. The resulting affinity-purified PezR1Ab recognised only the 130 kDa Pez band (Fig. 2A) and affinity-purified PezR2 detected predominantly the 130 kDa Pez band as well as a minor non-specific low molecular mass band (Fig. 2B). Both the affinity-purified Abs were subsequently used for detection of Pez by indirect immunofluorescence.

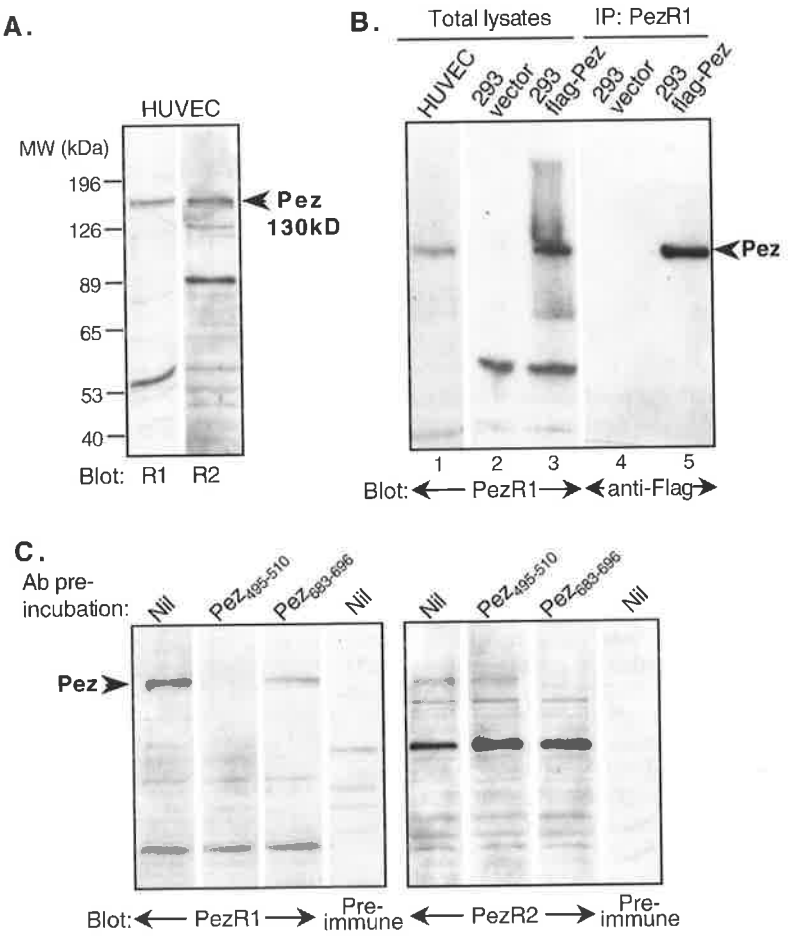

Fig. 1. Characterisation of anti-Pez antisera. (A) Western blot of HUVEC lysate with PezR1 and PezR2 antisera. The migration of prestained markers (MW) is shown. Each Ab recognises two major bands, of which only the 130 kDa Pez band is common. (B) Lanes 1-3: western blot of lysates from HUVEC or 293 cells transfected with empty vector or flag-tagged Pez using the PezR1 antiserum showing ectopically expressed Pez comigrating with endogenous Pez from HUVEC. Lanes 4 and 5: immunoprecipitation using the PezR1 antiserum was carried out using lysates of 293 cells transfected either with empty vector or flag-tagged Pez followed by western blotting with the anti-flag epitope Ab. (C) Western blot of HUVEC lysate with PezR1 (left) or PezR2 (right) antisera that was either untreated (Nil), preincubated with the specific immunising peptide ($Pez_{495-510}$ for PezR1, $Pez_{683-696}$ for PezR2) or the non-specific peptide ($Pez_{683-696}$ for PezR1, $Pez_{495-510}$ for PezR2), showing that the immunising peptide specifically blocks the Pez band. The right lane in each panel was blotted with the appropriate preimmune serum.

Endogenous Pez is nuclear or cytoplasmic depending on cell density

The subcellular localisation of Pez in primary HUVEC was examined by indirect immunofluorescence and confocal laser microscopy using both the affinity-purified PezR1 and PezR2 Abs. In confluent monolayers, as predicted by the presence of the FERM domain in the N terminus of Pez, both Abs stained predominantly the cytoplasmic compartment (Fig. 2A,B, confluent) and in some cells appeared to be concentrated at the plasma membrane at intercellular junctions (data not shown). When cells were plated sparsely, however, predominantly nuclear staining was observed using both Abs (Fig. 2A,B, sparse). An identical pattern of localisation was observed using the PezR1 whole serum whereas preimmune sera or sera preincubated with the corresponding immunising peptides showed either no staining or greatly reduced staining, respectively (data not shown). Because both affinity-purified Abs (one of which, PezR1, recognised only Pez by western blotting) showed an identical pattern of density-dependent staining, we concluded that Pez localises to the nucleus in sparsely plated cells but is cytoplasmic in confluent monolayers.

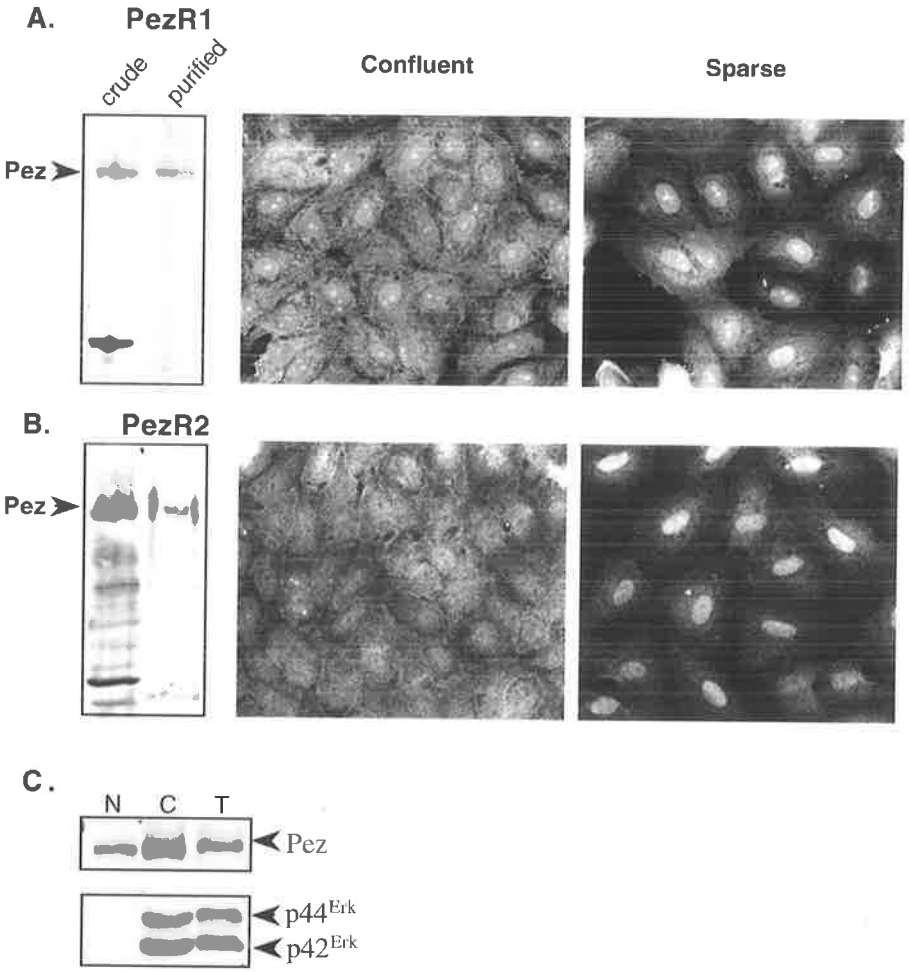

Fig. 2. Affinity purification of PezR1 and PezR2 Abs and their use in the subcellular localisation of endogenous Pez. (Left) Western blots of total HUVEC lysate to compare crude serum and affinity-purified PezR1 and PezR2 Abs. (Middle and right) Confocal micrographs showing indirect immunofluorescence of HUVEC plated as confluent (9×10^4 cells/well, middle) and sparse (2.5×10^4 cells/well, right) monolayers stained with either the affinity-purified PezR1 (A) or PezR2 (B) Abs. The staining with both Abs is predominantly diffuse cytoplasmic in the confluent monolayers and nuclear in sparse isolated cells. (C) Western blot of total HUVEC lysate (T) and after subcellular fractionation into crude nuclear (N) and cytoplasmic (C) extracts, using the PezR1 antiserum (top) and the anti-MAPK Ab (bottom). Pez was present in both nuclear and cytoplasmic fractions whereas the MAPK was detectable only in the cytoplasmic fraction.

To further verify that the nuclear staining observed by in situ immunofluorescence microscopy was due to the presence of Pez in the nuclear compartment, HUVEC were fractionated into nuclear and cytoplasmic extracts and western blotted with PezR1 Ab. The same 130 kDa band was detected in both the nuclear and cytoplasmic fractions (Fig. 2C). The nuclear fraction was shown to be free of cytoplasmic contamination by the complete absence of the cytoplasmic protein MAPK ($p44^{Erk}$ and $p42^{Erk}$) from the nuclear fraction. Thus Pez is indeed present in the nucleus.

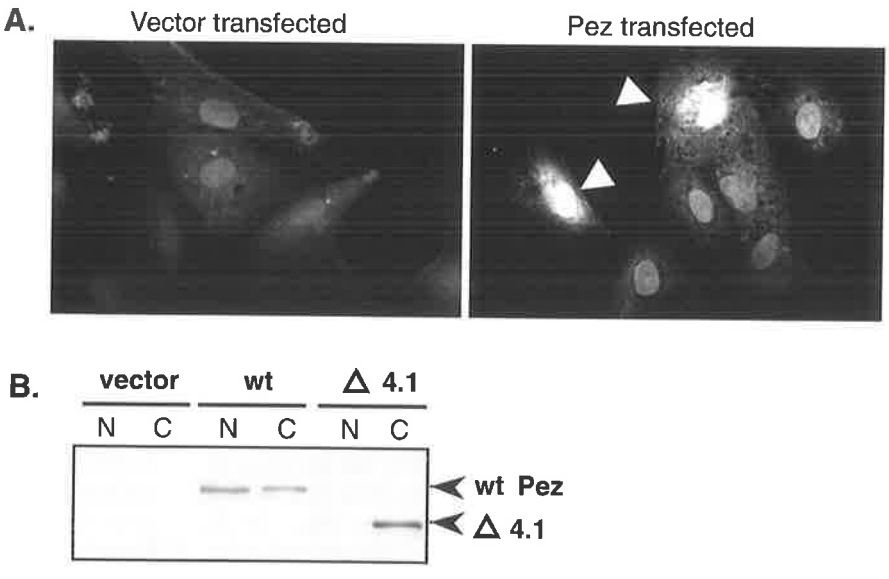

Fig. 3. Ectopically expressed Pez also localises to both the nucleus and cytoplasm. (A) Confocal microscopy of HUVEC transfected either with empty vector (left) or Flag-tagged Pez (right) and detected using the PezR1 antiserum. Pez-overexpressing cells (amongst a background of nontransfected cells showing endogenous Pez staining) are denoted by arrowheads. The figures shown are representative of at least three separate experiments, each using a different line of HUVEC. (B) Western blot of HEK293 nuclear (N) and cytoplasmic (C) lysates obtained after subcellular fractionation from cells transfected with either empty vector, wild-type (wt) Flag-tagged Pez or an N-terminal deletion mutant (Δ4.1). The ectopically expressed proteins were detected with an anti-Flag epitope Ab. Nothing was detected in the empty vector transfectants, wt Pez was present in both the nuclear and cytoplasmic fractions but the Δ4.1 mutant was only present in the cytoplasmic fraction.

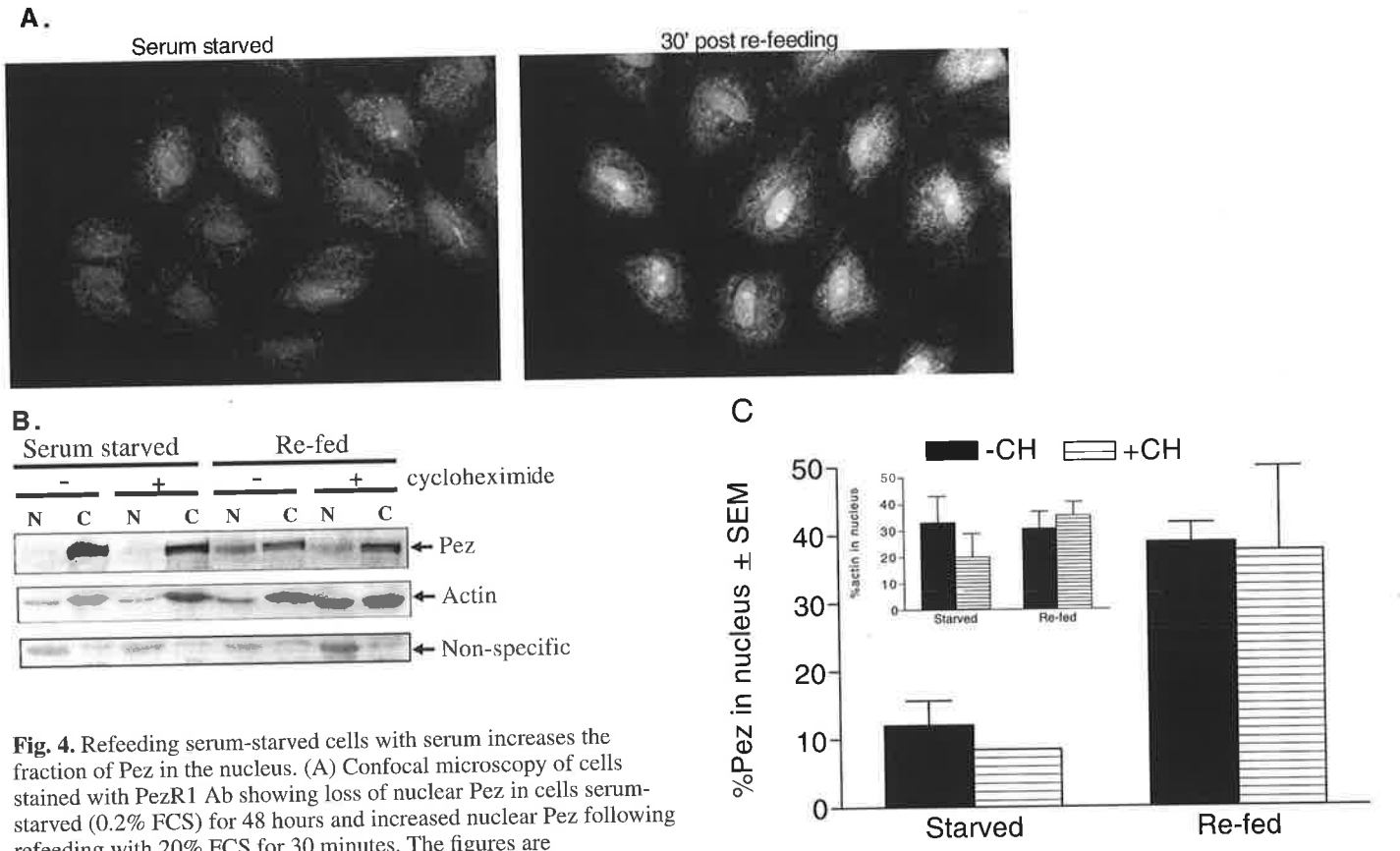

Fig. 4. Refeeding serum-starved cells with serum increases the fraction of Pez in the nucleus. (A) Confocal microscopy of cells stained with PezR1 Ab showing loss of nuclear Pez in cells serum-starved (0.2% FCS) for 48 hours and increased nuclear Pez following refeeding with 20% FCS for 30 minutes. The figures are representative of three separate experiments, each done in duplicate. (B) A representative western blot detected with the PezR1 Ab (top and bottom panels) used for quantitating the proportion of Pez in the nucleus. Serum-starved or refed cells (as in A) were fractionated into nuclear (N) and cytoplasmic (C) fractions. Lysates normalised for equal cell numbers were loaded onto each lane. Blots were also reblotted with an anti-actin Ab (middle panel) to further control for loading. The nonspecific band (bottom panel) is a low molecular mass nuclear protein also recognised by the PezR1 crude antiserum; it does not translocate into the cytoplasm when cells are serum-starved. In cells treated with cycloheximide, 10 μg/ml cycloheximide was added 15 minutes prior to refeeding with serum and left in for the entire duration of refeeding (30 minutes). (C) Quantitation of western blots carried out as described in B using a fluorimager and the Imagequant software. Following background subtraction, the percentage of Pez in the nucleus relative to total cellular Pez was plotted. No significant difference was observed in the percentage of nuclear Pez between refeeding in the presence or absence of cycloheximide (CH) (P=0.44, Student's t-test, n=2) but the percentage of nuclear Pez in refed cells compared to serum-starved cells was significantly different (P<0.01, Student's t-test, n=2). Inset shows quantitation of actin in the nuclear fraction relative to total cellular actin after the blots were reprobed with an anti-actin Ab, showing that there is no significant change in the compartmentalisation of actin upon refeeding with serum or treatment with cycloheximide.

To determine the subcellular localisation of ectopically expressed Pez, a Flag epitope-tagged Pez construct was transfected into HUVEC and the cells stained with the PezR1 Ab. By indirect immunofluorescence, cells transfected with Pez had greatly increased staining in both the nucleus and cytoplasm compared to endogenous Pez staining in empty vector transfected or untransfected cells (Fig. 3A). The presence of ectopic Pez in both compartments can also be demonstrated by western blotting following transfection into HEK293 cells using an Ab against the Flag epitope (Fig. 3B, wt). The presence of ectopic Pez in the nucleus cannot be attributed solely to overexpression because a truncated form with the N-terminal FERM domain deleted was not detected in the nucleus (Fig. 3B, Δ4.1). This further confirms that both endogenous and ectopically expressed Pez can localise to the nucleus and that nuclear localisation of Pez is likely to be dependent on sequences in the FERM domain.

Similar patterns of localisation were also observed using the PezR1 Ab in HeLa cells (data not shown), suggesting that this pattern of compartmentalisation was not confined to HUVEC.

Nuclear localisation of Pez is serum dependent

The above observation raised the possibility that Pez may be nuclear in proliferating cells but cytoplasmic in cells that are quiescent. HUVEC plated at low density were starved of serum (0.2% FCS, 48 hours) to induce quiescence and the localisation of Pez examined thereafter. By indirect immunofluorescence, nuclear Pez was significantly reduced in cells made quiescent by serum starvation but was increased following refeeding with 20% FCS for 30 minutes (Fig. 4A). To quantitate the amount of Pez in the nucleus following serum starvation and refeeding, nuclear and cytoplasmic fractions of cell lysates were examined for Pez content by western blotting and fluorimaging. From this analysis, only about 10% of total Pez

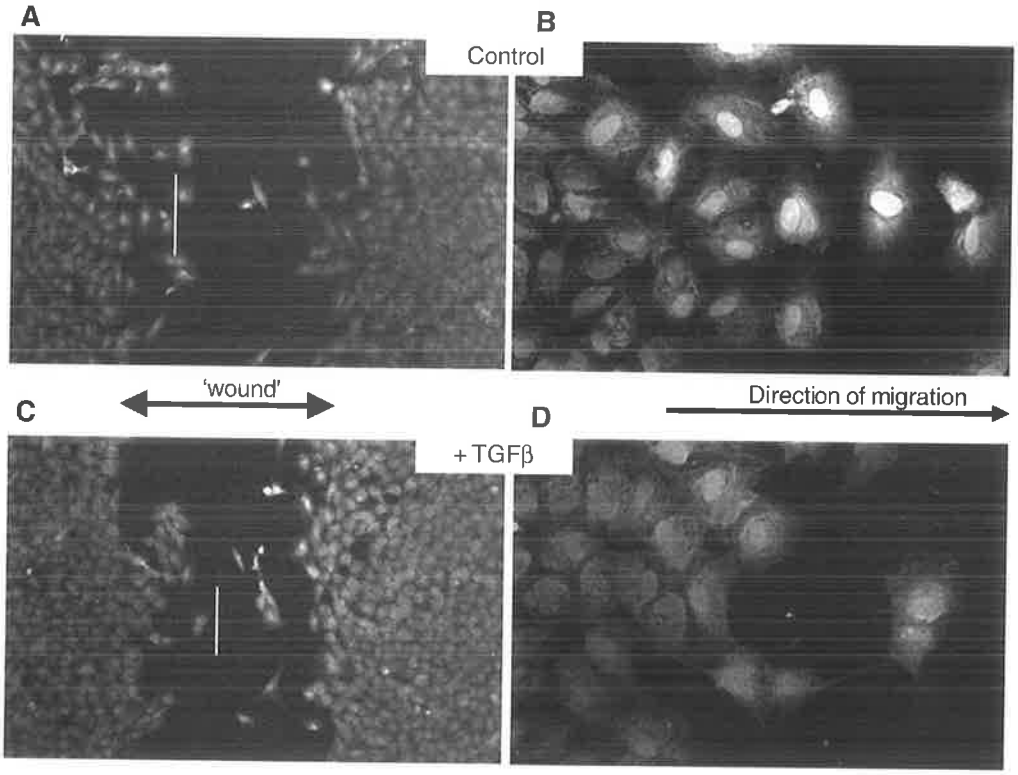

Fig. 5. TGFβ inhibits translocation of Pez to the nucleus in a 'wound' assay. Confocal microscopy of a confluent monolayer of HUVEC through which a 'wound' was made by denuding a linear region on the monolayer followed by staining with PezR1 antiserum 17-24 hours after 'wounding'. (A,B) Controls, no addition. (C,D) TGFβ (0.4 ng/ml) was added at the time the wound was made. (A and C) show the 'wound' into which a similar degree of cellular migration has occurred in both control and TGFβ cells. (B and D) are higher magnifications of the regions marked by a vertical line in A and C, respectively, showing translocation of Pez into the nucleus in the control but not TGFβ treated cells that have migrated into the 'wound'. The figures are representative of three separate experiments each done in duplicate.

remained in the nucleus after serum starvation but about 40-50% of total Pez was back in the nucleus as rapidly as 30 minutes after refeeding with serum (Fig. 4B,C), indicating that Pez is translocated back into the nucleus when quiscent cells (at G_0) are stimulated to proliferate upon the addition of serum. The increase in nuclear Pez in this short period of refeeding was unaffected by the presence of the protein synthesis inhibitor, cycloheximide, during refeeding, suggesting that the increase in nuclear Pez can occur in the absence of de novo protein synthesis. In contrast to Pez, there was no significant alteration in the proportion of actin in the nucleus when serum was added back to the cells and this was similarly unaffected by cycloheximide treatment (Fig. 4B,C, inset). Interestingly, the nuclear localisation of the non-specific band also recognised by the crude PezR1 antisera does not alter with serum starvation (Fig. 4B, non-specific) indicating that not all proteins translocate from the nucleus to the cytoplasm when cells become quiescent. The presence of this nonspecific band in the nucleus even after serum-starvation could account for some of the residual nuclear staining detected by immunofluorescence in Fig. 1A. We have also found that there is no significant alteration in the amount of total Pez in the cells after 30 minutes refeeding with serum (data not shown).

Pez translocates into the nucleus in cells at a 'wound' edge

At sites of vascular injury and during angiogenesis, endothelial cells migrate out of the intact confluent endothelium and proliferate. To mimic these events in vitro, a confluent monolayer of HUVEC was 'wounded' to generate a linear region denuded of cells (Fig. 5A,B). The cells from the confluent area subsequently migrate into the denuded area and proliferate. In cells at the 'wound' edge that have recently migrated into the 'wound', Pez is localised almost exclusively to the nucleus whereas cells in the confluent monolayer distal from the edge that have not migrated clearly showed Pez remaining in the cytoplasm (similar to Fig. 2A).

Translocation of Pez to the nucleus is inhibited by TGFβ

Because the cells at the 'wound' edge are both migratory and proliferative, the role of Pez in the nucleus of these cells could be related to either migration, proliferation or both. To differentiate between these possibilities, cells were treated at the time of generating the 'wound' with TGFβ, which inhibits proliferation by arresting cells at G_1 phase (Iavarone and Massague, 1997) but has no effect on cell migration. Following TGFβ treatment, cells at the 'wound'edge showed little or no increase in nuclear Pez compared to the cells in the confluent region distal from the 'wound', suggesting that TGFβ inhibited the translocation of Pez into the nucleus (Fig. 5C,D). There was no difference in the distance migrated or the number of cells migrating into the 'wound' between untreated and TGFβ-treated cells, confirming that TGFβ does not inhibit cell migration (Fig. 5A,C). These data further support our earlier observations that the translocation of Pez into the nucleus correlates with the transition from quiescence to proliferation.

DISCUSSION

Intracellular compartmentalisation is a powerful means of ensuring specificity of signal transduction pathways by limiting the access of activated, promiscuous enzymes to other downstream partners, while increasing the efficiency of reactions by grouping proteins involved in a particular cascade

together. Hence, the subcellular localisation of a PTP can be an indicator of the physiological role of the PTP.

Because a number of members of the FERM family of proteins are localised through their FERM domain to the plasma membrane-actin cytoskeleton interface (Tsukita and Yonemura, 1997, 1999), it was assumed that Pez would also be similarly localised. However, this had not been demonstrated for the endogenous protein. Using specific Abs to Pez, we demonstrated that the nuclear or cytoplasmic localisation of Pez was regulated by a number of factors including cell density and the presence or absence of serum. The presence of Pez in the nucleus of cells at low but not high density, its absence from the nucleus of serum-starved cells and its return to the nucleus when the cells are replenished with serum, all suggest that Pez is a nuclear protein during cell proliferation.

The resting endothelium that lines blood vessels is normally nonproliferative. At sites of vascular injury and during angiogenesis, however, endothelial cells can become migratory and proliferative. To address whether there is an alteration in the subcellular localisation of Pez when endothelial cells are induced from a quiescent state to migrate and proliferate, we used an in vitro 'wounding' assay to induce a small population of endothelial cells at the 'wound' edge to migrate and proliferate. Pez was indeed nuclear in the cells at the 'wound' edge that had migrated into the 'wound' but remained cytoplasmic in the confluent cells distal from the edge. TGFβ, which inhibits cell proliferation but not migration, inhibited the translocation of Pez to the nucleus of cells at the 'wound' edge, further strengthening the argument that Pez may play a nuclear role during cell proliferation. Although Pez is expressed in many different cell types to varying levels, its abundance in HUVEC suggests that it may be an important regulator of nuclear events in endothelial cells under situations where endothelial cell proliferation occurs.

Studies on the murine homologue of Pez, PTP36, showed that the epitope-tagged, ectopically expressed protein used in the studies was cytoplasmic in both HeLa cells and fibroblasts, with no report of its presence in the nucleus (Ogata et al., 1999a,b). We have observed, however, that Flag-epitope tagged Pez ectopically expressed in both HUVEC (Fig. 3A) and 293 cells (Fig. 3B) was predominantly present in the nucleus in cells at low density. The reason for the difference in subcellular localisation between Pez and PTP36 is not immediately obvious and requires further investigation. One reason may be that Pez and PTP36 are isoforms rather than orthologues. A precedence for differential localisation of isoforms of a member of the FERM family of proteins exists: Band 4.1 protein, commonly known for its role in cross-linking the plasma membrane of red blood cells to the spectrin cytoskeleton, also has a nuclear isoform (Krauss et al., 1997).

The presence of Pez in the nucleus of almost all of the cells in a low density asynchronous culture suggests that Pez is nuclear in proliferating cells through all the stages of the cell cycle. This remains to be confirmed by following the subcellular localisation of Pez through the stages of the cell cycle in synchronised cells. The data presented, taken together, suggest that Pez translocates into the nucleus at the G_0/G_1 transition and moves out of the nucleus when cells enter G_0. Cell proliferation is a complex, highly coordinated process that requires regulation of both the structural and biochemical changes that occur. Since changes occur in the activity of many nuclear proteins, understanding the function of Pez in the nucleus during cell proliferation will require identification of its nuclear substrates.

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REFERENCES

Iavarone, A. and Massague, J. (1997). Repression of the CDK activator Cdc25A and cell-cycle arrest by cytokine TGFβ in cells lacking the CDK inhibitor p15. *Nature* **387**, 417-422.

Krauss, S. W., Larabell, C. A., Lockett, S., Gascard, P., Penman, S., Mohandas, N. and Chasis, J. A. (1997). Structural protein 4.1 in the nucleus of human cells: dynamic rearrangements during cell division. *J. Cell Biol.* **137**, 275-289.

Laemmli,U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Madeo, F., Schlauer, J., Zischka, H., Mecke, D. and Frohlich, K. U. (1998). Tyrosine phosphorylation regulates cell cycle-dependent nuclear localization of Cdc48p. *Mol. Biol. Cell* **9**, 131-141.

Mui, A. L., Wakao, H., O'Farrell, A. M., Harada, N. and Miyajima, A. (1995). Interleukin-3, granulocyte-macrophage colony stimulating factor and interleukin-5 transduce signals through two STAT5 homologs. *EMBO J.* **14**, 1166-1175.

Neel, B. G. and Tonks, N. K. (1997). Protein tyrosine phosphatases in signal transduction. *Curr. Opin. Cell Biol.* **9**, 193-204.

Ogata, M., Takada, T., Mori, Y., Oh-hora, M., Uchida, Y., Kosugi, A., Miyake, K. and Hamaoka, T. (1999a). Effects of overexpression of PTP36, a putative protein tyrosine phosphatase, on cell adhesion, cell growth, and cytoskeletons in HeLa cells. *J. Biol. Chem.* **274**, 12905-12909.

Ogata, M., Takada, T., Mori, Y., Uchida, Y., Miki, T., Okuyama, A., Kosugi, A., Sawada, M., Oh-hora, M. and Hamaoka, T. (1999b). Regulation of phosphorylation level and distribution of PTP36, a putative protein tyrosine phosphatase, by cell-substrate adhesion. *J. Biol. Chem.* **274**, 20717-20724.

Smith, A. L., Mitchell, P. J., Shipley, J., Gusterson, B. A., Rogers, M. V. and Crompton, M. R. (1995). Pez: a novel human cDNA encoding protein tyrosine phosphatase- and ezrin-like domains. *Biochem. Biophys. Res. Commun.* **209**, 959-965.

Tsukita, S. and Yonemura, S. (1997). ERM proteins: head-to-tail regulation of actin-plasma membrane interaction. *Trends. Biochem. Sci.* **22**, 53-58.

Tsukita, S. and Yonemura, S. (1999). Cortical actin organization: lessons from ERM (Ezrin/Radixin/Moesin) proteins [In Process Citation]. *J. Biol. Chem.* **274**, 34507-34510.

Wall, R. T., Harker, L. A., Quadracci, L. J. and Striker, G. E. (1978). Factors influencing endothelial cell proliferation in vitro. *J. Cell Physiol.* **96**, 203-213.

Zhang, S. H., Eckberg, W. R., Yang, Q., Samatar, A. A. and Tonks, N. K. (1995). Biochemical characterization of a human band 4.1-related protein-tyrosine phosphatase, PTPH1. *J. Biol. Chem.* **270**, 20067-20072.

Zhang, S. H., Kobayashi, R., Graves, P. R., Piwnica-Worms, H. and Tonks, N. K. (1997). Serine phosphorylation-dependent association of the band 4.1-related protein-tyrosine phosphatase PTPH1 with 14-3-3beta protein. *J. Biol. Chem.* **272**, 27281-27287.

Zhang, S. H., Liu, J., Kobayashi, R. and Tonks, N. K. (1999). Identification of the cell cycle regulator VCP (p97/CDC48) as a substrate of the band 4.1-related protein-tyrosine phosphatase PTPH1. *J. Biol. Chem.* **274**, 17806-17812.