N4WBP5A (Ndfip2), a Nedd4-interacting protein, localizes to multivesicular bodies and the Golgi, and has a potential role in protein trafficking

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Summary

N4WBP5A (Ndfip2) belongs to an evolutionarily conserved group of Nedd4-interacting proteins with two homologues in mammalian species. We have previously shown that N4WBP5A expression in *Xenopus* oocytes results in increased cell-surface expression of the epithelial sodium channel. N4WBPs are characterized by one or two amino terminal PPxY motifs and three transmembrane domains. Here we show that both PPxY motifs of N4WBP5A mediate interaction with WW domains of Nedd4 and that N4WBP5A can physically interact with the WW domains of several Nedd4-family proteins. N4WBP5A is ubiquitinated and ubiquitination does not significantly affect the turnover of N4WBP5A protein. Ubiquitination of N4WBP5A is enhanced by Nedd4 and Nedd4-2 expression. N4WBP5A localizes to the Golgi, vesicles associated with the Golgi complex and to multivesicular bodies. We show that the ectopic expression of N4WBP5A inhibits receptor-mediated endocytosis of labelled epidermal growth factor. N4WBP5A overexpression inhibits accumulation of EGF in large endocytic/lysosomal vesicles suggestive of a role for N4WBP5A in protein trafficking. We propose that N4WBP5A acts as an adaptor to recruit Nedd4 family ubiquitin-protein ligases to the protein trafficking machinery.

Key words: Nedd4, Ubiquitination, WW domains, Multivesicular bodies

Introduction

Protein ubiquitination is an important sorting signal that acts within the endocytic and biosynthetic pathways. Direct ubiquitination of plasma membrane proteins is required for delivery into endocytic vesicles while the ubiquitination of components of the endocytic machinery might also be required for this initial step of endocytosis (reviewed by Weissman, 2001; Hicke, 2001). Ubiquitin acts as a sorting signal in the biosynthetic pathway within the Golgi complex. In the trans-Golgi network (TGN) ubiquitination can determine whether yeast proteins are delivered to the plasma membrane, where they perform their function as permeases, or to the vacuole, where they are degraded (Weissman, 2001; Hicke, 2001; Pickart, 2001). The exocytic release of viruses from infected mammalian cells is also a process that can be regulated by ubiquitination (Kikonyogo et al., 2001). Thus, ubiquitin performs essential roles at multiple steps within the endocytic and biosynthetic pathways.

The Nedd4 family of proteins, which form part of the HECT family of ubiquitin-protein ligases (E3), are assumed to be key enzymes mediating ubiquitin-dependent protein sorting and trafficking (Hicke, 2001; Kumar et al., 1992; Kumar et al., 1997; Harvey and Kumar, 1999). Nedd4-like E3s are characterized by the presence of 2-4 WW domains, which bind specific ligands, the most common of which is the PPxY (PY) motif, and in most instances by a calcium/lipid binding C2 domain (Harvey and Kumar, 1999; Sudol and Hunter, 2000; Rizo and Sudhof, 1998). In yeast, the Nedd4 homologue Rsp5p acts at multiple sites within protein trafficking pathways. Rsp5p functions at the initial step of endocytosis, is involved in vesicle trafficking within the endocytic compartment and has also been shown to be required for sorting in the biosynthetic pathway within the TGN (Dunn and Hicke, 2001; Shih et al., 2003; Beck et al., 1999; Helliwell et al., 2001). In *Drosophila,* Nedd4 binds and ubiquitinates Comm, which allows the internalization of the Robo receptor (Myat et al., 2002). The best known target of mammalian Nedd4 is the amiloride-sensitive epithelial sodium channel (ENaC), which plays a crucial role in fluid and electrolyte homeostasis (reviewed by Snyder, 2002). ENaC consists of three transmembrane subunits termed α, β and γ, all of which can bind Nedd4, and its closest homologue Nedd4-2 (Staub et al., 1997; Dinudom et al., 1998; Harvey et al., 1999; Abriel et al., 1999; Harvey et al., 2001; Dinudom et al., 2001; Kamynina et al., 2001; Fotia et al., 2003). Ubiquitination mediated by Nedd4/Nedd4-2 is believed to regulate ENaC endocytosis and downregulation (Staub et al., 1997; Dinudom et al., 1998; Harvey et al., 1999; Abriel et al., 1999; Harvey et al., 2001; Dinudom et al., 2001; Kamynina et al., 2001; Fotia et al., 2003).

Although the role of Rsp5p in protein secretory and

endocytic trafficking is well established, much less is known about mammalian Nedd4 and its targets. In a far-western screen using the WW domains of mouse Nedd4 as a probe, we had previously identified several PY-containing proteins that interact with the WW domains of Nedd4 (Jolliffe et al., 2000). One of these, N4WBP5 (Ndfip1)[†], is a novel Golgi-associated protein containing two PY motifs in the amino terminal region and three putative transmembrane (TM) domains in the carboxyl terminal half of the protein (Harvey et al., 2002). A closely related mammalian protein, which we named N4WBP5A (Ndfip2), was identified in database searches. In *Drosophila* these two proteins are represented by a single homologue (Harvey et al., 2002). Interestingly, N4WBP5 and N4WBP5A share significant homology with intracellular vesicle transmembrane proteins synaptoporin and secretory carrier-associated membrane proteins (SCAMPs) (Knaus et al., 1990; Bauerfeind et al., 1995; Singleton et al., 1997; Fernandez-Chacon and Sudof, 2000), suggesting that these proteins may play a role in Nedd4-mediated regulation of intracellular protein trafficking in mammals.

We have previously shown that in *Xenopus* oocytes ectopic expression of N4WBP5A causes increased ENaC expression at the cell surface (Konstas et al., 2002). As N4WBP5A activity in this system requires intact PY motifs it may regulate ENaC cell-surface expression by interaction with the *Xenopus* Nedd4 homologue. It is probable that sequestration of *Xenopus* Nedd4/Nedd4-2 to an intracellular site by N4WBP5A, which prevents its association with ENaC at the plasma membrane, would result in the observed effect. Thus, it is possible that the role of the N4WBP5s is to recruit Nedd4 and Nedd4 family members to distinct subcellular compartments. In this paper we have further characterized N4WBP5A, and we show that N4WBP5A can interact with a range of Nedd4 family proteins and that ubiquitination of N4WBP5A is enhanced by Nedd4 and Nedd4-2 expression. We define the subcellular localization of N4WBP5A and provide evidence that this protein potentially functions in endocytic protein trafficking.

Materials and Methods

Plasmids and cDNA constructs

The coding region of N4WBP5A was amplified by RT-PCR, with and without a carboxyl terminal FLAG-epitope, and cloned into the *Hin*dIII and *Eco*RI sites of pcDNA3 (Invitrogen) to generate pcDNA3-N4WBP5A-FLAG and pcDNA3-N4WBP5A. pcDNA3-N4WBP5A-myc was PCR amplified from pcDNA3-N4WBP5A, with primers containing an amino terminal c-myc-epitope and cloned into the *Hin*dIII and *Eco*RI sites of pcDNA3. N4WBP5A-GFP was constructed by cloning the coding region of N4WBP5A into the *Hin*dIII and *Kpn*I sites 5′ of the EGFP coding sequence of pEGFP-N1 (Clontech).

One or both PY motifs in pcDNA3-N4WBP5A-Flag were mutated by PCR mutagenesis such that the terminal Tyr residues of the PY motifs were altered to Ala residues (Y56A; Y82A). pcDNA3-N4WBP5A K4 was generated by PCR mutagenesis such that the Lys residues in pcDNA3-N4WBP5A were altered to Ala or Arg (K96,98,221A; K179R) in pcDNA3-N4WBP5A. The expression constructs used to generate the various WW domain proteins fused to glutathione S-transferase (GST) have been described previously (Dinudom et al., 1998; Harvey et al., 1999; Harvey et al., 2001). The N4WBP5A-GST bacterial expression construct used for antibody production and far-western analysis was generated by PCR amplification of the N4WBP5A cDNA encoding the amino terminal 135 residues, lacking the initiation methionine, and cloned into pGEX-2TK (Amersham Pharmacia) such that N4WBP5A was fused to the carboxyl terminal of GST. Construction of the wild-type and cysteine mutant Nedd4 expression plasmids pCXN2-Nedd4 has been previously described (Kumar et al., 1997; Harvey et al., 2002). Construction of the Nedd4-2 expression plasmid pcDNA3-Nedd4-2 has been previously described (Fotia et al., 2003). The HA-ubiquitin expression plasmid was kindly provided by Dirk Bohmann (University of Rochester, Rochester, NY).

Cell lines and transfections

HEK 293T cells were grown in RPMI 1640/10% FCS, whereas other cell lines were grown in DMEM/10% FCS at 37°C with 5% CO_2. Transfections were performed using FuGENE 6 (Roche) according to the manufacturer's instructions.

Antibodies and reagents

Commercial antibodies and labelled factors were obtained from the following suppliers: monoclonal anti-FLAG M2 (Sigma-Aldrich), anti-CD63 (Zannettino et al., 2003), anti-EGFR (ab3103, Abcam), anti-β-Cop (Sigma-Aldrich), anti-HA (Roche), anti-GM130 (clone 35, BD Transduction Laboratories), anti-LAMP-1 (CD107a, BD Transduction Laboratories), anti-Nedd4 (clone 15, BD Transduction Laboratories), anti-c-myc antibody (clone 9E10, Roche), anti-rabbit-FITC (Chemicon International), anti-mouse Alexa Fluor 568 (Molecular Probes), anti-mouse R-phycoerythrin-Cy5 (PE) (Southern Biotechnologies), anti-rat-Texas red (Rockland Immunochemicals for Research), anti-mouse-AP and anti-rabbit-AP (Chemicon Int.), anti-rat-AP (Pierce Chemical Co.) and anti-mouse and anti-rabbit gold (10 nm and 15 nm) conjugated antibodies (British Biocell). EGF Alexa Fluor 488 and TFN Alexa Fluor 594 (Molecular Probes). The production of anti-N4WBP5 and anti-N4WBP5A antibodies have been previously described (Harvey et al., 2002; Konstas et al., 2002).

Immunoprecipitation, immunoblotting and far-western analysis

Where indicated, cells were treated before lysis with 20 μM MG132 (Affiniti Research Products). Immunoprecipitations were performed by harvesting cells in lysis buffer [50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 10 mM EDTA, Complete Protease Inhibitor Cocktail (Roche)], preclearing lysates with Protein G Sepharose (Amersham Biosciences) for 2 hours, incubating lysates overnight at 4°C with the appropriate antibody (5 μg/ml) and protein G Sepharose. Immunoprecipitates were washed twice in lysis buffer, once in RIPA buffer [50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP40, 0.5% deoxycholic acid, 0.1% SDS, 10 mM EDTA, Complete Protease Inhibitor Cocktail (Roche)] where indicated and once in PBS, eluted and subjected to SDS-PAGE and transferred to PVDF. Primary antibodies for western analysis were used as follows: anti-FLAG M2 antibody (2 μg/ml), anti-N4WBP5A (0.75 μg/ml) and anti-HA (1/500 dilution). Secondary alkaline phosphatase conjugated antibodies (1/2000 dilution) were used; detection of bound antibody was achieved using ECF (Amersham Biosciences) and using a Typhoon 9410 (Molecular Dynamics) and ImageQuant software (Amersham Biosciences).

GST fusion proteins were produced and purified as described previously (Harvey et al., 1999). ^{32}P-labelled protein probes were produced by directly labelling the GST fusion protein using protein kinase A (New England Biolabs). Glutathione beads containing bound fusion protein were incubated with protein kinase A and ^{32}P-

[†]Genome nomenclature committee has recently suggested Ndfip1 and Ndfip2 for N4WBP5 and N4WBP5A, respectively.

ATP in a buffer containing 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 12 mM $MgCl_2$ and 1 mM DTT (4°C, 1 hour). Beads were washed in PBS and labelled protein was eluted with glutathione buffer. Membranes were blocked in Hyb 75 (Jolliffe et al., 2000) and then hybridized with the ^{32}P-labelled GST fusion protein probe (4°C, 4 hours) in Hyb 75. Membranes were washed in Hyb75 and exposed to X-ray film.

Golgi-budding assay

A stacked Golgi-enriched membrane fraction was prepared by density gradient centrifugation from rat liver homogenates and used with cytosol in a vesicle budding assay as described previously (Heimann et al., 1999; Wylie et al., 2003). Briefly, Golgi membranes were incubated with rat liver cytosol, GTPγ S, and HKM buffer for 30 minutes at 37°C. A mixture of rat liver cytosol and cytosol from transfected cells was used to assay the binding of recombinant proteins to the Golgi membranes. After budding, the remnant Golgi cisternae were separated by centrifugation at 17,500 ***g*** for 10 minutes, and the Golgi-derived vesicles were then separated from the remaining cytosol by centrifugation over a 20% sucrose cushion at 100,000 ***g*** for 90 minutes. The vesicle pellet was resuspended in 20% sucrose in HKM buffer and then loaded at the top of a 20-50% discontinuous sucrose gradient and centrifuged at 100,000 ***g*** for 18 hours at 4°C. Fractions were collected from the bottom by needle puncture and analysed by SDS-PAGE.

Immunohistochemistry and uptake assays

Immunofluorescence was performed by fixing cells in 4% paraformaldehyde for 15 minutes at room temperature and permeabilising in 0.2% Triton/PBS for 2 minutes. Primary and secondary antibodies for immunofluorescence were diluted in 4% fetal calf serum in PBS (5-10 μg/ml). For EGF uptake assays HeLa cells were serum starved for 2 hours at 37°C 16 hours after transfection, incubated for 1 hours at 4°C with Alexa Fluor 488 EGF (200 ng/ml) or Alexa Fluor 594 TFN (10 μg/ml), and uptake performed for up to 40 minutes at 37°C. Surface-bound EGF and TFN was stripped from the cells by treatment in 0.2 M acetic acid (pH 2.5), 0.5 M NaCl for 1 minute on ice. Cells were fixed and permeabilized as described above for anti-myc immunohistochemistry. Confocal images were captured using a BioRad Radiance 2100 confocal microscope (Bio-Rad Microscience, UK) equipped with three lasers: Argon ion 488 nm (14 mw), Green HeNe 543 nm (1.5 mw) and Red Diode 637 nm (5 mw), and an Olympus IX70 inverted microscope. The objective used was a 40× W UPLAPO with NA=1.3. The dual labelled cells were imaged with two separate channels (PMT tubes) in a sequential setting. Green fluorescence was excited with an Ar 488 nm laser line and the emission viewed through a HQ515/30 nm narrow band barrier filter in PMT1. Red fluorescence was excited with a HeNe 543 nm laser line and the emission viewed through a long pass barrier filter (E570LP) in PMT2. Automatically all signals from PMTs 1 and 2 were merged. Image analysis was performed with Confocal Assistant software for Microsoft Windows™ (Todd Clark Brelje, USA) and LaserPix V4 (Bio-Rad) software.

Electron microscopy

Cells were fixed for 1 hour with 4% paraformaldehyde (EM grade, ProSciTech), pH 7.4 and scraped from the dishes, resuspended in 10% gelatin and solidified at 4°C. Small blocks were infiltrated with PVP/sucrose (15%, 1.7 M, Sigma-Aldrich) overnight, and then frozen onto cryo stubs in liquid nitrogen. Sections (80 nm) were cut on a Reichert FCS cryomicrotome (Leica Microsystems) at –120°C. For immunolabelling, sections were quenched with 0.02 M glycine, blocked with 1% BSA/PBS and incubated with primary antibody followed by anti-mouse or anti-rabbit IgG gold conjugated antibodies diluted in 1% BSA/PBS. For double-labelling, sections were then blocked in 1% glutaraldehyde and the labelling regime repeated using a different sized secondary gold conjugate. Sections were viewed and photographed on a JEOL 1010 electron microscope (JEOL Australasia) at 80 kV.

Flow cytometric analysis

Eighteen hours after transfection HeLa cells were serum starved for 2 hours, harvested in 2 mM EDTA and washed twice in wash solution (PBS, 0.1% BSA, 0.1% sodium azide) at 4°C. Cells were blocked on ice in wash solution supplemented with 10% rabbit serum. 1×10^6 cells were incubated on ice with 1 μg primary antibody for 45 minutes in wash solution supplemented with 10% rabbit serum. Cells were washed twice in wash solution at 4°C and incubated with secondary antibody (1/50 dilution) in wash solution supplemented with 10% rabbit serum on ice for 45 minutes. Cells were washed twice and fixed in 1% PFA. Cells were analysed using an EPICS-XL flow cytometer (Coulter). Twenty thousand events were collected per sample as list mode data and analysed using RXP software (Beckman Coulter).

Results

Both PY motifs in N4WBP5A bind Nedd4 WW domains

Previously we have shown that endogenous Nedd4 and its close homologue Nedd4-2 interact with N4WBP5A (Konstas et al., 2002). To determine which PY motifs of N4WBP5A interact with the WW domains of Nedd4, we used a far-western approach (Fig. 1A). Cells were transfected with either wild-type N4WBP5A-FLAG (WT), or mutant N4WBP5A-FLAG in which either PY motif (PY1, PY2) or both PY motifs (PY1,2) were mutated. The N4WBP5A-FLAG proteins were immunoprecipitated with anti-FLAG antibody, subjected to SDS-PAGE and transferred to nitrocellulose. The membrane was probed with a ^{32}P-labelled GST-fusion protein containing the 3 WW domains of mouse Nedd4. As can be seen in Fig. 1A, the GST fusion protein containing the WW domains 1-3 of Nedd4 interacts with wild-type N4WBP5A. This interaction was significantly reduced when PY motif 1 was mutated, moderately reduced when PY motif 2 was mutated and abrogated when both PY motifs were mutated. These results confirm that the physical association of N4WBP5A and Nedd4 is mediated via PY/WW interactions, with PY motif 1 contributing most to this interaction, which is similar to the interaction between N4WBP5 and Nedd4 (Harvey et al., 2002).

To assess which of the WW domains of Nedd4 contribute to this interaction, far-western analysis was performed with individual WW domains of Nedd4. A nitrocellulose membrane containing immunoprecipitated wild-type (WT) or N4WBP5A-PY1,2 (PY1,2) was probed with ^{32}P-labelled individual WW domains of Nedd4 expressed as GST fusion proteins. WW1 of Nedd4 did not interact with N4WBP5A, whereas WW domains 2 and 3 of Nedd4 interacted with wild-type N4WBP5A, with the strongest interaction between WW3 and N4WBP5A (Fig. 1B). This is similar to the binding studies reported for N4WBP5 (Harvey et al., 2002). The WW domains of Nedd4 did not interact with N4WBP5A in which both PY motifs had been mutated. Thus, the association of N4WBP5A and Nedd4 is predominantly mediated PY motif 1 of N4WBP5A and WW domain 3 of mouse Nedd4.

N4WBP5A interacts with the WW domains of multiple Nedd4 family proteins

Far-western analysis was further used to investigate the possible interaction of N4WBP5A with other Nedd4 family members (Fig. 1C). Recombinant proteins consisting of GST-fusions of the WW domains of human NEDD4, NEDD4-2, KIA0332, SMURF1, WWP2, AIP-4 and mouse Itch were immobilized on nitrocellulose and probed with the ^{32}P-labelled N4WBP5A-GST. The N4WBP5A probe bound to each of these proteins with the exception of Smurf1 under in vitro conditions (Fig. 1C). A similar interaction pattern was seen with N4WBP5 (Harvey et al., 2002), indicating that in vivo N4WBP5 and N4WBP5A may physically associate with a range of Nedd4-family members via PY/WW-mediated interactions.

N4WBP5A is potentially ubiquitinated by Nedd4 family proteins

The ability of N4WBP5A to interact with Nedd4 family members, and the fact that N4WBP5 is ubiquitinated, suggested that N4WBP5A might also be ubiquitinated. To investigate this possibility wild-type N4WBP5A and a mutant version of N4WBP5A in which all four Lys residues, which may act as ubiquitin acceptors, were mutated were used in immunoprecipitation experiments. HEK 293T cells were cotransfected with either wild-type N4WBP5A (5A WT) or mutant N4WBP5A (5A K4) and HA-ubiquitin (Ub-HA) or vector as a control. Immunoprecipitations were carried out with an affinity purified polyclonal antiserum raised to the amino terminal half of N4WBP5A (Fig. 2A, lanes 2-7) or a control antibody (Fig. 2A, lane 1). In Fig. 2A, lanes 5 and 7, cells were treated with the proteasomal inhibitor MG132 (20 μM) before lysis. To enhance the stringency of these immunoprecipitations, immunoprecipitated proteins were washed once in RIPA buffer after two washes in lysis buffer. The upper panel in Fig. 2A, probed with the N4WBP5A antibody, shows the relative amounts of immunoprecipitated protein. The N4WBP5A K4 mutant protein has a different mobility in SDS-PAGE to that of wild-type N4WBP5A. When the same blot was probed with an anti-HA antibody (A, lower panel), HA-tagged ubiquitinated forms of wild-type N4WBP5A (lanes 4, 5) corresponding to mono-ubiquitinated (42 kDa) and di-ubiquitinated (50 kDa) N4WBP5A could be seen in addition to polyubiquitinated forms of N4WBP5A. This was not the case for the N4WBP5A K4 mutant (lanes 6, 7). The fact that N4WBP5A can be ubiquitinated and can physically interact with Nedd4 family proteins suggests that N4WBP5A may be ubiquitinated by one or more Nedd4 family members in vivo.

The role of N4WBP5A ubiquitination was assessed in pulse-chase experiments in transfected HEK 293T cells. Cells were transfected with wild-type N4WBP5A or the Lys mutant N4WBP5A K4 and the stability of the proteins

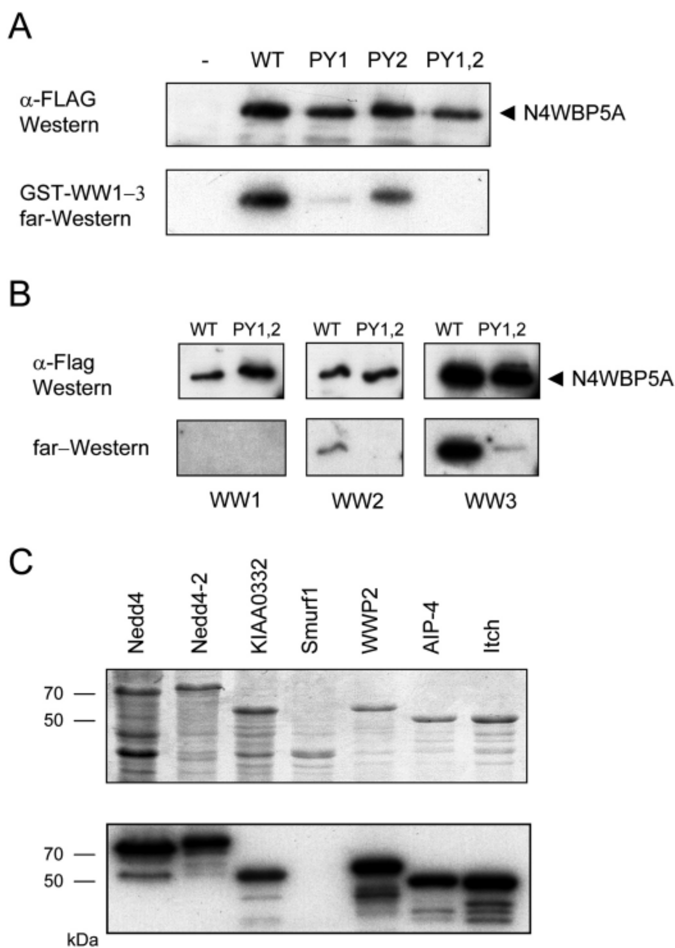


Fig. 1. N4WBP5A interacts with Nedd4 family members. (A) The interaction between N4WBP5A and the WW domains of Nedd4 is mediated via PY motifs 1 and 2. COS cells were transfected with vector (–), wild-type pcDNA3-N4WBP5A-FLAG (WT), or pcDNA3-N4WBP5A-FLAG in which PY motif 1 was mutated (Y56A; PY1) or PY motif 2 was mutated (Y82A; PY2), or pcDNA3-N4WBP5A-FLAG-PY1,2 (PY1,2). FLAG-tagged proteins were immunoprecipitated with anti-FLAG antibody and immunoprecipitates subjected to SDS-PAGE and blotted. The blot was probed with a ^{32}P-labelled GST-Nedd4 protein containing WW domains 1-3 of mouse Nedd4. Upper panel: anti-FLAG western blot showing the relative amount of immunoprecipitated FLAG-tagged proteins. Lower panel: far-western of the above blot probed with ^{32}P-labelled Nedd4 GST-WW1-3. (B) The interaction between N4WBP5A and Nedd4 is mediated via WW domains 2 and 3. COS cells were transfected with wild-type pcDNA3-N4WBP5A-FLAG (WT), or pcDNA3-N4WBP5A-FLAG in which both PY motifs 1 and 2 were mutated (Y56A, Y82A; PY1,2). FLAG-tagged proteins were immunoprecipitated with anti-FLAG antibody and immunoprecipitates subjected to SDS-PAGE and blotted. The blot was probed with a ^{32}P-labelled Nedd4-GST protein containing WW domain 1, 2 or 3 of Nedd4. Upper panel: anti-FLAG western blot showing the relative amount of immunoprecipitated FLAG-tagged proteins. Lower panel: far-western of the above blot probed with ^{32}P-labelled Nedd4 GST-WW1, GST-WW2 or GST-WW3.
(C) N4WBP5A interacts with the WW domains of Nedd4 family proteins. *Escherichia coli* extracts expressing various GST-WW proteins of the human genes *NEDD4*, *NEDD4-2*, *KIAA0332*, *SMURF1*, *WWP2* and *AIP-4* or the mouse gene *Itch* were blotted and probed with a ^{32}P-labelled GST-N4WBP5A protein comprising the amino terminal 136 amino acids of N4WBP5A, containing the PY motifs, fused to the carboxyl terminal of GST. Upper panel: Coomassie-stained gel of GST WW domain fusion proteins. Lower panel: far-western blot of a duplicate gel blotted and probed with the ^{32}P-labelled GST-N4WBP5A protein.

compared over a 24 hour period. N4WBP5A is a long-lived protein with a half-life of approximately 6 hours. The stability of the Lys mutant was not significantly different to that of wild-type N4WBP5A (results not shown), indicating that ubiquination of N4WBP5A is unlikely to significantly affect turnover of the protein.

The ability of Nedd4 and Nedd4-2 to enhance ubiquitination of N4WBP5A was assessed by immunoprecipitation. HEK 293T cells were cotransfected with either wild-type N4WBP5A (5A WT) or mutant N4WBP5A (5A PY1,2), HA-ubiquitin (Fig. 2B, all lanes) and increasing amounts of Nedd4 (wild-type N4 or catalytically inactive, N4 cys) or Nedd4-2 (N4-2) or vector (Fig. 2B, lane 1). Immunoprecipitations were carried out with anti-N4WBP5A. As shown in Fig. 2B (lower panel), co-expression of catalytically active Nedd4 or Nedd4-2 enhanced ubiquitination of N4WBP5A (Fig. 2B, lanes 3-6). N4WBP5A-PY1,2, which is unable to interact significantly with Nedd4-family proteins, showed a low level of ubiquitination similar to that seen with vector cotransfection, that was not enhanced by Nedd4 co-expression (Fig. 2B, lanes 7, 8). This indicates that the enhanced ubiquitination of N4WBP5A requires physical interaction with the Nedd4 protein, strongly suggesting that Nedd4 directly ubiquitinates N4WBP5A.

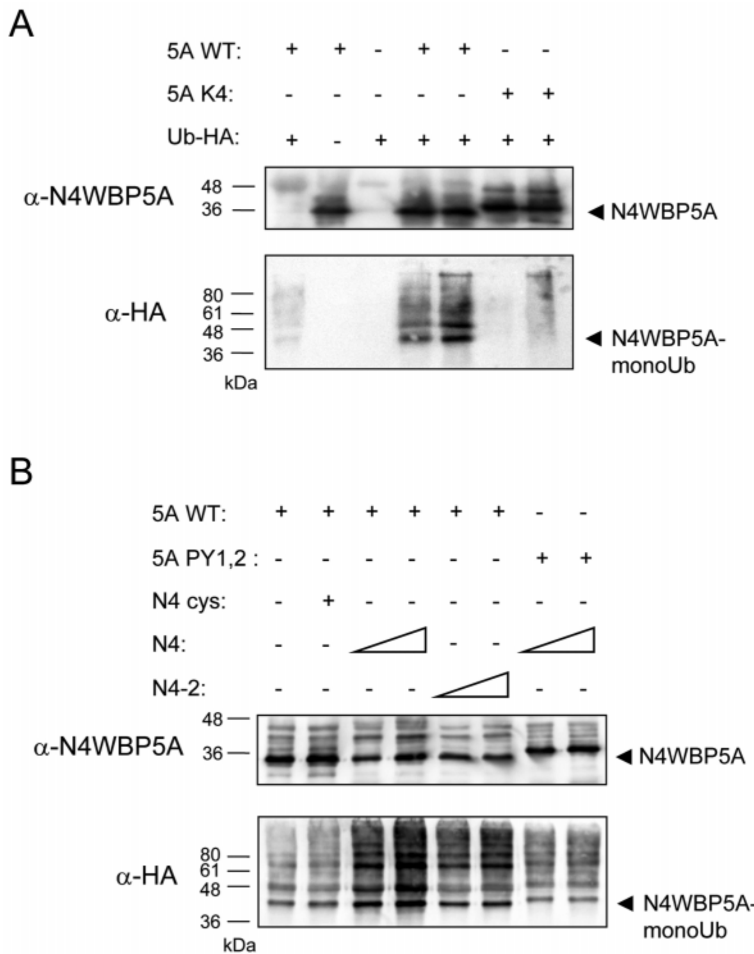


Fig. 2. N4WBP5A is ubiquitinated by Nedd4 and Nedd4-2. (A) As indicated above the lanes, immunoprecipitations were carried out from 293T cells transfected with pcDNA3-N4WBP5A-FLAG (5A WT), or pcDNA3-N4WBP5A lysine mutant (5A K4) and vector or HA-ubiquitin (Ub-HA) using a control antibody (lane 1) or anti-N4WBP5A antibody (lanes 2-7). In lanes 5 and 7, cells were treated with MG132 for 4 hours before lysis. Upper panel: anti-N4WBP5A western blot showing immunoprecipitated N4WBP5A proteins. Lower panel: anti-HA western blot showing ubiquitinated immunoprecipitated proteins. (B) Immunoprecipitations were carried out from 293T cells transfected with pcDNA3-N4WBP5A (5A WT), or pcDNA3-N4WBP5A-PY1,2 (5A PY1,2), HA-ubiquitin (Ub-HA), and increasing amounts of Nedd4 (N4, 0.5 and 1.0 μg), Nedd4 cysteine mutant (N4 cys, 1.0 μg), Nedd4-2 (N4-2, 0.5 and 1.0 μg) or vector (lane 1) using the anti-N4WBP5A antibody. Upper panel: anti-N4WBP5A western blot showing immunoprecipitated N4WBP5A proteins. Lower panel: anti-HA western blot showing ubiquitinated immunoprecipitated proteins.

N4WBP5A localizes to Golgi vesicles and multivesicular bodies (MBVs)

The subcellular localization of endogenous and ectopically expressed N4WBP5A was investigated using immunostaining on intact cells and ultrathin cryosections. The anti-N4WBP5A antibody was used to detect the subcellular localization of endogenous N4WBP5A in epithelial M-1 cells (Fig. 3A-F) and neuroblastoma N18 cells (not shown). Unlike N4WBP5, which primarily localizes to the Golgi complex in most cell types (Harvey et al., 2002), in M-1 and N18 cells endogenous N4WBP5A localized predominantly in large vesicular structures within the cytoplasm (Fig. 3A-F). There appears to be little overlap between N4WBP5A and the Golgi matrix protein GM130 (Fig. 3B,C). The vesicular staining pattern of N4WBP5A suggested that N4WBP5A may be localized to post-Golgi vesicles or endosomal compartments. There was partial overlap of N4WBP5A with the late endsomal/lysosomal marker LAMP1 (Fig. 3E,F), indicating that N4WBP5A is partially localized to a late endosomal compartment. In HeLa and M1 cells (data not shown), ectopically expressed N4WBP5A-myc localizes to vesicles in the area of the Golgi (Fig. 3H,I) and significant colocalization with the late endosomal marker CD63 was seen (Fig. 3K,L). These results indicate that ectopically expressed N4WBP5A has a similar localization to that of the endogenous protein.

Immunogold labelling at the electron microscopy level was carried out to further identify the subcellular compartment where N4WBP5A is localized. In both N18 (not shown) and HeLa cells (Fig. 4A-C, Table 1), a portion of N4WBP5A labelling was found associated with Golgi stacks and with vesicles around the stacks. Labelled vesicles were associated with the TGN, as evidenced by the presence of clathrin-coated vesicles in some fields. The majority of N4WBP5A-myc labelling was primarily localized to late-stage endosomes with the characteristic ultrastructure of multivesicular bodies (Fig. 4B,C). N4WBP5A-myc labelling was present on limiting membranes but was most intense on internal vesicles (Fig. 4C). Interestingly, in N4WBP5A-myc-expressing cells, a proportion of endogenous Nedd4 protein was colocalized with N4WBP5A in MVBs (Fig. 4A,B). This was not seen in untransfected cells (data not shown), suggesting that N4WBP5A can recruit Nedd4 to distinct subcellular compartments.

Table 1. Labelling density of gold particles on N4WBP5A-myc transfected HeLa cells

MVB	Golgi	Rest of cell
88.5±11.3	71.9±17.2	1.05±0.16

Numbers represent gold particles per μm^2 (mean ± s.e.m.) in multi-vesicular bodies (MVB) and over the Golgi complex compared with the rest of the cell. HeLa cells were transiently transfected with BP5A-myc as described and were immunolabelled with c-myc antibody followed by 10 nm goat anti-mouse gold. For each field of view (*n*=17), gold particles were counted and the total surface areas of each region were measured. Sections incubated with goat anti-mouse gold alone (no primary antibody) had negligible labelling (1 gold particle/10 μm^2).

An in vitro Golgi budding assay was used to test whether N4WBP5 and N4WBP5A are recruited to vesicles. The assay was performed by incubating isolated stacked Golgi membranes with cytosol from rat liver to detect N4WBP5 or from transfected cells to detect recombinant N4WBP5A. Immunoblotting for β-Cop was used as a marker of Golgi-derived vesicles. Both proteins bound to the Golgi stack membranes, N4WBP5 stayed associated with the Golgi stacks while N4WBP5A-myc was also recovered on budded vesicles (Fig. 4D). Density gradient separation of the vesicles, which segregates different populations of vesicles (Wylie et al., 2003), revealed that N4WBP5A was concentrated in some fractions, suggesting it is specifically recruited to a subset of vesicles, in keeping with the distribution seen by immunogold labelling.

N4WBP5A potentially functions in protein trafficking

Given the similarity of N4WBP5A to synaptoporin and the SCAMPS, molecules involved in protein trafficking, and its localization in cellular compartments involved in trafficking, we assessed the role of N4WBP5A in receptor-mediated endocytosis. We tested the effect of N4WBP5A-myc overexpression on uptake of labelled epidermal growth factor (EGF) and transferrin (TFN) in HeLa cells. Following 16 hours of transfection, EGF uptake was monitored for up to 30 minutes using EGF-Alexa Fluor 488. Surface-bound EGF was removed, and the cells fixed, permeabilized and stained with an anti-myc antibody to detect transfected cells. The amount of endocytosed EGF varied within the population of cells and may reflect levels of expression of the EGF receptor. Following EGF uptake for 5 minutes, low levels of labelled EGF were localized in small vesicles close to the plasma membrane (Fig. 5A). After uptake for 10 minutes EGF labelled small vesicles throughout the cytoplasm (Fig. 5B), and by 20 minutes EGF

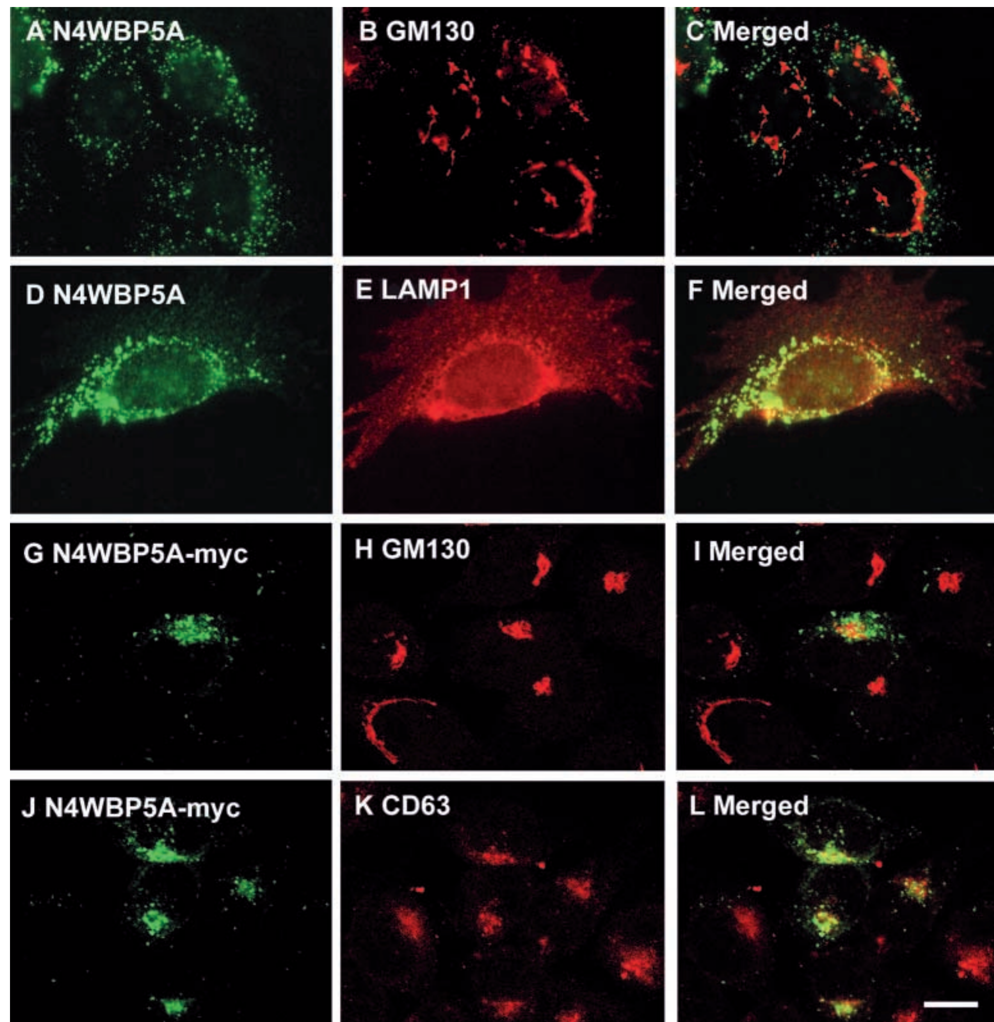
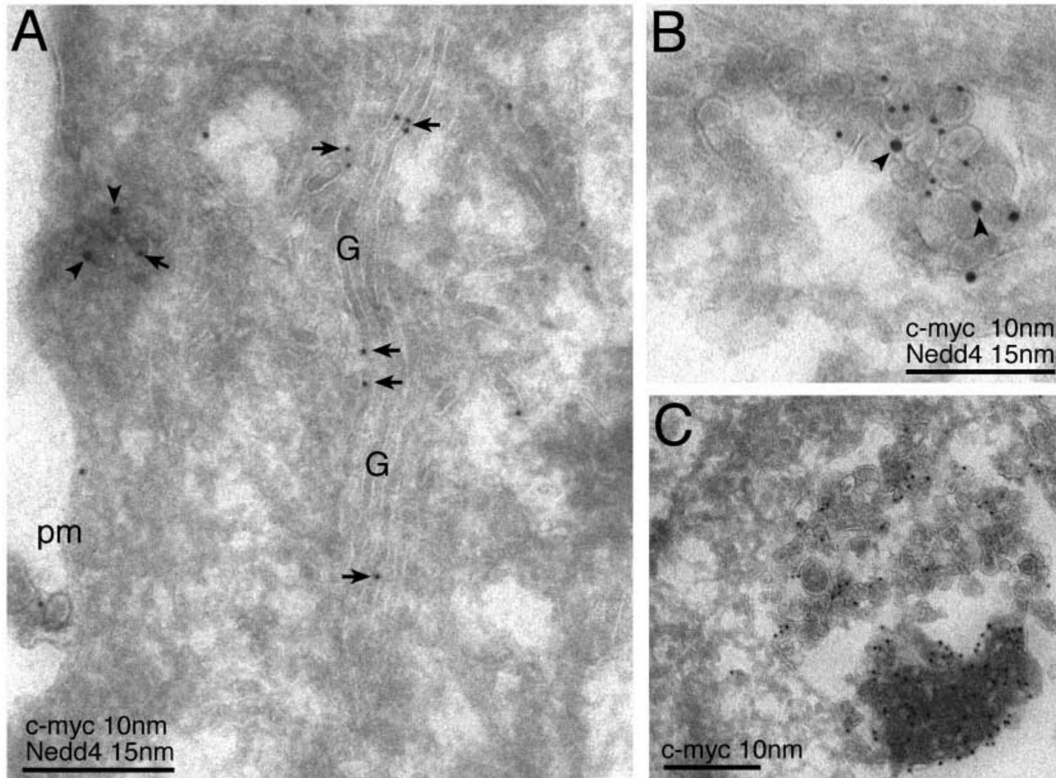


Fig. 3. N4WBP5A localizes to cytoplasmic vesicular structures. (A-F) Endogenous N4WBP5A protein detected in M-1 cells by immunofluorescence. (A,D) Localization of N4WBP5A; (B) localization of the Golgi matrix protein, GM130; (C) colocalization of N4WBP5A and GM130; (E) localization of the lysosomal protein, LAMP1 and (F) colocalization of N4WBP5A and LAMP1. (G-L) Ectopically expressed N4WBP5A-myc protein in HeLa cells detected by immunofluorescence. (G,J) Localization of N4WBP5A-myc; (H) localization of the Golgi matrix protein, GM130; (I) colocalization of N4WBP5A-myc and GM130; (K) localization of the late endosomal protein, CD63 and (L) colocalization of N4WBP5A-myc and CD63. Bar, 10 μm.



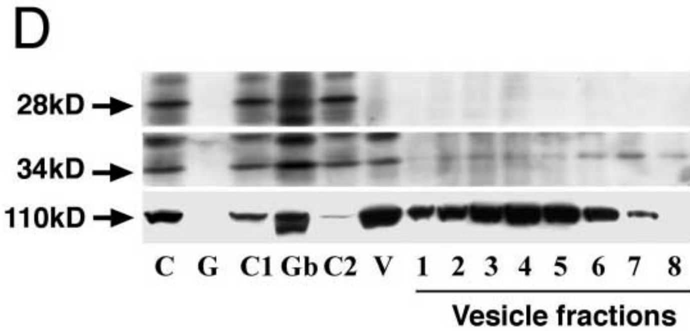


Fig. 4. N4WBP5A localizes to multivesicular bodies and Golgi vesicles. HeLa cells transiently transfected with N4WBP5A-myc were fixed with paraformaldehyde and processed for cryo-electronmicroscopy. Ultra-thin sections were double labelled with monoclonal anti-myc antibody and Nedd4 (A,B) using 10 nm or 15 nm goat anti-mouse or goat anti-rabbit gold conjugates. (A) Localization of N4WBP5A-myc (arrows) in the TGN and Nedd4 (arrowheads) in the TGN (G, TGN; pm, plasma membrane). (B) Localization of N4WBP5A-myc and Nedd4 (arrowheads) in MVBs. (C) Localization of N4WBP5A-myc in a MVB showing abundant labelling on the internal vesicles and some labelling on the limiting membrane. Bar, 200 nm. (D) Western blot analysis of N4WBP5 and N4WBP5A in Golgi-derived vesicles. Golgi membranes (G) were incubated with rat liver cytosol (C), GTPγS and HKM buffer. A mixture of rat liver cytosol and cytosol from transfected cells was used to assay the binding of recombinant proteins to the Golgi membranes. After budding, the remnant Golgi cisternae (Gb) were separated by centrifugation, and the Golgi-derived vesicles (V) were separated from the remaining cytosol (C2). The vesicle pellet was prepared on a 20-50% discontinuous sucrose gradient and fractions (1-9) collected and analysed by western blot using appropriate antibodies.

began to localize in larger vesicles, which tended to be more perinuclear in localization (Fig. 5C). Following EGF uptake for 10 minutes or longer, a clear difference was seen between transfected and untransfected cells. Cells expressing N4WBP5A (outlined cells) consistently contained less EGF compared with the majority of untransfected cells (Fig. 5D-L). The amount of EGF-Alexa fluor 488 endocytosed in 15 minutes by untransfected and transfected cells was compared. The average total intensity (green fluorescence) per cell above background in transfected cells (quantified using LaserPix Version 4.0 software, Bio-Rad) was expressed as a percentage of that measured in untransfected cells. In transfected cells the value was 58% of that of untransfected controls (Fig. 6). By 20 minutes EGF accumulated in large vesicles that were localized in the cytoplasm and in the perinuclear region of untransfected cells (Fig. 5G,I). In N4WBP5A transfected cells EGF uptake appeared to be inhibited at an early stage of endocytosis. When EGF uptake was performed for 25 minutes the EGF-containing endosomes in transfected cells were small and were most similar to the early endosomes labelled with EGF in untransfected cells following uptake performed for 5 to 10 minutes (Fig. 5). In transfected cells both the fluorescence intensities and size of EGF-containing vesicles were reduced (Fig. 7A,B) when compared with untransfected cells (Fig. 7C,D).

It has been reported that Hgs is required for ligand-independent degradation of the EGFR (Katz et al., 2002). Ubiquitination of Hgs by Nedd4 was shown to inactivate the endocytic activity of Hgs on EGFR (Katz et al., 2002). Thus, it is possible that the observed effect of N4WBP5A overexpression on EGF uptake may be caused by sequestration of endogenous Nedd4 by N4WBP5A, causing a reduction in EGFR at the plasma membrane in the absence of EGF. The levels of plasma membrane EGFR were assessed by flow cytometry in HeLa cells transfected with N4WBP5A-GFP or EGFP-N1 as a control. Cell-surface EGFR was detected by indirect immunofluorescence using an anti-EGFR antibody and secondary PE-conjugated antibody. The amount of cell-surface EGFR represented by PE positivity was not significantly altered by N4WBP5A-GFP expression compared with either untransfected cells or cells transfected with EGFP-N1 (Fig. 8). Thus, reduced EGFR at the plasma membrane caused by

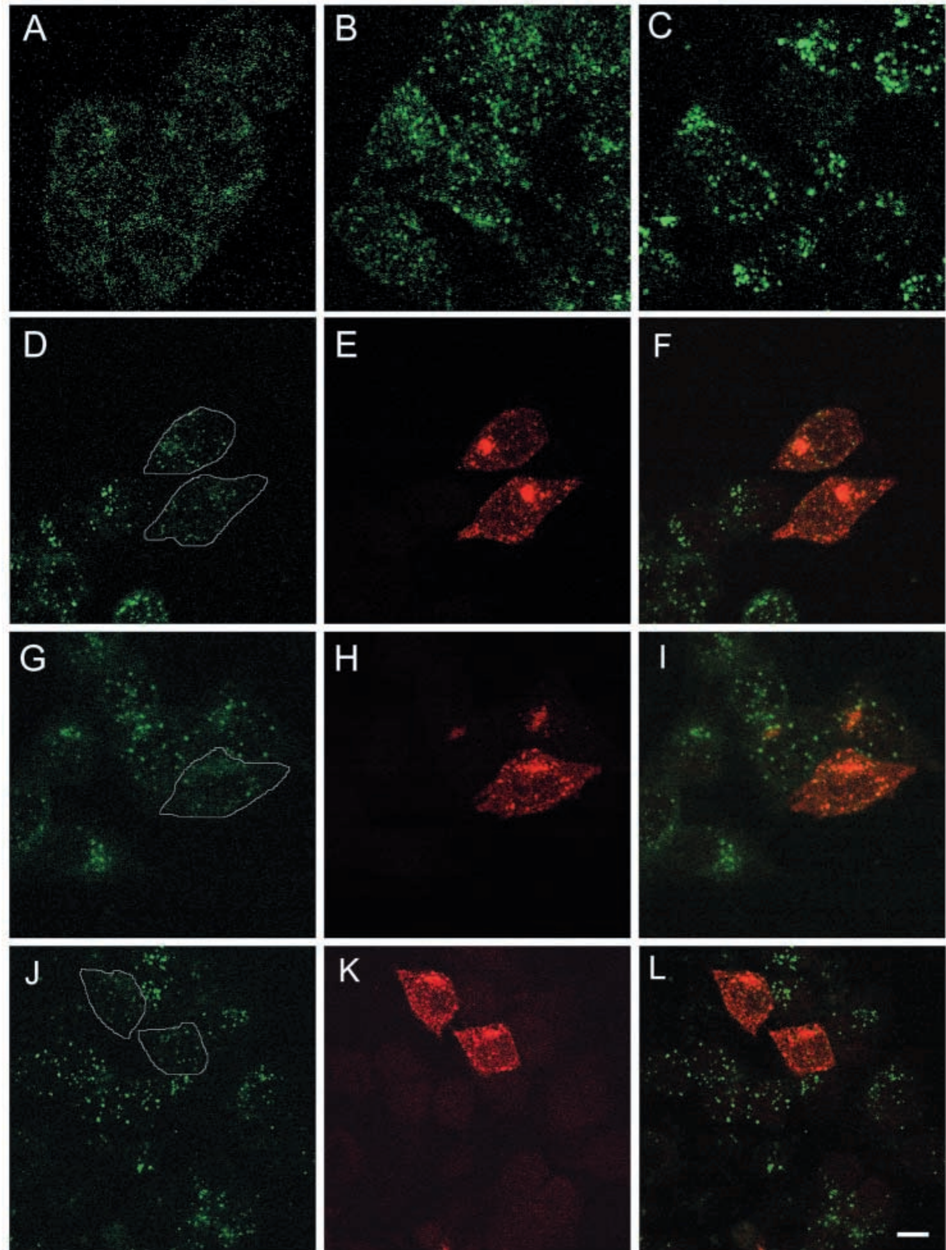


Fig. 5. Overexpression of N4WBP5A inhibits receptor-mediated endocytosis. HeLa cells untransfected (A-C) or transfected with N4WBP5A-myc (D-L) were serum starved, cultured with EGF-Alexa Fluor 488 (200 ng/ml, green) for 1 hour at 4°C and EGF uptake was performed at 37°C for up to 25 minutes. Surface-bound EGF-Alexa fluor 488 was stripped from the cells, cells were fixed and permeabilized and immunohistochemistry performed with an anti-myc antibody and anti-mouse Alexa Fluor 568 secondary antibody (red). (A) EGF uptake for 5 minutes; (B) 10 minutes; (C) 20 minutes. (D-F) EGF uptake for 15 minutes. (G-I) EGF uptake for 20 minutes. (J-L) EGF uptake for 25 minutes. (A-C,D,G,J) EGF localization (green, transfected cells outlined). (E,H,K) Transfected cells expressing N4WBP5A (red) and merged images (F,I,L) showing transfected cells and endocytosed EGF Alexa Fluor 488. Bar, 10 μm.

N4WBP5A overexpression does not result in the observed effect on EGF uptake.

Overexpression of N4WBP5A did not inhibit the endocytic uptake of TFN, a marker of the recycling endocytic pathway, in HeLa cells. When uptake of labelled TFN was performed for 20 (Fig. 9A-C) and 40 (Fig. 9D-F) minutes no observable difference between transfected and untransfected cells could be seen in either the amount or localization of endocytosed TFN (Fig. 9). Thus, N4WBP5A specifically affects endocytosis within the degradative pathway and may be specific for that of the EGFR.

Discussion

We have provided evidence that N4WBP5A can interact with the WW domains of Nedd4/Nedd4-2 and several other Nedd4 family members. N4WBP5A is ubiquitinated and its ubiquitination is enhanced by Nedd4/Nedd4-2 expression. The localization of N4WBP5A to MVBs, Golgi and Golgi-derived vesicles is distinct from the closely related protein N4WBP5, which primarily localizes to the Golgi complex (Harvey et al., 2002). MVBs are the endocytic compartment in which some of the sorting of cell-surface receptors is carried out. Recycling receptors (e.g. TFNR) and receptors destined for degradation in the lysosome (e.g. EGFR) are initially endocytosed into the same endocytic compartment and are then segregated in MVBs, the endosomal compartment proximal to the lysosome (Katzmann et al., 2002). Recycling receptors remain on the limiting membrane of MVBs and recycle to the plasma membrane, while receptors destined for degradation accumulate on the intravesicular membranes and are subsequently delivered to the lysosome for degradation (Katzmann et al., 2002). MVBs thus perform essential functions for the endocytic process. Our EGF uptake experiments suggest that N4WBP5A may affect EGFR trafficking at the stage of MVB delivery. This is consistent with the presence of N4WBP5A on internal vesicles in MVBs.

Our results show that the overexpression of N4WBP5A inhibits accumulation of EGF in late stage endosomes/lysosomes without affecting endocytosis of TFNR. The levels of EGFR at the plasma membrane are not altered in N4WBP5A transfected cells indicating that the defect in EGF uptake occurs downstream of receptor binding. Following ligand binding EGFR is ubiquitinated by the ubiquitin ligase Cbl and is endocytosed (Katzmann et al., 2002). EGFR ubiquitination appears to be primarily required for MVB sorting since

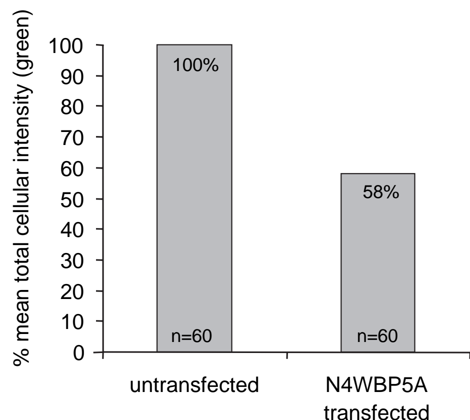


Fig. 6. Quantitative analysis of N4WBP5A expression on EGF uptake. Graph of the total average intensity (green fluorescence) above background (threshold 75) per N4WBP5A transfected cell (*n*=60) expressed as a percentage of the total average intensity (green fluorescence) above background per untransfected cell (*n*=60) quantitated using LaserPix V4.0 software (Bio-Rad) following EGF uptake for 15 minutes (calculated from independent experiments).

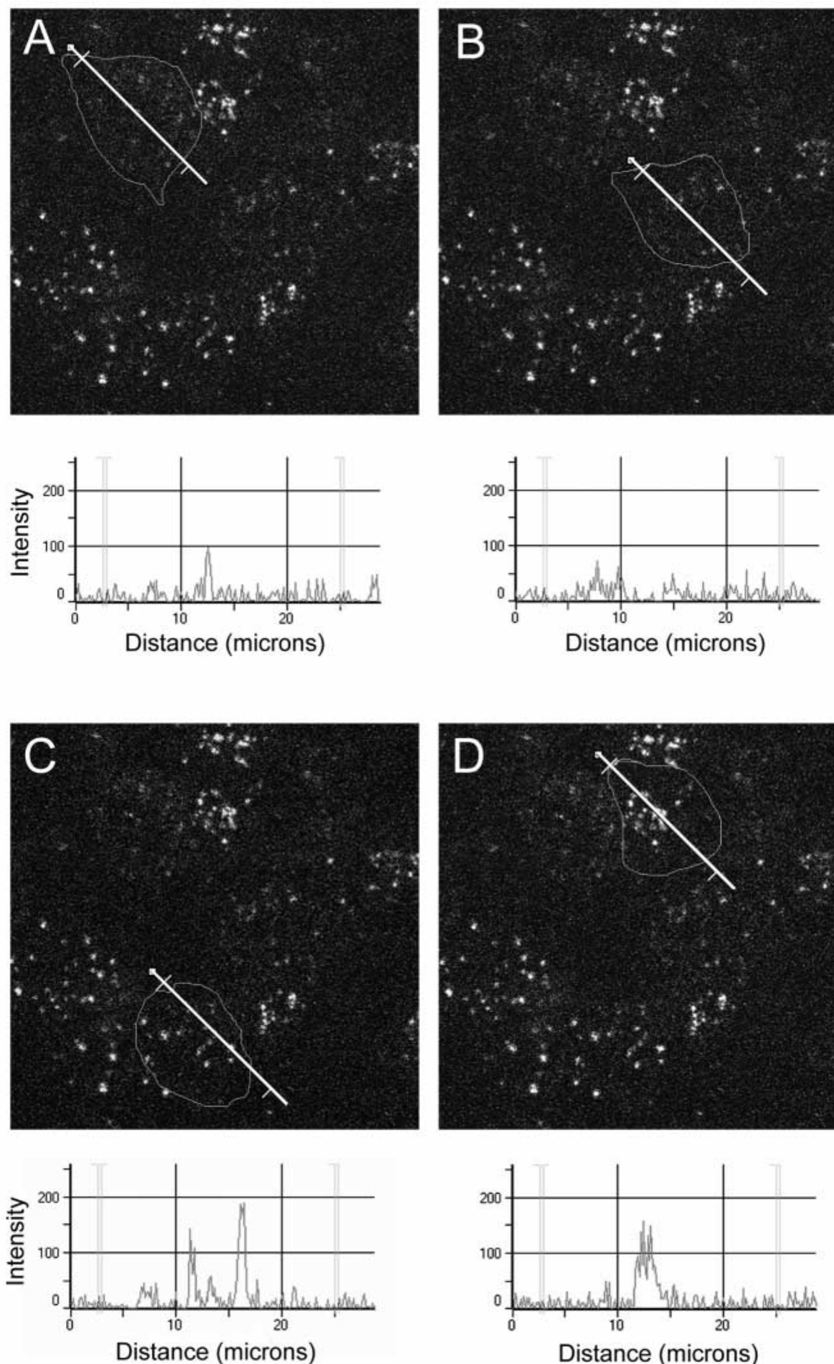
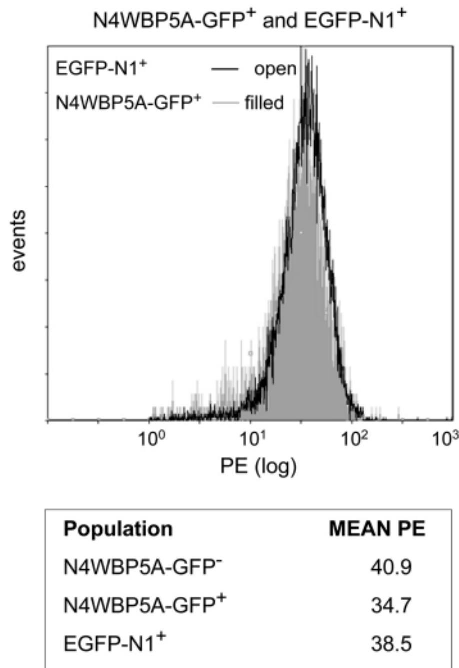


Fig. 7. EGF localizes in early stage endosomes in N4WBP5A transfected cells. EGF uptake was performed in HeLa cells transfected with myc-N4WBP5A for 20 minutes as described in Fig. 5. The line profile tool [LaserPix V4.0 software (Bio-Rad)] was used to measure the fluorescence intensity and size of EGF-containing endosomes in transfected cells (A,B) and untransfected cells (C,D). Graphs showing examples of the fluorescence intensity versus distance in microns measured with the line profile are shown below.

overexpression of Cbl enhances EGFR degradation without affecting the rate of internalization (Levkowitz et al., 1998). It has also been shown that internalized EGFRs that are not ubiquitinated recycle to the plasma membrane, escaping degradation (Levkowitz et al., 1998). The role of Nedd4 in the regulation of EGFR endocytosis is complex. Nedd4 family proteins have been shown recently to directly ubiquitinate Cbl causing its proteasomal degradation and resulting in inhibition of Cbl-mediated EGFR downregulation (Magnifico et al., 2003). Both Hgs and Eps15 are involved in EGFR regulation that can be controlled by Nedd4. Nedd4-mediated ubiquitination of Hgs has been shown to inactivate its endocytic activity in the absence of EGF (Katz et al., 2002), while the effect of mono-ubiquitination of Eps15 on its endocytic acitivity is not currently known (Polo et al., 2002). Compared with EGFR, ubiquitination of the TFNR is not required for its endocytic removal from the plasma membrane and it can either recycle directly to the plasma membrane from the early endocytic compartment or indirectly via the perinuclear recycling centre (Ghosh and Maxfield, 1995). A possible explanation for the N4WBP5A-mediated effect on EGF endocytosis is that the function of component(s) of the trafficking machinery required for delivery to or sorting within the MVB are disrupted by N4WBP5A overexpression. If ubiquitination of the EGFR is affected by N4WBP5A overexpression, endocytosed EGFR may recycle inappropriately to the cell surface and result in the observed effect. The specificity of the effect for EGF indicates that the component(s) involved do not function in the recycling pathway utilized by TFN. Sequestration of such components by N4WBP5A may interfere with their function. The identification of N4WBP5A-



Population	MEAN PE
N4WBP5A-GFP⁻	40.9
N4WBP5A-GFP⁺	34.7
EGFP-N1⁺	38.5

Fig. 8. Overexpression of N4WBP5A does not affect cell-surface levels of the EGFR. HeLa cells were transfected with N4WBP5A or EGFP-N1 as a control and analysed for cell-surface levels of EGFR by indirect immunoflourescence using flow cytometry. Cell-surface EGFR (PE positivity) was measured on untransfected cells, or cells transfected with N4WBP5A-GFP or EGFP-N1. The histogram shows the EGFR levels (PE Log scale) on EGFP-N1$^+$ (open black line) and N4WBP5A-GFP$^+$ (grey filled) cells against relative number of events. The mean PE values for the various populations are shown below.

interacting proteins, in addition to Nedd4 family members, is likely to provide insight into the mechanism responsible for this observation.

In a previous study we have shown that ectopic expression of N4WBP5A in *Xenopus* oocytes results in increased cell-surface expression of ENaC subunits and this was dependent on the intact PY motifs of N4WBP5A (Konstas et al., 2002). In further experiments we showed that the effect on ENaC was probably due to sequestration of Nedd4 by N4WBP5A, making it unavailable to bind to and ubiquitinate ENaC (Konstas et al., 2002). Given that the results of this study show that ectopic N4WBP5A expression results in a significant proportion of cellular Nedd4 localizing to MVBs, it is possible that the normal function of N4WBP5A is to act as an adaptor for Nedd4 family proteins recruiting them to specific subcellular sites, such as components of the trafficking machinery. The emerging role of ubiquitin in protein trafficking has linked Nedd4 family members to many of these processes in eukaryotes. The data in this paper suggest that through interaction with adaptor molecules, such as the N4WBP5s, Nedd4 family members may play as yet uncharacterized roles in additional trafficking events.

Constitutive overexpression of N4WBP5A in mammalian cells (L.M.S.-W. and S.K., unpublished), or *Saccharomyces cerevisiae* leads to an inhibition of cell growth (A. L. Munn., L.M.S.-W. and S.K., unpublished). Interestingly, the *S. cerevisiae* protein Bsd2p shares significant homology with N4WBP5 and N4WBP5A (A. L. Munn, L.M.S.-W. and S.K., unpublished), and as such is a putative yeast homologue of the N4WBP5s. Like the N4WBP5s, Bsd2p has three TM domains, and a PY motif in the amino terminal region. Bsd2p has been shown to negatively regulate the stability of the metal ion transporters Smf1p and Smf2p (Liu and Culotta, 1999; Portnoy et al., 2000). Bsd2p, localized in the endoplasmic reticulum, is required for delivery of Smf1p and Smf2p to the vacuole for rapid degradation in the presence of intracellular heavy metals such as manganese by an unknown mechanism (Liu and Culotta, 1999; Portnoy et al., 2000). We propose that Bsd2p acts as an adaptor which recruits the yeast Nedd4 family protein Rsp5p to Smf1p and Smf2p in the ER. Under high metal ion concentrations we propose that Rsp5p ubiquitinates Smf1p and Smf2p, causing their delivery to the vacuole for degradation. In future experiments it would be

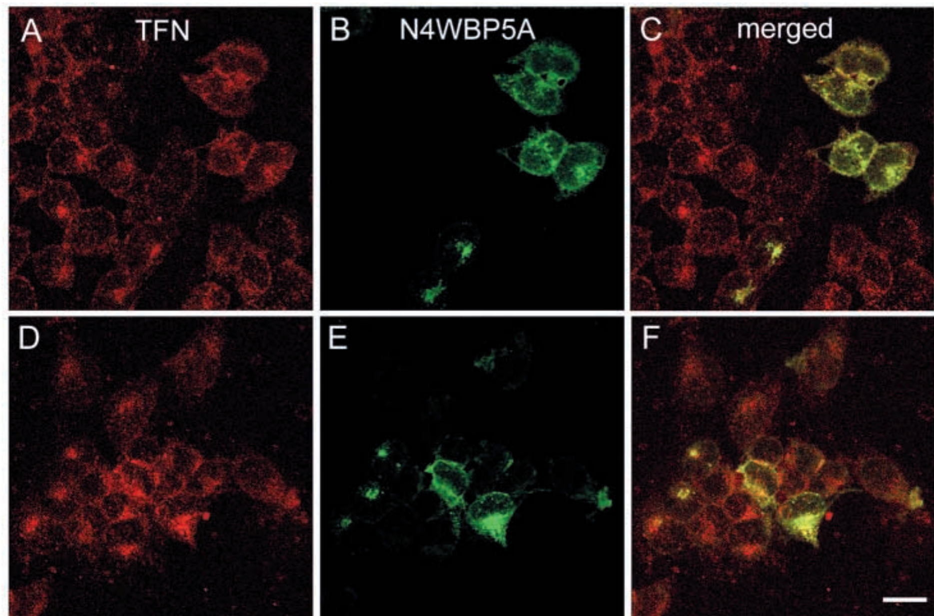


Fig. 9. TFN uptake is not inhibited by N4WBP5A overexpression. TFN uptake was performed in HeLa cells transfected with myc-N4WBP5A for 20 (A-C) and 40 minutes (D-F). Cells were serum starved for 2 hours and cultured with TFN-Alexa Fluor 594 (10 μg/ml, red) for 30 minutes at 4°C and TFN uptake was performed at 37°C for up to 20 and 40 minutes. Surface-bound TFN-Alexa Fluor 594 was stripped, cells were fixed and permeabilized and immunohistochemistry performed with an anti-myc antibody and secondary antibody conjugated to Alexa Fluor 488. The figure shows endocytosed TFN-Alexa Fluor 594 (A,D), myc-N4WBP5A transfected cells (B,E) and merged images (C,F). Bar, 20 μm.

interesting to determine if N4WBP5 and/or N4WBP5A proteins can compensate for the loss of Bsd2p in yeast.

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