



# **The Cloning and Functional Characterisation of Murine**

## **Phosphatidylinositol 3-kinase gamma**

By

Sumone Chakravarti B.Sc (Hons)

Department of Molecular Biosciences

Discipline of Microbiology & Immunology

Adelaide University, Australia

A thesis submitted to Adelaide University in fulfilment of the

requirements for the degree of Doctor of Philosophy

August 2001

## ABSTRACT

The family of PI3-kinases are an important group of lipid kinases involved in cellular signalling. All members of the family are responsible for phosphorylating the 3-hydroxyl groups of inositol phospholipids. Their structural conformation and substrate specificity separates members into three distinct classes, these being the Class I, II and III PI3-kinases. The Class I family is the best-characterised of all the members and can be further subdivided into 2 groups, the Class IA and Class IB PI3-kinases. The Class IA PI3-kinases are activated through the downstream recruitment of protein tyrosine kinases. In contrast, the Class IB PI3-kinases are dependent on heterotrimeric G proteins for their activation. This study focuses on the only known member of the Class IB PI3-kinases, PI3 $\gamma$ .

The chemokine gene superfamily is a group of chemotactic cytokines originally identified on their ability to directly recruit distinct and overlapping subsets of leukocytes, and therefore has a critical role in the maintenance, activation and regulation of the immune system. To date, all chemokine receptors are G protein-coupled receptors. Activation of chemotactic receptors results in a transient increase in the level of 3-phosphorylated phospholipids, products of the PI3kinase family. More recently, this increase has been attributed to PI3 $\gamma$ .

The present study addresses the role of PI3 $\gamma$  in lymphocyte migration *in vitro* and *in vivo*, in response to chemotactic factors. A novel isoform of PI3 $\gamma$ , MmPI3 $\gamma$ 1111, was isolated from a murine macrophage cDNA library. Comparison of the sequence of MmPI3 $\gamma$ 1111 with the previously cloned human and porcine

orthologues demonstrated above 90% identity. Consistent with the human and porcine PI3kys, MmPI3ky1111 possesses all four conserved domains common to the Class IB subfamily. Uniquely, MmPI3ky1111 contained an additional 11 amino acids in the catalytic domain. During the latter stages of this study a murine orthologue of PI3ky, MmPI3ky1100, was independently cloned. MmPI3ky1100 did not contain the additional 11 amino acids found in MmPI3ky1111.

MmPI3ky1111 was over-expressed in HEK 293 cells and examined for lipid and protein kinase activity. Consistent with MmPI3ky1100, MmPI3ky1111 produced PI 3 P *in vitro*, hence lipid kinase activity. MmPI3ky1111 also demonstrated autophosphorylation, a characteristic common to all known Class IB PI3-kinases. Furthermore, phosphorylation of MmPI3ky1111 downregulated the ability of MmPI3ky1111 to act as a lipid kinase.

Mutation of the lysine residue (position 833) critical for the activation of all Class I PI3-kinases resulted in a loss of both the lipid and protein kinase activities of MmPI3ky1111. The catalytically-inactive form of MmPI3ky1111 was stably transfected into a B lymphocyte cell line (B300.19) expressing the CCR6 chemokine receptor. B300.19-huCCR6 cells expressing the catalytically-inactive form of MmPI3ky1111, MmPI3kyKR, demonstrated a marked decrease in the ability to migrate towards huMIP-3 $\alpha$ , the ligand for huCCR6, in an *in vitro* chemotaxis assay. Subcutaneous air pouches were used to examine the role of MmPI3ky1111 in *in vivo* B lymphocyte migration. However, in contrast to the *in vitro* data obtained, the *in vivo* model did not provide clear support for a role for MmPI3ky1111 in lymphocyte

migration. The biological significance of these data, and future research directions are addressed in this thesis.

## **ABSTRACT**

## **DECLARATION OF ORIGINALITY**

## **ACKNOWLEDGMENTS**

## **LIST OF ABBREVIATIONS**

## **CHAPTER 1: INTRODUCTION**

1.1 History of the Phosphatidylinositol 3-kinase family	1
1.1.2 The Phosphatidylinositol 3-kinase Family	1
1.1.3 Class I Phosphatidylinositol 3-kinases	3
1.1.4 Class IA Phosphatidylinositol 3-kinases	3
1.1.4.1 Structure of the Class IA Regulatory Subunits	3
1.1.4.2 Structure of the Class IA Catalytic Subunits	8
1.1.4.3 Activity and Regulation of the Class IA Phosphatidylinositol 3-kinases	9
1.1.4.3.1 Activation by Translocation	10
1.1.4.3.2 Interactions with Protein Tyrosine Kinases	10
1.1.4.3.3 Protein Phosphorylation	12
1.1.5 History of the Class IB Phosphatidylinositol 3-kinases	13
1.1.5.1 The Class IB Catalytic subunit- PI3K $\gamma$	15
1.1.5.2 Activity and Regulation of Class IB Phosphatidylinositol 3-kinases	16
1.1.6 Cellular Role of PI3-kinases and their Lipid Products	20
1.1.6.1 Chemical Inhibitors	20
1.1.6.2 Dominant-negative Over-Expression	21
1.1.6.3 Gene Knockout Animal Models	22
1.1.6.4 Cellular Role of Phosphatidylinositol 3-kinase Lipid Products	24
1.1.6.5 Phosphatidylinositol 3 Phosphate	24

1.1.6.6 Phosphatidylinositol 3,4 bisphosphate	25
1.1.6.7 Phosphatidylinositol 3,4,5 Trisphosphate	26
1.1.7 Summary of the phosphatidylinositol 3-kinases	26
1.2 The Chemokine Gene Superfamily	29
1.2.1 Structure and Function of members of the Chemokine Superfamily	29
1.2.1.1 Chemokines involved in myeloid cell trafficking and function.	30
1.2.1.2 Chemokines involved in lymphoid cell trafficking and function.	31
1.2.2 Chemotactic Factor Receptors	37
1.2.3 The Chemokine Receptors	39
1.2.4 Chemotactic Factor Receptor Signalling	39
1.2.5 Chemotactic factor signalling and PI3ky activation	41
1.3 Rationale, Hypothesis and Aims	44

## **CHAPTER 2: REAGENTS AND TECHNIQUES**

<b>2.1 Molecular techniques</b>	<b>46</b>
2.1.1 Bacterial strains, growth media and buffers	46
2.1.2 Preparation of Competent Cells	47
2.1.3 Preparation of Phenol	47
2.1.4 Phenol/Chloroform extraction	48
2.1.5 Agarose Gel Electrophoresis	48
2.1.6 Bacterial Transformation	49
2.1.7 Mini-Preparation (small scale) of Plasmid DNA	49
2.1.8 Medium - Scale Preparation of Plasmid DNA	50
2.1.9 Restriction Endonuclease Digestion	51

2.1.10 Dephosphorylation of vector DNA	50
2.1.11 DNA Ligations	52
2.1.12 BRESA-Clean™	52
2.1.13 Mutagenesis	53
2.1.13.1 Primer design	53
2.1.13.2 Mutagenesis Reaction	54
2.1.14 cDNA Library Screening	54
2.1.14.1 Preparation of Host cells	54
2.1.14.2 Library Plating	55
2.1.14.3 Plaque Lifts	55
2.1.14.4 DNA Denaturation and Fixing	56
2.1.14.5 Radiolabelling DNA probes	56
2.1.14.6 Hybridisation	57
2.1.15 RNA Isolation	57
2.1.16 Generation of First Strand cDNA - Reverse Transcription	58
2.1.17 PCR - Taq Polymerase Based Amplification	59
2.1.18 PCR - Amplification using PFU polymerase	59
2.1.19 DNA Sequencing - Dye Terminator Sequencing	60
2.1.20 Primer design	60
2.1.21 Primers	61
2.1.22 DNA Constructs	62
2.1.23 Molecular weight markers	63
2.1.24 Molecular Analysis Programs	64

<b>2.2 <i>In vitro</i>/cellular techniques</b>	<b>64</b>
2.2.1 Agonists	64
2.2.2 Maintenance of cell lines	64
2.2.3 Cryopreservation of cell lines	65
2.2.4 Thawing of cryopreserved cells	66
2.2.5 Transient Transfection of Adherent Cell Lines - FuGENE 6	66
2.2.6 Electroporation of B300.19 B Lymphocytes	66
2.2.7 One Colour Labelling for Flow Cytometry	67
2.2.8 One Colour Flow Cytometry	68
2.2.9 Protein Precipitation	68
2.2.10 SDS-PAGE and Protein Transfer	69
2.2.11 Western Analysis	70
2.2.12 Lipid Kinase Assay	70
2.2.12.1 Preparation of Samples	70
2.2.12.2 Preparation of Lipid Substrates	71
2.2.12.3 Enzyme Reaction	71
2.2.12.4 Lipid Extraction	72
2.2.12.5 Thin Layer Chromatography	72
2.2.13 Protein Kinase Assay	73
2.2.13.1 Preparation of Samples	73
2.2.13.2 Enzyme Reaction	73
2.2.14 Chemotaxis	74
2.2.15 Calcium Mobilisation Assay	75
2.2.16 Protein A Chromatography	75



<b>2.3 <i>In vivo</i> techniques</b>	<b>76</b>
2.3.1 Animals and Animal Care Procedures	76
2.3.2 Generation of Air Pouches	76
2.3.3 Intravenous injection of cultured cells	77
2.3.4 Statistical analysis	77

### **CHAPTER 3: THE CLONING OF MURINE PHOSPHATIDYLINOSITOL**

#### **3-KINASE GAMMA**

3.1 Introduction	78
3.2 Isolation of putative clones	80
3.3 Sequence analysis of putative clones	81
3.4 Construction of the MmPI3K $\gamma$ open-reading frame	82
3.5 Discussion	85

### **CHAPTER 4: *IN VITRO* CHARACTERISATION OF MmPI3k $\gamma$ 1111**

4.1 Introduction	87
4.2 <i>In vivo</i> expression of MmPI3k $\gamma$ 1111	89
4.3 Generation of polyclonal antibodies specific for PI3k $\gamma$	90
4.4 Over-expression of MmPI3k $\gamma$	92
4.5 Lipid kinase activity MmPI3k $\gamma$ 1111	93
4.6 Protein kinase activity of MmPI3k $\gamma$ 1111	94
4.7 The relationship between phosphorylation status of MmPI3k $\gamma$ 1111 and its lipid kinase activity	95
4.8 Discussion	97

## **CHAPTER 5: THE ROLE OF MmPI3ky1111 IN LYMPHOCYTE**

### **MIGRATION *IN VITRO* AND *IN VIVO***

5.1 Introduction	104
5.2 Mutagenesis of MmPI3ky1111	106
5.3 Lipid Kinase activity of MmPI3kyKR	108
5.4 Protein kinase activity of MmPI3kyKR	108
5.5 Development of a retroviral dominant-negative expression system	109
5.6 Generation of B lymphocytes stably-transfected with pcDNA3::MmPI3kyKR	112
5.7 Protein and lipid kinase activity of the MmPI3kyKR	113
5.8 Effect of transfection with MmPI3kyKR on <i>in vitro</i> chemotaxis of B300.19-huCCR6 cells in response to human MIP-3 $\alpha$	114
5.9 CCR6 expression on B300.19-huCCR6::MmPI3kyKR cells	115
5.10 Calcium mobilisation of B300.19-huCCR6 cells stably-transfected with pcDNA3::MmPI3kyKR	116
5.11 <i>In vivo</i> recruitment of B300.19-huCCR6 cells	117
5.12 The effect of transfection with MmPI3ky1111 on <i>in vivo</i> migration of B300.19-huCCR6 cells	119
5.13 Discussion	122

## **CHAPTER 6: GENERAL DISCUSSION**

6.1 Discussion	129
----------------	-----

## **CHAPTER 7: BIBLIOGRAPHY**

7.1 Bibliography

139

**APPENDIX I**

**APPENDIX II**