Studies of a Phosphatidylinositol 3-kinase complex linked to vesicular trafficking in human cells.

by

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ABSTRACT

Phosphoinositide 3-kinases (PI 3-kinases) are signal transducing molecules that catalyse the addition of phosphate to the 3-OH position of the inositol ring of phosphoinositides. PI 3-kinase has emerged as an important mediator in the events leading to mitogenesis, cytoskeletal re-arrangement, receptor internalisation and glucose transport. In mammalian cells numerous PI 3-kinases have been discovered, including those regulated by Receptor Tyrosine Kinases (RTK) and heterotrimeric G proteins. These PI 3-kinases can generate Phosphatidylinositol (PtdIns)$3P$, PtdIns$(3,4)P_2$ and PtdIns$(3,4,5)P_3$ as potential second messengers. In contrast, genetic and biochemical studies have shown that yeast contain only one PI 3-kinase, termed Vps34p. This enzyme uses PtdIns as its only substrate and is not activated by any known ligand. Vps34p is found associated with Vps15p, a 170kDa serine/threonine protein kinase. This heterodimeric complex is required for the efficient sorting and delivery of proteins to the yeast vacuole. A human homologue of the yeast Vps34p protein, PtdIns 3-kinase has been identified and shown to form a complex with a protein of 150kDa (termed p150), raising the possibility that a human Vps15p homologue exists.

In this study the cDNA encoding the p150 protein was isolated and used to express a 150kDa protein in insect (Sf9) and mammalian (COS-7) cells. p150 was shown to display extensive amino acid homology with Vps15p and sequence comparisons between both proteins have been used to delineate potential functional domains. The data presented in this thesis also show that recombinant p150 associates with PtdIns 3-kinase both *in vitro* and *in vivo* . Using immunofluorescence techniques, p150 expressed in COS-7 cells was localised to perinuclear punctate compartments. To investigate which of the p150 domains are responsible for its subcellular localisation and association with PtdIns 3-kinase, deletion and amino acid substitution studies were undertaken. Additional biochemical studies investigating the 3'-phosphoinositides produced in cells expressing PtdIns 3-kinase demonstrated elevated levels of PtdIns$3P$ and PtdIns$(3,5)P_2$. The increase in PtdIns$3P$ production by the p150/PtdIns 3-kinase complex in the presence of phosphatidylinositol transfer protein (PI-TP) also suggests a role for PI-TP in PtdIns substrate presentation to PtdIns 3-kinase.

The results presented in this study demonstrate that p150 is the human Vps15p homologue, suggesting an evolutionary conservation between yeast and humans in the basic molecular mechanisms of vesicle transport. Based on the data presented, mechanisms by which the p150/PtdIns 3-kinase complex and its 3'-phosphoinositide products might be implicated in membrane trafficking events are proposed.

To my father,
Zacharias Panaretou
(8/4/35 - 13/1/98)
'Αιωνια η μνημη'

Acknowledgements

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Abbreviations

Accepted abbreviations in this thesis are listed in Biochem. J. (1998) **329**, 1-16. In addition the following abbreviations are used:

AMCA	7-Amino-4-methylcoumarin
ARF	ADP-ribosylation factor
βARK	β-adrenergic receptor kinase
BCR	Break point cluster region
bp	Basepairs
BSA	Bovine serum albumin
CDK	Cyclin dependent kinase
CGN	*Cis*-Golgi network
CHO	Chinese hamster ovary
COP	Coatamer protein
CPY	Carboxypeptidase Y
C-terminal	Carboxy-terminal
DAG	Diacylglycerol
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulphoxide
dNTPs	Deoxyribonucleoside triphosphates
DTT	dithiothreitol
EEmAb	'EFMPME' epitope tag specific mouse monoclonal antibody
EST	Expressed sequence tag
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetra-acetic acid
EGF	Epidermal growth factor
ERK	Extracellular signal-related kinase
FcγR	Fc gamma receptor
FCS	Foetal calf serum
FGF	Fibroblast growth factor
FITC	Fluorescein Isothiocyanate
fMLP	N-formylmethionyl-leucyl-phenylalanine
GAP	GTPase activating protein
GroPIns	Glycerophosphatidylinositol
GST	Glutathione S-transferase
HBSS	Hanks Balanced Salt Solution
HEPES	N-2-hydroxyethyl piperazine-N'-2-ethane sulphonic acid

HPLC	High pressure liquid chromatography
Ig	Immunoglobulin
IL-	Interleukin-
IPTG	Isopropyl-2-D-thiogalactopyranoside
IRS-1	Insulin receptor substrate
kb	Kilobase
kDa	Kilodalton
KLH	Keyhole limpet haemocyanin
LPA	Lysophosphatidic acid
LPS	Lipopolysaccharide
LY294002	[2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one]
MAPK	Mitogen-activated protein kinase
MBP	Myelin basic protein
MGN	*Medial*-Golgi network
MHC	Major histocompatibility complex
mT	Middle T
NRK	Normal rat kidney
N-terminal	Amino-terminal
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PDGF	Platelet-derived growth factor
PEG	Polyethylene glycol
PH	Pleckstrin homology
PI	Phosphatidylinositide
PI 3-kinase	Phosphatidylinositide 3-kinase
PI-TP	Phosphoinositide transfer protein
PKC	Protein kinase C
PLC	Phospholipase C
PLD	Phospholipase D
PMA	Phorbol 12-myristate 13-acetate
PMSF	Phenylmethylsulphonyl fluoride
PtdCho	Phosphatidylcholine
PtdEtn	Phosphatidylethanolamine
PtdIns	Phosphatidylinositol
PtdIns 3-kinase	Phosphatidylinositol 3-kinase
PtdOH	Phosphatidicacid
PtdIns3P	Phosphatidylinositol-3-phosphate
PtdIns(3,4)P_2	Phosphatidylinositol-3,4-bisphosphate
PtdIns(3,5)P_2	Phosphatidylinositol-3,5-bisphosphate

PtdIns(3,4,5)P_3	Phosphatidylinositol-3,4,5-trisphosphate
PtdIns4P	Phosphatidylinositol-4-phosphate
PtdIns(4,5)P_2	Phosphatidylinositol-4,5-bisphosphate
PTK	Protein tyrosine kinase
PVDF	Polyvinylidene difluoride
RPMI 1640	Roswell Park Memorial Institute 1640 medium
SDS	Sodium dodecylsulphate
Sf9	Spodoptera frugiperda-9
Sos	Son of sevenless
SSC	Saturated sodium citrate
SH1/2/3	Src homology 1/2/3
SV40	Simian virus 40
TCA	Trichloroacetic acid
TGN	*Trans*-Golgi network
TRITC	Tetramethylrhodamine B Isothiocyanate
Tween 20	Polyoxyethylenesorbitan monolaurate

Abbreviations for amino acids

Amino Acid	Three Letter Abbreviation	One-letter Symbol
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V
Lysine	Lys	K

CHAPTER 1

Introduction

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1.1 General Introduction

The activity of all cells is controlled by external signals that elicit intracellular responses. DNA synthesis and cell growth are regulated in part by a large number of polypeptide growth factors, cytokines and hormones, which exert their mitogenic effects by interaction with specific cell surface receptors on responsive cells. The binding of a ligand to its receptor elicits a cascade of events including protein phosphorylation, stimulation of second messenger generating systems, ion fluxes and enhanced DNA synthesis. These complex and often interacting signalling events co-ordinate the multifunctional cellular programmes that culminate in cell proliferation or differentiation. This acquisition and subsequent release of signalling information is the process of signal transduction (Egan and Weinberg, 1993).

Transmembrane signalling systems at the plasma membrane relay information (i.e. extracellular stimuli) from the cell surface to the cytoplasm via a myriad of different protein-protein interactions and second messenger cascades. This process is in contrast to vesicle-mediated membrane traffic which was initially considered to be a constitutive process that was not influenced by layers of regulation. However, certain signal transduction pathways such as those involving regulated exocytosis, have been demonstrated to exert their effects in the cytoplasm and have a profound influence upon membrane traffic.

The importance of membrane traffic is highlighted by the fact that eukaryotic cells are characterised by the presence of numerous functionally distinct membrane-enclosed organelles. The functional identity of intracellular organelles is largely defined by the unique set of proteins residing within them. Consequently, the accurate and efficient delivery of proteins to a given organelle is of fundamental importance in establishing and maintaining their structure and function (Stack *et al.*, 1995c).

The observation that each step in membrane traffic must be tightly controlled and co-ordinated with other cellular events, has given rise to the idea that protein and vesicle sorting during membrane trafficking may represent a form of intracellular signal transduction. Membrane traffic requires regulation to maintain constant organelle size and composition. Therefore, traffic to the Golgi complex must equal traffic from the Golgi and similarly, traffic to the plasma membrane should balance traffic from the cell surface.

In addition, the level of membrane traffic must also be regulated to meet the changing physiological needs of the cell. For example, a growing cell requires much more membrane synthesis than a quiescent cell. Membrane traffic also varies in response to various hormones and growth factors that bind particular receptors. There is a growing recognition that the machinery for responding to extracellular signals and the machinery for controlling membrane traffic may be much more closely connected than has been previously appreciated (Bomsel and Mostov, 1992). This suggests a role for membrane traffic in cellular regulation, consequently, the identification of molecules involved in membrane transport will make them an interesting and important area for study, providing a greater insight into the mechanisms of cellular regulation.

1.2 Phospholipids as signalling molecules

1.2.1 Phosphoplipids in mammalian membranes

Eukaryotic cells are composed of a complex set of membrane-enclosed organelles that are distinguished by the unique lipid compositions of their corresponding membranes. The plasma membrane of many mammalian cells for example contains, in addition to other lipids, four major phospholipids; phosphatidylcholine (PtdCho), sphingomyelin, phosphatidylserine (PtdSer) and phosphatidylethanolamine (PtdEtn) (van Meer, 1989). Not only can the lipid composition of individual organelles differ, but the lipid bilayer itself can also be assymetric in lipid content (Alb *et al.*, 1996). The cytoplasmic leaflet of the mammalian plasma membrane is highly enriched compared with the outer leaflet in PtdSer and PtdEtn. Maintenance of such assymetry is a crucial physiological activity since the exposure of PtdSer on the cell surface appears to mark a mammalian cell for clearance from the body (Savill *et al.*, 1993). A fifth phospholipid has been identified in mammalian cells. Lysobisphosphatidic acid (LBPA), is found only in late endosomes/lysosomes and is consequently a marker lipid for these organelles (Kobayashi *et al.*, 1998; Schmid and Cullis, 1998). LBPA is postulated to have a structural role in the maturation of late endosomes, its tendency not to form a bilayer (due to its small negatively charged headgroup) could help in developing the complex intralumenal membrane system observed in endosomes. Alternatively, LBPA is resistant to phospholipases so it may stabilise late-endosomal/lysosomal membranes against degradation (Huterer and Wherrett, 1990). At present the precise role of this late-endosome specific lipid is unknown but is an example of how specific lipid compositions might be required for proper organelle function.

Of the total phospholipid pool, phosphatidylinositol (PtdIns) is a minor component, comprising 5%-10% of all phospholipids in cell membranes (Moreau and Cassagne, 1994; van Meer, 1989). This acidic molecule contains a myo-inositol head group *in*
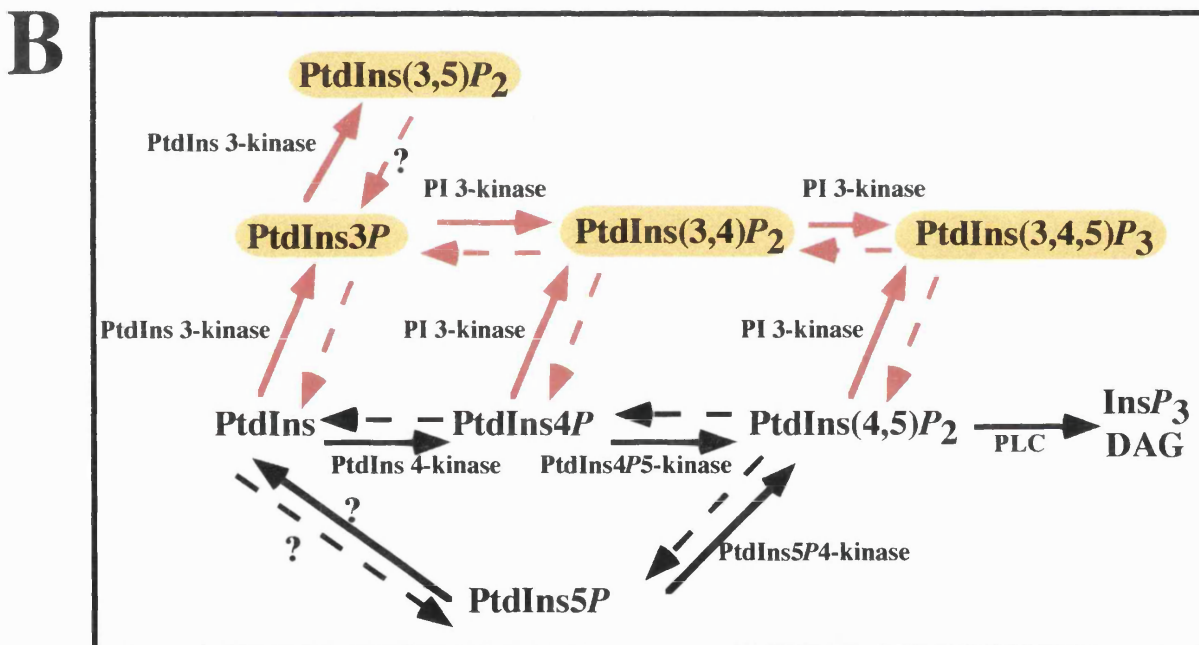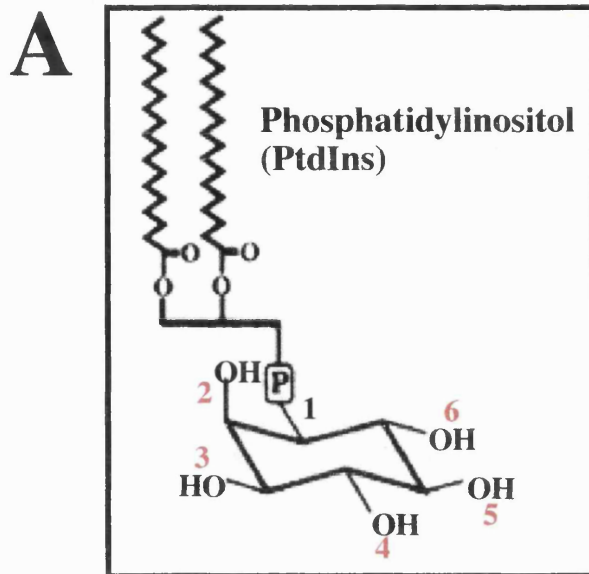
Figure 1.1 Synthesis of phosphoinositides.

A. Structure of phosphatidylinositol (PtdIns), depicting numbering of the inositol ring.

B. The lipid products generated by the 3'-phosphoinositide pathway are shown in yellow and the 3'-phosphoinositide pathway is depicted in red to differentiate from the phosphoinositides involved in the canonical PI turnover pathway shown in black.The enzymes thought to catalyse the different reactions are shown. The solid arrows indicate enzymes which phosphorylate the inositol ring, dashed line indicatephosphatase activities, examples of which are the type II 5'-phosphatases (Zhang *et al.*, 1995, Jackson *et al.*, 1995) and 3'-phosphatase (Woscholski *et al.*, 1995). Question marks indicate postulated pathways, for which enzyme activities have not yet been identified.
(PLC; phospholipase C, DAG; diacylglycerol)

vivo and its structure is represented in figure 1.1A. The relatively small amount of PtdIns found in membranes suggested that this lipid is unlikely to have a structural role in maintaining the lipid bilayer, a function that has been attributed to other phospholipids. The observation of rapid PtdIns phosphorylation following cellular stimulation indicated that the resulting phosphoinositides (PIs) may be implicated in mediating signal transduction events.

The metabolic map describing the relationship between PtdIns, PtdIns4P and PtdIns(4,5)P_2 in intact cells was defined in the early 60's by Ballou and co-workers (Brockerhoff and Ballou, 1962). In this canonical PI turnover pathway, PtdIns is sequentially phosphorylated on the D-4 and D-5 position of the myo-inositol ring (figure 1.1B) by PtdIns 4 kinase and PtdIns 4P 5-kinase respectively, to generate PtdIns(4,5)P_2, the major substrate of Phospholipase C (PLC). A number of different PtdIns 4-kinase and PtdIns 4P 5-kinase isoforms exist which are responsible for the biosynthesis of PtdIns(4,5)P_2 (Gehrmann and Heilmayer, 1998; Hsuan *et al.*, 1998). The rapid activation of PLC in response to ligand-receptor interaction results in the hydrolysis of PtdIns(4,5)P_2 by PLC and the generation of two second messengers, inositol 1,4,5-triphosphate (InsP_3) and diacylglycerol (DAG) (Downes and Macphee, 1990; James and Downes, 1997). InsP_3 promotes release of Ca^{2+} from intracellular stores (Berridge and Irvine, 1989) and DAG activates protein kinase C (PKC) (Nishizuka, 1995). Both the rise in intracellular Ca^{2+} and PKC activity have been demonstrated to be important steps in propagating signals from the cell surface to the nucleus (Berridge, 1993; Nishizuka, 1988).

1.2.2   3'-phosphoinositides as second messengers

For many years the canonical pathway of PI metabolism was thought to be the only one initiated in response to extracellular stimuli. However, subsequent studies showed that several extracellular agonists and growth factors, stimulated the rapid and transient formation of two novel, highly polar phosphoinositides. Both these phosphoinositides were phosphorylated on the D-3 position of the inositol ring. In response to extracellular stimuli PtdIns(3,4,5)P_3 was generated first, shortly followed by PtdIns(3,4)P_2 (Auger *et al.*, 1989b; Stephens *et al.*, 1991; Traynor Kaplan *et al.*, 1988). In contrast to PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2, the intracellular levels of PtdIns3P were largely unaltered upon cellular stimulation. PLC enzymes can hydrolyse PtdIns, PtdIns4P and PtdIns(4,5)P_2 (Lin *et al.*, 1990), however, the 3'-phosphorylated inositol lipids were not found to be substrates for hydrolysis by any known PLC and represented only 1%-2% of the total phosphatidylinositol polyphosphate pool (Lips *et al.*, 1989; Majerus *et al.*, 1990; Serunian *et al.*, 1989). As a result, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 were proposed to function directly as second messenger molecules, whereas the constitutive production of

PtdIns3P was proposed to play a more central, metabolic role (Fry, 1994; Stephens *et al.*, 1993b). Although 3'-phosphorylated lipids are not substrates for PLCs, their catabolism seems to occur through the action of inositol lipid phosphatases. Degradation of PtdIns(3,4,5)P_3 occurs mainly through 5-phosphatase activity, contributing to the corresponding delayed increase in PtdIns(3,4)P_2 observed in intact cells. The type II 5-phosphatase dephosphorylates inositol polyphosphates as well as PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 (Hansen *et al.*, 1987; Zhang *et al.*, 1995b). In addition, a 5-phosphatase was identified that selectively dephosphorylated PtdIns(3,4,5)P_3 (Jackson *et al.*, 1995). In contrast to PtdIns(3,4,5)P_3, PtdIns3P and PtdIns(3,4)P_2 appear to be metabolised mainly by a 3-phosphatase activity (Woscholski *et al.*, 1995). The speed with which agonist driven accumulations of PtdIns(3,4,5)P_3 can be cleared from cells, even in the continued presence of agonists (Jackson *et al.*, 1992; Traynor Kaplan *et al.*, 1989) further supported the notion that this phospholipid might represent a signalling molecule. The observed dephosphorylation of PtdIns(3,4,5)P_3 by a 5'-phosphatase however, also raised the possibility that this degradative reaction might also have a role in causing PtdIns(3,4)P_2 accumulation, contributing to the formation of another potential signalling lipid.

Studies on the PI specific kinase activities present in murine fibroblasts, led to the identification of at least two distinct PI kinase activities on the basis of their sensitivity to non-ionic detergents and inhibitors such as adenosine (Whitman *et al.*, 1987). These two PI kinase activities were termed type I (inhibited by non-ionic detergent and resistant to inhibition by adenosine) and type II (activated by detergents and inhibited by adenosine). The type I enzyme was found to be specifically associated with many oncogene products, such as polyoma middle T/pp60^{c-src} complex and growth factor receptors (Cantley *et al.*, 1991). The type II enzyme a PtdIns 4-kinase, catalysed the formation of PtdIns4P whilst the type I enzyme a PI 3-kinase, was found to catalyse the novel addition of phosphate at the D-3 position of the inositol ring using PtdIns as substrate *in vitro*, generating PtdIns3P (Whitman *et al.*, 1988). A similar lipid kinase activity which associated with the activated platelet derived growth factor (PDGF) receptor tyrosine kinase was found to also use PtdIns4P and PtdIns(4,5)P_2 to produce PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 respectively (Auger *et al.*, 1989b).

The identification of elevated levels of 3'-phosphorylated lipids by agonist stimulated cells and the discovery of PI 3-kinase activity in activated protein tyrosine kinase immunoprecipitates, implicated the 3'-phosphoinositide pathway as playing an important role in the transduction of mitogenic and oncogenic signals (Downes and Carter, 1991; Stephens *et al.*, 1993b). As a result, the identification of the lipid kinase(s) responsible for these signalling events attracted great interest, particularly if pharmaceutical

attenuation of the novel lipid kinase activity could inhibit mitogenesis and consequently control oncogenesis.

1.2.3 Characterisation of the first PI 3-kinase

PI 3-kinase was first identified as a lipid kinase activity associated with an 85 kDa phosphoprotein in immunoprecipitates from cells transformed with a number of polyoma middleT mutants (Courtneidge and Heber, 1987). These studies facilitated the purification of PI 3-kinase activity from bovine brain which corresponded to a heterodimeric complex (Morgan *et al.*, 1990). This complex was composed of an 85 kDa adaptor (p85) and a 110 kDa catalytic subunit (p110) (Hiles *et al.*, 1992; Otsu *et al.*, 1991). Cloning of the PI 3-kinase catalytic subunit and analysis of its amino acid sequence, revealed similarity within its catalytic domain to Vps34p, a yeast protein that was shown to be required for efficient vesicle trafficking and subsequently demonstrated to also possess PI 3-kinase activity (Herman and Emr, 1990; Schu *et al.*, 1993). The sequence alignment of the p110 and Vps34p lipid kinase domains allowed the use of polymerase chain reaction (PCR) based strategies, which combined with biochemical characterisation and protein purification approaches, have led to the isolation of novel PI 3-kinase family members (Domin and Waterfield, 1997; Vanhaesebroeck *et al.*, 1997a). These PI 3-kinase enzymes can be catagorised on the basis of their structure and probable mechanism of regulation. The following section describes the classification of the PI 3-kinase catalytic and adaptor subunits into three main classes (Figure 1.2):

1.3   Three classes of PI 3-kinase

1.3.1   Class I PI 3-kinases

These enzymes all form a heterodimeric complex with an adaptor protein which renders them responsive to ligand stimulation. *In vitro*, they can utilize PtdIns, PtdIns4P and PtdIns(4,5)P_2 as substrate (Irvine, 1992). *In vivo* however, the class I PI 3-kinases appear to preferentially phosphorylate PtdIns(4,5)P_2 since stimulation of these enzymes induces the generation of PtdIns(3,4,5)P_3 as the first 3'-phosphorylated lipid (Stephens *et al.*, 1993b). The reason for this discrepancy is presently unclear, although it could be due to the differences, *in vivo* and *in vitro* of substrate availability or lipid presentation. The class I PI 3-kinase can be subdivided based on the form of adaptor subunit with which the catalytic subunit associates.

1.3.1.1   Class I$_A$ PI 3-kinases

The class I$_A$ PI 3-kinases form a heterodimeric complex consisting of a 110-120 kDa catalytic subunit and an adaptor protein which contains two src homology 2 (SH2) domains and one src homology 3 (SH3) domain. Mammalian class I$_A$ catalytic subunits

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Class	Catalytic Subunit	Schematic Representation	Substrate Specificity	Adaptor/ Binding Partner	Regulation
I_A	p110α, β, δ (m) Dp110 (Dm) AGE-1 (Ce) PIK1 PIK2 (Dd)		PtdIns PtdIns4P PtdIns(4,5)P_2	p85α, β (m) p55α, γ (m) p50α (m) p60 (Dm)	Tyrosine kinases and Ras
I_B	p110γ (m) PI3K3 (Dd)			p101 (m)	G protein βγ subunits and Ras
II	PI3K-C2α/ Cpk-m/p170 (m) PI3K C2β (m) PI3K C2γ (m) PI3K_68D/cpk (Dm) PI3K-C2 (Ce)		PtdIns PtdIns4P (PtdIns(4,5)P_2)	Unknown	Unknown
III	Vps34p (Sc) PtdIns 3-kinase (m) (see figure 1.2B for other species specific homologues)		PtdIns	Vps15p (Sc)	Unknown

● p85 binding, ■ Ras binding, ▮ PIK domain, ⬭ Kinase domain, ⬮ C2 domain, ▮ Proline rich region

Figure 1.2A Classification of PI 3-kinase family members.
The assignment of catalytic subunits to a particular class is based on sequence homology within the catalytic domain (Zvelebil *et al*, 1996). Structural motifs (p85 binding site, Ras binding site, PIK domain, kinase domain, C2 domain and proline rich regions) are indicated. To date, PI3K-C2α is the only class II member which has been shown to phosphorylate PtdIns(4,5)P_2. Abbreviations used are: m, mammalian; Ce, *Caenorhabditis elegans* ; Dd, *Dictyostelium discoideum*; Dm, *Drosophila melanogaster* ; Sc, *Saccharomyces cerevisiae* .
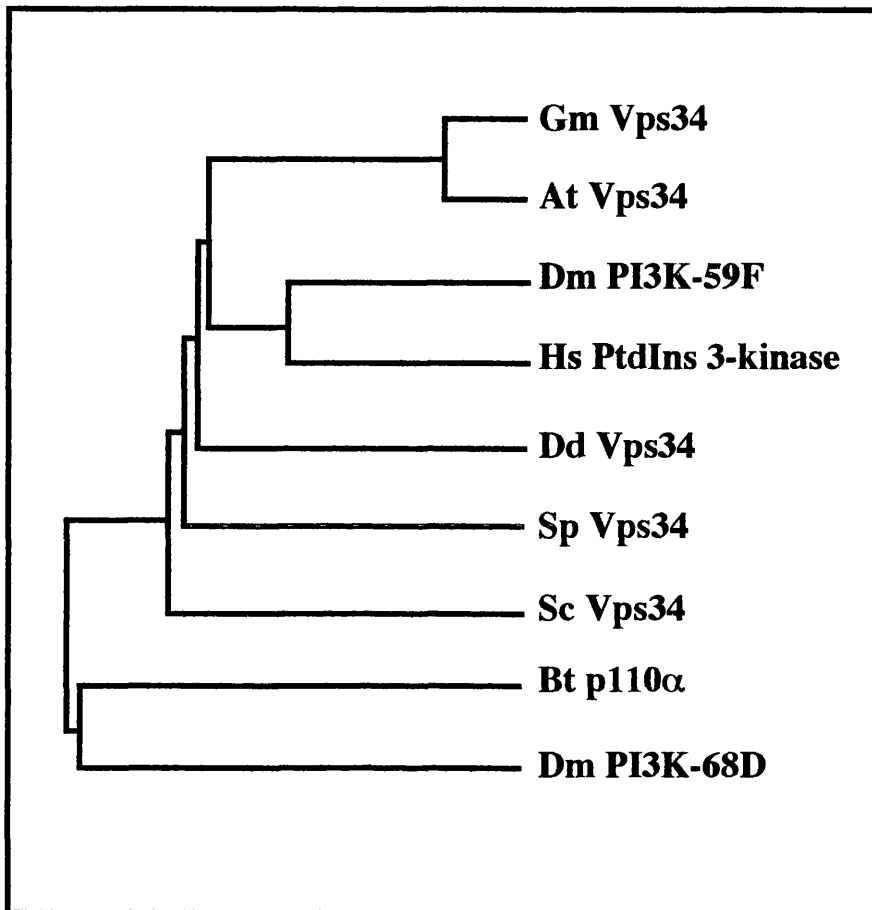
Figure 1.2B Dendogram to show evolutionary relationship between members of the class III PI 3-kinases.

Prototype members of class I (Bt p110α) and class II (DmPI3K-68D) are also shown. The dendogram was generated using the PILEUP program, UWGCG package (Devereux *et al*, 1984).
Abbreviations: Gm, *Glycine. max*; At,. *Arabidopsis thaliana*;
Dm, *Drosophila melanogaster*; Hs, *Homo sapiens* (man);
Dd, *Dictyostelium discoideum*; Sp, *Schizosaccharomyces pombe*;
Sc, *Saccharomyces cerevisiae*; Bt, *Bos taurus*.

include p110α, p110β and p110δ (Hiles *et al*, 1992; Hu *et al*, 1993; Vanhaesebroeck *et al*. 1997b). Both p110α and p110β demonstrate a ubiquitous tissue distribution, while the expression of p110δ is restricted to leukocytes thereby implying a specificity of function (Chantry *et al.*, 1997; Vanhaesebroeck *et al.*, 1997b). Homologous molecules have been identified from several other species including *Drosophila melanogaster* (Dm p110), *Dictyostelium discoideum* (PIK1, PIK2) and *Caenorhabditis elegans* (AGE-1) (Leevers *et al.*, 1996; Morris *et al.*, 1996; Zhou *et al.*, 1995). All these catalytic subunits have an N-terminal region which contains the interaction sites for the p85 adaptor subunit and the small GTP binding protein Ras (Dhand *et al.*, 1994a; Rodriguez Viciana *et al.*, 1996). The C-terminus contains a PI kinase (PIK) domain and the 'core' catalytic domain. The PIK domain is a region conserved amongst all PI 3-and PI 4-kinases and although its role is presently unclear, it is likely to be involved in substrate presentation (Flanagan *et al.*, 1993).

All members of the class I_A catalytic subunits bind the p85 adaptor subunit through which they can be recruited to receptor signalling complexes upon stimulation of tyrosine kinase activity (Domin *et al.*, 1996). The first adaptors to be identified were p85α and β (Otsu *et al.*, 1991). Both proteins contain two SH2 domains separated by an inter-SH2 domain through which the adaptor interacts with p110 (Dhand *et al.*, 1994a; Klippel *et al.*, 1993). The p85 SH2 domains bind phosphorylated tyrosine residues within a specific sequence context of pYXXM (Fantl *et al.*, 1992; Felder *et al.*, 1993; Songyang *et al.*, 1993). Consequently, growth factor receptors or other molecules can become binding partners for p85 upon their phosphorylation by intrinsic or associated tyrosine kinase activity. This event is postulated to bring the PI 3-kinase to the vicinity of its lipid substrate in the plasma membrane and is considered to be a major regulatory mechanism of lipid kinase activity (Domin and Waterfield, 1997; Fry, 1994)

At the N-terminus of p85 there is a src homology 3 (SH3) domain (Gout *et al.*, 1993) followed by a break point cluster region (BCR) which has homology with the GAP (GTPase activating protein) domain of the break point cluster region gene product (Musacchio *et al.*, 1996). Although BCR domains have been identified in a number of proteins which display GAP activity towards the Rho family GTPases, no equivalent biochemical activity has, as yet, been found for the BCR domain of p85. Two proline rich regions flank the BCR domain of p85. Since SH3 domains can bind such regions, the p85 SH3 domain may interact with either of these polyproline motifs in an intramolecular manner, to confer an allosteric change in the adaptor (Kapeller *et al.*, 1994). Such conformational change, perhaps upon receptor binding, might modulate the kinase activity of the catalytic subunit. Alternatively, the polyproline motifs may have a

role in facilitating intermolecular interactions between other SH3 domain containing molecules including other p85 molecules.

A number of smaller adaptor proteins have been identified which also bind the class I_A catalytic subunits. These molecules are homologous to p85 but lack its SH3 domain, one of the two proline rich regions and the BCR domain. Although some are generated by alternate splicing of the p85α gene, mouse p55^{PIK} and its bovine homologue p55γ (p85γ) are encoded by independent genes (Antonetti *et al.*, 1996; Pons *et al.*, 1995)

In addition to lipid kinase activity, class I_A PI 3-kinases also possess an intrinsic ser/thr protein kinase activity (Carpenter *et al.*, 1993; Dhand *et al.*, 1994b). This serves to phosphorylate the associated adaptor within the inter-SH2 domain of p85 in the case of p110α and p110β (Dhand *et al.*, 1994b), or mediates autophosphorylation in the case of p110δ (Vanhaesebroeck *et al.*, 1997b). Although such protein kinase activity might also participate in mediating intracellular signalling events, the insulin receptor protein tyrosine kinase substate IRS-1 is the only exogenous protein reportedly phosphorylated by p110α (Lam *et al.*, 1994). Phosphorylation of p85α Ser^{608} which is located within its inter-SH2 domain has been proposed to regulate the lipid kinase activity of the catalytic subunit. Phosphorylation of p85α by p110α has been shown to attenuate lipid kinase activity *in vitro* (Dhand *et al.*, 1994b). The biological significance of the shortened p85 isoforms (p55^{PIK}, p55γ) is at present unclear. Although they do not display selectivity in binding the catalytic subunit, these adaptors however, do show a tissue specific distribution. It is therefore possible that their structural modifications may be of regulatory significance. Interestingly, many of the smaller adaptor subunits lack a residue equivalent to p85 Ser^{608} which might contribute some functional importance. In a recent study, a 65 kDa mutant of the p85α regulatory subunit was isolated which included the initial 571 residues of the wild type p85α protein linked to a region conserved in the eph tyrosine kinase family (Jimenez *et al.*, 1998). This mutated protein was obtained from a transformed cell line and it induces the constitutive activation of the p110 PI 3-kinase which contributes to cellular transformation. These data further implicate the PI 3-kinase enzyme in mammalian tumour development (Jimenez *et al.*, 1998) and highlight the importance of the adaptor subunit in regulating the activity of the catalytic subunit.

1.3.1.2   Class I_B PI 3-kinases

Studies using platelets and neutrophils, have resulted in the identification of a form of PI 3-kinase which can act downstream of receptors which signal through heterotrimeric G-proteins (Kucera and Rittenhouse, 1990; Stephens *et al.*, 1993a; Tang and Downes, 1997). Fractionation of neutrophil and U937 cell lysates resulted in the isolation of a PtdIns(4,5)P_2 selective kinase activity which was chromatographically distinct from the

class I_A enzymes (Stephens *et al.*, 1994b). This PI 3-kinase complex was found to be activated by heterodimeric GTPase $\beta\gamma$ subunits (G$\beta\gamma$), possessed a native molecular mass of 220 kDa and did not contain a p85 like adaptor subunit. The subsequent cloning of p110γ was used to define a PI 3-kinase catalytic subunit which could be activated by both the Gα and G$\beta\gamma$ subunits of heterotrimeric G proteins (Stoyanov *et al.*, 1995), whereas the G-protein activated PI 3-kinase purified from myeloid cells was found to be activated by G$\beta\gamma$ subunits alone (Stephens *et al.*, 1994b). This PI 3-kinase was then cloned from porcine neutrophils, and expressed as a 120 kDa protein highly related to p110γ. In addition a 97 kDa adaptor termed p101 (based on electrophoretic mobility) was isolated and cloned (Stephens *et al.*, 1997). The binding of p110γ to the p101 adaptor, rendered the enzyme considerably more sensitive to activation by G$\beta\gamma$ subunits. Transient expression of the p101/p110γ heterodimer together with G$\beta\gamma$ subunits in COS-7 cells resulted in a 25-fold increase in PtdIns(3,4,5)P_2 levels (Stephens *et al.*, 1997). In addition, p101/p110γ was also shown to be regulated by cell surface receptors that regulate intracellular signalling pathways. Lysophosphatidic acid (LPA) which is known to activate G-protein linked cellular responses was added to COS-7 cells transiently expressing p101/p110γ. This resulted in a stimulated PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 accumulation (Stephens *et al.*, 1997). These data indicate that the p110γ PI 3-kinase in complex with p101 can contribute to the ability of heterotrimeric G protein activated receptors to stimulate PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 accumulation.

1.3.1.3 Regulation of Class I PI 3-kinase activity

Most of what is known about PI 3-kinase activation and regulation has been gained from the study of the class I molecules. The reason for this is mainly chronological since the p110 catalytic subunit was the first PI 3-kinase to be purified and cloned (Hiles *et al.*, 1992). The class I PI 3-kinases have been demonstrated to play a role in numerous receptor mediated signalling events (Fry, 1994). Ligand stimulation results in either the activation of intrinsic receptor tyrosine kinase activity (e.g the PDGF or EGF receptors), the recruitment of an associated tyrosine kinase (e.g the src-like kinases) or the activation of heterotrimeric G proteins.

When tyrosine residues become phosphorylated and are presented within a pYXXM consensus sequence they serve as docking sites for the class I_A adaptor subunit SH2 domains (Kapeller and Cantley, 1994). This occurs upon ligand stimulation which promotes tyrosine phosphorylation by activated kinases. As a result, the p85 SH2 domains bind the pYXXM sites of receptor or associated tyrosine kinases, in addition to tyrosine phosphorylated cytosolic proteins such as the insulin receptor substrates IRS-1 and IRS-2 (Sun *et al.*, 1993; Yenush and White, 1997). This adaptor mediated translocation of PI 3-kinase to receptor tyrosine kinases and their substrates positions the

p110 catalytic subunit close to membranes that contain its lipid substrate. Furthermore, phosphopeptide binding to the p85 adaptor stimulates the associated p110 lipid kinase activity (Rordorf Nikolic et al., 1995). The SH2 domains of p85 have also been shown to specifically bind PtdIns(3,4,5)P_3 (Rameh et al., 1995). The in vivo relevance of this result is supported by the observation that inhibition of PtdIns(3,4,5)P_3 production results in enhanced association of the p85 subunit of PI 3-kinase with tyrosine-phosphorylated insulin receptor and IRS-1. These results suggested that PtdIns(3,4,5)P_3, a product of PI 3-kinase activity, bound directly to the SH2 domains of p85 and caused dissociation of the p85/p110 complex from tyrosine-phosphorylated proteins (Toker and Cantley, 1997). This may represent one way in which PI 3-kinase activity could be down regulated in a negative feedback loop.

The class I_A PI 3-kinases also interact with Ras in a GTP dependent manner, so far this interaction has only been described in detail for the p85α/p110α heterodimer. In vitro, incubation of GTP-Ras with p85α/p110α results in a modest increase in PI 3-kinase activity (Rodriguez Viciana et al., 1996). Co-expression studies of p85α/p110α with various Ras mutants indicated that Ras can regulate p85α/p110α lipid kinase activity in vivo suggesting that activation of the p85α/p110α PI 3-kinase occurs downstream of Ras activation (Marte et al., 1997; Rodriguez Viciana et al., 1994). Evidence from experiments using PDGF receptor mutants also suggests that accumulation of GTP bound Ras is required for full activation of class I_A PI 3-kinases by PDGF (Klinghoffer et al., 1996). The interaction of Ras with p110α might result in the allosteric activation and/or contribute to the recruitment of p110α to the plasma membrane. Taken together, these data indicate that PI 3-kinases might be another class of Ras effector molecules alongside proteins such as the Raf ser/thr kinases (Marshall, 1996) raising the possibility that the mitogenic and transforming actions of Ras are mediated through both the mitogen activated protein (MAP) kinase pathway (Cano and Mahadevan, 1995) and PI 3-kinase. A constitutively active mutant of p110α has been shown to stimulate Ras-dependent Xenopus oocyte maturation and fos transcription, suggesting that PI 3-kinase activation precedes Ras activation in this system (Hu et al., 1995). The activation of the p85α/p110α PI 3-kinase complex upstream of Ras in a Gβγ-mediated MAP kinase signalling pathway has also been reported (Hawes et al., 1996), consequently, whether PI 3-kinase functions upstream or downstream of Ras requires further clarification.

At present, it is unclear how heterotrimeric G-proteins activate the class I_B PI 3-kinase. Whilst the Gβγ subunits of heterotrimeric G-proteins can directly stimulate p110γ lipid kinase activity, this stimulation is considerably enhanced in the presence of the p101 adaptor (Stephens et al., 1997). The p110γ catalytic subunit contains an N-terminal pleckstrin homology (PH) domain (section 1.3.1.4.2) (Zvelebil et al., 1996). Since the

PH domains of Gβγ-regulated proteins such as β-adrenergic receptor kinase have been identified to bind Gβγ (Gaudet *et al.*, 1996; Touhara *et al.*, 1994), it has been speculated that the PH domain of p110γ mediates a Gβγ activation of lipid kinase activity (Leopoldt *et al.*, 1998). Using monoclonal antibodies that blocked the PH domain of p110γ, Leopoldt *et al*, have shown that the PH domain was not required for Gβγ-mediated stimulation (Leopoldt *et al.*, 1998). Furthermore, results obtained with deletion mutants of 110γ indicated that Gβγ bound to an N-terminal region as well as to a region near or within the C-terminal catalytic domain of p110γ. N-and C-terminal stretches of p110γ were therefore suggested to form a common Gβγ effector region (Leopoldt *et al.*, 1998).

The activation of lipid kinase activity by Gβγ is not restricted to the class I$_B$ PI 3-kinases. The p85-associated class I$_A$ PI 3-kinase, p110β, has been shown to be activated by Gβγ subunits (Kurosu *et al.*, 1997). It has also been demonstated that this PI 3-kinase catalytic subunit (and possibly other class I$_A$ members) can be synergistically activated by both Gβγ and phosphotyrosine containing peptides derived from growth factor receptors or IRS-1 (Kurosu *et al.*, 1997; Tang and Downes, 1997). This may represent a mechanism by which the effects of tyrosine kinase and G-protein-linked receptors might be co-ordinated. In addition, the class I$_B$ PI 3-kinase, p110γ, has been shown to mediate Gβγ dependent regulation of MAPK and stress activated Jun kinase (JNK) signalling pathways (Lopez Ilasaca *et al.*, 1997; Lopez Ilasaca *et al.*, 1998), suggesting that p110γ acts as an intermediate connecting G protein-coupled receptors to MAPK/JNK cascades.

1.3.1.4   Downstream targets attributed to class I PI 3-kinases

It is generally thought that PI 3-kinase lipid products interact with certain proteins and modulate their localisation and/or activity. For many of these proteins, the interacting lipids are mainly PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2. Although both phospholipids can be produced by the class I PI 3-kinases, PtdIns(3,4)P_2 can also be generated by the more recently characterised class II PI 3-kinases *in vitro* (section 1.3.2). Consequently, more work is required to correlate particular PI 3-kinase isoforms with specific downstream signalling events.

1.3.1.4.1   p70^{S6} kinase

p70^{S6} kinase becomes activated upon mitogenic stimuli and plays an important role in the progression of cells from G1 to S phase of the cell cycle. It phosphorylates the 40S ribosomal protein S6, during mitogenic responses but might also be involved in the regulation of other cellular processes (Proud, 1996). The role of S6 phosphorylation is still not fully understood, but correlates with an increase in translation, probably from specific mRNAs encoding proteins essential for G1 transition (Brown and Schreiber, 1996). Although activation of p70^{S6} kinase is achieved by a ser/thr protein kinase

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activity independent of the Raf/MAPK pathway (Ferrari *et al.*, 1992), evidence suggests that p70^{S6} kinase is an important downstream target of PI 3-kinase. Overexpression of receptor mutants which fail to bind the p85 adaptor and use of the PI 3-kinase inhibitors wortmannin, a fungal metabolite (Arcaro and Wymann, 1993) and LY294002, a bioflavinoid quercetin analogue (Vlahos *et al.*, 1994) both block ligand induced activation of p70^{S6} kinase (Chung *et al.*, 1994). Furthermore, constitutive PI 3-kinase activity results in the phosphorylation of p70^{S6} kinase and antisera which target p70^{S6} kinase and block the mitogenic activity of PI 3-kinase (McIlroy *et al.*, 1997; Weng *et al.*, 1995).

1.3.1.4.2 Akt/PKB kinase

The Akt ser/thr kinase [also termed protein kinase B (PKB)] is the cellular homologue of the retroviral oncogene v-akt and is activated upon ligand stimulation of receptor tyrosine kinases (Bellacosa *et al.*, 1991; Burgering and Coffer, 1995). Potential targets of Akt include glycogen synthase kinase-3 (GSK3), and p70^{S6} kinase (Burgering and Coffer, 1995; Cross *et al.*, 1995a). Akt has also been demonstrated to phosphorylate the BCL-2 family member BAD, resulting in a suppression of apoptosis and promoting cell survival (Datta *et al.*, 1997). The Akt protein contains an N-terminal pleckstrin homology (PH) domain (Lemmon *et al.*, 1996) in addition to the protein kinase domain.

The PH domain was originally identified as an internal repeat in the phosphoprotein pelckstrin (Haslam *et al.*, 1993). The PH domain is present in a large number of proteins involved in cellular signalling and can mediate either protein-protein or protein-lipid interactions (Lemmon *et al.*, 1996). Several PH-domain-containing proteins including pleckstrin, PLCδ1, dynamin and son of sevenless (Sos) have been shown to bind to PtdIns(4,5)P_2 and/or Ins(1,4,5)P_3 directly (Chen *et al.*, 1997a; Lemmon *et al.*, 1995; Rameh *et al.*, 1997a; Toker and Cantley, 1997). PH domains have also been demonstrated to bind 3'-phosphorylated lipids. The PH domain of Bruton's tyrosine kinase has a high affinity for PtdIns(3,4,5)P_3 (Salim *et al.*, 1996). In addition the PH domain of PLCγ also binds PtdIns(3,4,5)P_3 and is targeted to the plasma membrane on PI 3-kinase activation (Falasca *et al.*, 1998).

The PH domain of Akt has been shown to bind PtdIns(3,4,)P_2 and PtdIns(3,4,5)P_3 *in vitro* (James *et al.*, 1996). The binding of these lipids does not activate Akt protein kinase activity directly (James *et al.*, 1996). Instead, Akt is phosphorylated and activated by an upstream kinase only when PtdIns(3,4,5)P_3 is bound. This suggested that the PH domain of Akt restricts access to the Akt phosphorylation site and that this was relieved upon binding of PtdIns(3,4,5)P_3, allowing the phosphorylation event to occur (Stokoe *et al.*, 1997). Akt phosphorylation is achieved by the 3'-phosphoinositide-dependent protein kinase 1 (PDK1) and the as yet uncharacterised PDK2 (Alessi *et al.*, 1997).

PDK1 has been cloned (Stephens *et al.*, 1998) and the encoded protein, like Akt, contains a PH and protein kinase domain. PDK1 binds PtdIns(3,4,)P_2 and PtdIns(3,4,5)P_3 *in vitro* and PtdIns(3,4,5)P_3 binding stimulates PDK1 protein kinase activity, which allows phosphorylation of Thr^{308} of Akt (Alessi *et al.*, 1997). This phosphorylation contributes to the activation of Akt protein kinase activity. A model for Akt activation (figure 1.3) suggests that Akt binds PtdIns(3,4)P_2 or PtdIns(3,4,5)P_3, resulting in the translocation of Akt to the plasma membrane (Stephens *et al.*, 1998). The binding of PtdIns(3,4,5)P_3 to the PH domain of Akt exposes Thr^{308} for phosphorylation by PDK1 which is also activated by PtdIns(3,4,5)P_3. Ser^{473} of Akt is probably phosphorylated by PDK2 which might also be regulated by PtdIns(3,4,5)P_3. This results in a fully activated Akt protein kinase.

The dependence of Akt phosphorylation and activation upon 3'-phosphoinositides demonstrates a requirement for PI 3-kinase activity. The expression of PDGF receptor mutants which do not bind the SH2 domains of p85 fail to stimulate both PI 3-kinase and Akt activity (Burgering and Coffer, 1995; Franke *et al.*, 1995). Similarly, growth factor induced activation of Akt is inhibited by the PI 3-kinase inhibitor wortmannin and also by the expression of a dominant-negative form of PI 3-kinase, Δp85, in which 35 amino acids from the region responsible for binding the p110 catalytic subunit (the inter SH2 domain) has been deleted from the p85 adaptor (Franke *et al.*, 1995). These results have been used to show that the class I_A PI 3-kinases may be implicated in Akt signalling, however the observation that the transient expression of the p101/p110γ complex in COS-7 results in an LPA-stimulated activation of Akt (Stephens *et al.*, 1997) suggests that class I_B PI 3-kinases may also have a role in Akt mediated signalling events.

1.3.1.4.3 Rac

The Ras related GTP binding protein Rac can be activated by agonists (such as PDGF and insulin), leading to the assembly of a meshwork of actin filaments at the cell periphery to produce lamellipodia and membrane ruffles. In this way, Rac GTPase is able to link surface receptors to the re-organisation of the actin cytoskeleton. Studies have shown that activated PI 3-kinase increases the level of the GTP-bound, active form of Rac, which in turn induces the changes in the actin cytoskeleton responsible for membrane ruffling (Hawkins *et al.*, 1995). However, PI 3-kinase is unable to induce the Rac signalling pathways that regulate gene transcription from the serum response element (SRE) and Rac-mediated stress-activated protein kinase (JNK) activation (Reif *et al.*, 1996). Hence, PI 3-kinase signals can induce Rac-mediated cytoskeletal changes without activating Rac-mediated transcription factor pathways. Rac has also been shown to interact with p85 *in vitro* (Bokoch *et al.*, 1996) and the dominant negative form of p85
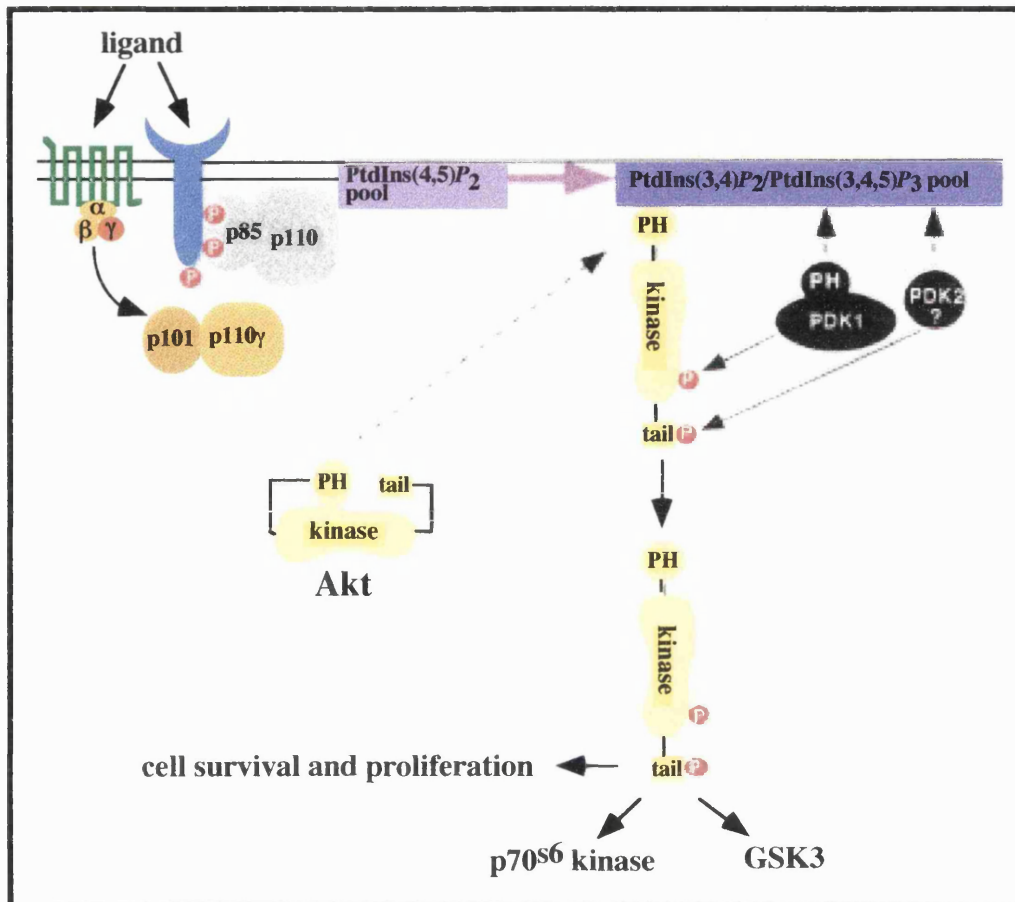
Figure 1.3 **A proposed model for Akt activation**

Stimulation of growth-factor receptor tyrosine kinases or possibly heterotrimeric G-protein linked receptors results in the activation of class I_A (p85/p110) or class I_B (p101/p110γ) PI 3-kinases, leading to the production of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3. PtdIns(3,4,5)P_3 binds to the PH domain of Akt, forcing its translocation to the plasma membrane (broken arrow), enabling its phosphorylation on Thr[308] by PDK 1. Full activation of Akt requires the phosphorylation of Ser[473] by the as yet uncloned PDK 2. Membrane localisation and activity of PDK 1, and perhaps PDK 2 are probably also regulated by binding to the 3'-phosphoinositides generated by the activation of class I PI 3-kinases. After its phosphorylation, the activated Akt can phosphorylate specific intracellular targets resulting in a variety of biological effects including supressing apoptosis and up-regulating protein synthesis and glycogen synthesis.

(Δp85) was found to block membrane ruffling that would normally have been induced by PDGF stimulation (Hawkins *et al.*, 1995). This suggests that the class I_A PI 3-kinases are most likely responsible for Rac induced actin reorganization.

1.3.1.4.4 Protein kinase C and related kinases

Several studies have provided evidence that some PKCs can be activated by PtdIns(3,4,5)P_3 and PtdIns(3,4,)P_2 (Palmer *et al.*, 1995; Toker *et al.*, 1994). The family of related PKCs are subdivided according to cofactor requirements and dependence on lipid activators (Akimoto *et al.*, 1996). Synthetic phosphoinositides, particularly PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2, have been used by laboratories to demonstrate selective activation of PKC isoforms particularly PKC-ϵ, PKC-η (Toker *et al.*, 1994) and atypical PKC-ζ (Nakanishi *et al.*, 1993) as well as the PKC-related kinase PRK1 (Palmer *et al.*, 1995). However, the results show that activation of PKCs by phosphoinositides *in vitro* is highly dependent on the assay conditions used. Studies of PKC-λ have provided *in vivo* evidence supporting the presence of a signalling pathway from receptor tyrosine kinases to PKC-λ through the p110α PI 3-kinase (Akimoto *et al.*, 1996). PKC-ϵ has also been shown to be activated downstream of PI 3-kinase in stimulated cells (Moriya *et al.*, 1996). At present the physiological relevance of activating PKC downstream of PI 3-kinase is unknown.

1.3.1.5 Biochemical processes implicating class I PI 3-kinase activity

1.3.1.5.1 Cell survival

Studies investigating the nerve growth factor (NGF) mediated survival of rat pheochromocytoma (PC)12 cells, have shown that the ability of NGF to prevent apoptosis (programmed cell death) can be inhibited by both wortmannin and LY294002. In addition, PDGF prevented apoptosis of PC12 cells expressing the wild type PDGF receptor, but not of cells expressing a mutant receptor that failed to activate PI 3-kinase (Yao and Cooper, 1995). These results suggest that cell survival appeared to be mediated by a PI 3-kinase dependent signalling pathway.

Further investigations have shown that PI 3-kinase can also meditate type 1 insulin like growth factor (IGF-1) dependent survival of Rat-1 and COS-7 cells (Dudek *et al.*, 1997). A role for Akt/PKB (section 1.3.1.4.2) in such IGF-1 mediated cell survival has also been indicated since overexpression of Akt prevents apoptosis in cells that have had PI 3-kinase activity inhibited (Kulik *et al.*, 1997). Through the use of Ras mutants that selectively stimulate PI 3-kinase and Akt but not the Raf/MAPK, Kauffmann Zeh *et al*,

were are able to prevent *c-myc* induced cell death in Rat-1 cells (Kauffmann Zeh *et al.*, 1997).

Taken together, the results from these studies suggest that there is a cell survival pathway that involves Ras-dependent stimulation of PI 3-kinase, which activates Akt independently of MAPK and p70^{S6} kinase. The exact mechanisms that lead to the prevention of apoptosis through PI 3-kinase stimulated Akt are at present undetermined. In addition, an element of confusion has arisen with a recent report from Yin *et al*. 1998 In this investigation, the PI 3-kinase adaptor subunit, p85, is shown to be a protein that participates in the cell death process. This apoptotic response is induced by oxidative stress and the role of p85 in this event does not seem to involve a catalytic PI 3-kinase subunit (Yin *et al.*, 1998). These results have been used to suggest that, although the lipid kinase activity of PI 3-kinase has been implicated in cell survival, p85 may also function independently of a p85/p110 PI 3-kinase complex in a p53-mediated apoptotic response.

1.3.1.5.2   Ageing

A *C.elegans* gene termed *age-1* or *daf-23* which is thought to encode a class I$_A$ PI 3-kinase, was identified in a genetic screen for mutants that promote longevity (*age* mutants) and for mutants that affect dauer larvae formation (*daf* mutants) (Klass *et al.*, 1983; Riddle and Alberts, 1997). Under uncrowded conditions with ample food, wild type *C.elegans* develop rapidly through four larval stages to become adult worms with a life span of 2-3 weeks. In contrast, when food is scarce and the population density is high an alternative third stage larvae (L3), the dauer larve is formed. Dauer larvae are developmentally arrested. When favourable conditions return, dauers recover and develop into adults with a normal lifespan (Vanhaesebroeck *et al.*, 1997a)

Null mutations in the *age-1* putative PI 3-kinase result in *C.elegans* that form dauer larvae constitutively, suggesting that *age-1* normally supresses dauer formation under favourable growth conditions. Alternatively, *age-1* may be required for L3 development such that in the absence of *age-1* activity, dauer larva development occurs by default. *C.elegans* with reduced levels of the *age-1* PI 3-kinase do not form dauer larvae, but develop into adults with significantly extended lifespans (Kenyon, 1996). The analysis of genetic interaction between *age-1* and various *daf* mutants has facilitated ordering of their putative gene products into a complex pathway affecting dauer formation and lifespan. Characterisation of these genes at the molecular level should clarify the way in which the *age-1* putative PI 3-kinase functions as a signalling molecule implicated in life span determination (Riddle and Alberts, 1997).

1.3.2 Class II PI 3-kinases

The catalytic subunits of the class II enzymes are the largest (170-220 kDa) and the most recently identified form of PI 3-kinase. Distinctively, they all contain a C-terminal C2 domain (figure 1.2A). The C2 domain was originally defined as the second of four conserved regions within mammalian PKC where it conferred Ca^{2+} sensitive phospholipid binding (Kaibuchi *et al.*, 1989). This property however, is not displayed by all C2 domains since only the first (C2A) but not the second (C2B) of two C2 domains within the synaptic vesicle protein synaptotagmin behaves in this manner (Li *et al.*, 1995a). In contrast, the synaptotagmin C2B domain mediates both a Ca^{2+} dependent dimerisation of this protein (Zhang *et al.*, 1994) and a Ca^{2+} independent association with the clathrin AP-2 complex (section 1.5.4.3.2)

The first class II PI 3-kinase, PI3K 68_D, was characterised in *Drosophila melanogaster* (MacDougall *et al.*, 1995). Subsequently two murine enzymes m-cpk and p170 (Molz *et al.*, 1996; Virbasius *et al.*, 1996) and two human enzymes PI3K-C2α and HsC2-PI3K (also known as PI3K-C2β) (Brown *et al.*, 1997; Domin *et al.*, 1997) were characterised. The two mouse proteins are both likely to be products of the same gene and are the murine homologue of PI3K-C2α. A sixth member of the class II PI 3-kinases has been cloned from the cDNA library of regenerating rat liver and is termed PI3K-IIγ (Ono *et al.*, 1998). This class II isoform has a liver specific tissue distribution. The increased expression of PI3K-IIγ during liver regeneration suggests that this PI 3-kinase may function mainly in highly differentiated hepatic cells (Ono *et al.*, 1998). Examination of lipid substrate specificity for the class II enzymes showed that PI3K_68D, p170 and PI3K-IIγ phosphorylate only PtdIns and PtdIns4P *in vitro* (MacDougall *et al.*, 1995; Virbasius *et al.*, 1996). This offered the attractive possibility that structural differences between members of each class of PI 3-kinase might reflect the type of 3' phosphorylated phosphoinositide they produce. This hypothesis was revised when the lipid substrate specificity of human PI3K-C2α was determined. In the presence of phosphatidylserine, PI3K-C2α also phosphorylated PtdIns(4,5)P_2, albeit at a relatively low level compared to PtdIns and PtdIns4P (Domin *et al.*, 1997). Although it is unclear whether this is a property displayed by other class II enzymes, it highlights possible differences due to *in vitro* lipid substrate presentation with consequences for *in vivo* substrate preference. As a result, since PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 can be generated by PI3K-C2α *in vitro*, it is possible that this enzyme could be implicated in the activation of Akt/PKB (section 1.3.1.4.2) or other proteins which are downstream targets of these 3' phosphorylated lipids.

The members of the class II PI 3-kinase family can also be distinguished from other PI 3-kinases by their response to PI 3-kinase inhibitors. PI3K-C2α and murine p170 are

refractory to both wortmannin (IC$_{50}$ 170-200nM) and LY294002 (Domin *et al.*, 1997; Virbasius *et al.*, 1996). Experiments examining the effect of wortmannin on cellular lipid levels showed that after 10 minutes of incubation with the drug, levels of PtdIns3P decreased by 70% and PtdIns(3,4,5)P_3 was undetectable. Interestingly, the levels of PtdIns(3,4)P_2 did not decrease in response to wortmannin (Shpetner *et al.*, 1996). These results suggested that under conditions of exponential growth, 30% of cellular PtdIns3P and most of the cellular pool of PtdIns(3,4)P_2 were generated by a wortmannin-insensitive PI 3-kinase. It is possible that PI3K-C2α and murine p170 may be candidates for such wortmannin-insensitve PI 3-kinase activity. In contrast, *Drosophila* PI3K 68_D, like the class I PI 3-kinases, is sensitive to the PI 3-kinase inhibitors wortmannin (IC$_{50}$ 2-5nM) and LY294002 (MacDougall *et al.*, 1995).

The C2 domain of PI3K_68D binds phospholipids in a Ca^{2+}-independent manner, similar to the synaptotagmin C2B domain. At present however, the function of the C2 domain within the class II PI 3-kinase family members remains unclear (MacDougall *et al.*, 1995). Currently there is no indication that the C2 domain confers a Ca^{2+} sensitivity to this class of enzyme. Nevertheless the phospholipid binding capacity of this domain may allow it to function in a manner similar to the PH domain, whereby the C2 domain provides a membrane binding function. This may facilitate membrane localisation for the class II PI 3-kinases for which an adaptor molecule remains elusive.

1.3.3 Class III PI 3-kinases

Class III PI 3-kinases have a substrate specificity restricted to PtdIns and are consequently known as PtdIns 3-kinases. The first class III PtdIns 3-kinase identified was the *Saccharomyces cerevisiae* protein Vps34p (Schu *et al.*, 1993; Stack and Emr, 1994). Since Vps34p is the only PtdIns 3-kinase present in yeast, members of this class are considered to be the earliest evolutionary form of PI 3-kinase. The gene encoding Vps34p was isolated using a genetic screen which identified vacuolar protein sorting (*VPS*) mutants (section 1.5.2) (Stack *et al.*, 1995c). Vps34p and its PtdIns 3-kinase activity were identified as essential in the trafficking of newly formed soluble hydrolases from the Golgi to the vacuole, the functional equivalent of the mammalian lysosome (Stack *et al.*, 1995a). Vps34p is found in complex with Vps15p, a 170 kDa ser/thr kinase which both activates and recruits Vps34p to intracellular membranes (Stack and Emr, 1994; Stack *et al.*, 1993). It is postulated that the Vps34p PtdIns3P product fulfils a housekeeping role in constitutive membrane trafficking and vesicle morphogenesis (De Camilli *et al.*, 1996; Stack *et al.*, 1995c). To date, Vps34p homologues (figure 1.2B) have been found in a number of eukaryotes including *Drosophila*, soybean, *Arabidopsis* and *Dictyostelium* (Hong and Verma, 1994; Linassier *et al.*, 1997; Welters *et al.*, 1994; Zhou *et al.*, 1995). The biochemical characterisation of a PtdIns 3-kinase activity in

mammalian cells (Stephens *et al.*, 1994a) and the cloning of a human PtdIns specific lipid kinase (Volinia *et al.*, 1995), suggested that this Vps34p homologue could have an evolutionarily conserved function in mammalian membrane trafficking. The function of yeast Vps34p and the human PtdIns 3-kinase are discussed in further detail in sections 1.5.3.1 and 1.7.1.3

1.4 Related PI 3-kinases

The PI 3-kinase related proteins are generally large (> 200 kDa) and contain a C-terminal region homologous to the PI 3-kinase catalytic domain (Zvelebil *et al.*, 1996). They include the ataxia telangiectasia-mutated gene product (ATM), the targets of rapamycin (TOR) and the DNA-dependent protein kinase (DNA-PK).

Ataxia telangiectasia is an inherited, autosomal recessive disorder that is characterised by cerebellar degeneration, immunodeficiency, cancer predisposition and radiation sensitivity. Cloning of the ATM gene was used to identify a 5.9 kb cDNA clone that encoded a putative protein of 200 kDa (Savitsky *et al.*, 1995). The ATM coding sequence was shown to contain a 400 amino acid C-terminal region that had 40% identity with the lipid kinase domain of the p110α PI 3-kinase. Although containing sequence similarity at the amino acid level to PI 3-kinase, ATM has not been shown to have a lipid kinase activity.

The yeast TOR1 and TOR2 proteins (Helliwell *et al.*, 1994) and their mammalian counterparts FRAP (Brown *et al.*, 1994) and RAFT (Sabatini *et al.*, 1994) are the targets of the immunosuppressant-immunophilin complex rapamycin-FKBP. TOR1 and TOR2 are 281 kDa proteins that control translation initiation and cell cycle G$_1$-phase progression in response to nutrients (Barbet *et al.*, 1996; Di Como and Arndt, 1996). The mammalian TOR proteins (RAFT and FRAP) have been implicated in a mitogen stimulated pathway that leads to activation of p70^{S6} kinase. Both the yeast and mammalian TOR protein contain a C-terminal region that is homologous to the lipid kinase domain of PI 3-kinase. However, no lipid kinase activity has been attributed to the TOR proteins, instead the TOR proteins demonstrate an intrinsic protein/autokinase activity (Brown *et al.*, 1995a).

DNA-PK is involved in DNA double-stranded break repair and comprises a DNA-targeting component called Ku and a 460 kDa catalytic subunit DNA-PK (Hartley *et al.*, 1995). Like ATM and the TOR proteins the C-terminal region of DNA-PK contains a region of amino acid sequence similarity with the lipid kinase domain of PI 3-kinase. Subsequent studies however have shown that DNA-PK has no detectable activity towards lipids but has the ability to phosphorylate proteins instead. Since it is possible

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that phosphate can be transfered from ATP to Ser^{608} of p85 by the PI 3-kinase p110α, this suggests that the putative lipid kinase domains of DNA-PK and the TOR proteins are in fact protein kinases rather than lipid kinases.

1.5   Vacuolar protein sorting in yeast

The use of biochemical, molecular and genetic approaches on genetically tractable organisms such as yeast has led to the identification of many cellular components that direct vesicle-mediated transport from one intracellular site to another in secretory or endocytic systems. Selective genetic screening in *S.cerevisiae* has resulted in the identification of a large number of genes required for the delivery of proteins to the lysosome-like vacuole. Analysis of the products of these *VPS* (vacuolar protein sorting) genes have revealed that mammalian homologues exist, suggesting that divergent cell types have converged on a common set of transport components (Stack *et al.*, 1995c) The identification of the yeast *VPS* protein Vps34p as a class III PtdIns 3-kinase (section 1.3.3) implies the existence of a mammalian homologue and consequently implicates a PtdIns 3-kinase activity in membrane trafficking functions.

1.5.1   Biosynthesis of soluble vacuolar proteins

The cytoplasmic environment of eukaryotic cells can be subdivided into a number of functionally distinct membrane-enclosed organelles. The accurate delivery of proteins to specific intracellular organelles is essential to establish and maintain the functional integrity of these compartments. This in turn is a prerequisite for retaining proper cellular function as a whole. The secretory pathway is utilised in eukaryotic cells to sort and transport proteins from their cytoplasmic site of synthesis to various locations within the cell and to direct proteins for secretion (Palade, 1975). Mechanisms therefore, must exist within the secretory pathway that recognise and divert proteins to their appropriate intracellular or extracellular destination. The lysosome/vacuole represents one of the major destinations of protein and membrane flow in the secretory pathway. The essential characteristics of the secretory pathway appear to be highly conserved in all eukaryotic cells (Pryer *et al.*, 1992), therefore, vacuolar protein sorting in yeast has provided a model system for studying eukaryotic protein sorting pathways in general and is comparable to mammalian systems.

In yeast, soluble (lumenal) vacuolar proteins such as carboxypeptidase Y (CPY), proteinase A (PrA) and proteinase B (PrB) transit the early stages of the secretory pathway (Raymond *et al.*, 1992) before being sorted in a late Golgi compartment away from proteins destined for the cell surface (Graham and Emr, 1991). Entry into and progression through the secretory pathway is accompanied by compartment-specific post-translational modifications of the vacuolar proteins. Such modifications of CPY can serve

2 3

as experimental indicators of its relative location in the secretory pathway on route to the vacuole. CPY is synthesised as a prepro precursor molecule and is translocated into the lumen of the ER where its signal sequence is cleaved off to form proCPY (Blachly Dyson and Stevens, 1987). N-linked core glycosylation of proCPY occurs in the ER, which results in a 67 kDa form known as p1CPY. This enzyme is then delivered to the Golgi complex via transport vesicles in a process that requires the action of SEC (secretory) gene products acting at the ER to Golgi step of the secretory pathway (figure 1.4). After arrival in the Golgi, extension of the core oligosaccharides takes place with an increase in the apparent molecular mass of CPY to 69 kDa. This form, p2CPY is sorted away from proteins destined for the cell surface in a late Golgi compartment that corresponds to or is distal to the compartment containing the Kex2 protease (a late Golgi marker) (Graham and Emr, 1991). This late Golgi compartment in yeast corresponds to the *trans*-Golgi network (TGN) in mammalian cells. Finally, p2CPY is proteolytically processed in the vacuole to generate the 61 kDa enzymatically active mature form referred to as mCPY. The delivery of CPY to the vacuole is not affected by mutations in SEC genes whose products facilitate vesicular traffic between the Golgi and cell surface (Rothblatt *et al*, 1994) which indicates that CPY is not transported to the cell surface and subsequently endocytosed into the vacuole (Stevens *et al.*, 1982). Biochemical fractionation studies have demonstrated that p2CPY is transported to the vacuole via an endosomal compartment between the Golgi and vacuole. Soluble lysosomal hydrolases (such as Cathepsin D) exist in mammalian cells and are processed in an analogous fashion (Kornfeld and Mellman, 1989). The sorting signal of yeast vacuolar hydrolases resides in their primary amino acid sequence (this is unlike mammalian lysosomal hydrolases which require a carbohydrate sorting signal for their delivery, section 1.6). Extensive deletion and site-directed mutagenesis studies have shown that alteration of amino acids 24-27 of proCPY results in the missorting and secretion of p2CPY (Valls *et al.*, 1990). These studies indicated that secretion represents the default pathway that CPY follows in the absence of signals directing it to the vacuole. This conclusion was further supported by the observation that p2CPY was secreted from the cell in mutants lacking Golgi to vacuole transport function and was subsequently used as a tool in delineating the role of these gene mutants in the secretory pathway.

1.5.2 Mutants in vacuolar protein sorting

Alteration of the vacuolar sorting signal in wild type CPY or in CPY-invertase fusion proteins results in the appearance of the enzyme at the cell surface. Such mislocalisation of vacuolar hydrolases to the cell surface is the basis of several genetic selections that have resulted in the isolation of a large number of yeast mutants specifically defective in the delivery of proteins to the vacuole. Genetic selections using a CPY-invertase fusion protein that is efficiently targeted to the vacuole in wild type cells led to the isolation of

more than 600 *vpt* (vacuolar protein targeting defective) mutants (Bankaitis *et al.*, 1986). A selection scheme based on the detection of secreted CPY enzyme activity identified 600 *vpl* (vacuolar protein localisation defective) mutants (Rothman and Stevens, 1986). These mutants are now refered to as vacuolar protein sorting defective (*vps*) mutants. Screens for yeast mutants defective in protease activity in the vacuole (*pep* mutants) have also identified mutants in the vacuolar protein sorting pathway (Stack *et al.*, 1995c). Complementation analyses of the *vpt*, *vpl*, and *pep* mutants determined that they had extensive genetic overlap and resulted in the generation of a collective set of more than 40 *vps* mutants (Rothman *et al.*, 1989). The existence of such a large number of genes, indicates that sorting proteins to the vacuole is a complex process. Possible molecular activities involved in vacuolar protein sorting include a) receptor(s) for vacuolar protein precursors, b) proteins that direct the segregation and packaging of receptor-ligand complexes into vesicular carriers, c) factors responsible for vesicle formation and membrane fusion, d) vesicle coat proteins, e) proteins involved in the targeting and recognition of vesicle carriers with their target organelles (such as the endosome or vacuole), f) receptor recycling mechanisms and g) proteins involved in the maintenance of vacuolar structure and function.

1.5.2.1 *vps* Mutants

Morphological characterisation of the *vps* mutants using light and electron microscopy revealed that they could be categorised into six classes, A-F (Banta *et al.*, 1988). Most of the *vps* mutants fall into class A. In this class, the *vps* mutants contain one to three large vacuoles similar to those seen in wild type cells, which suggests that they are competent for assembly and maintenance of a morphologically normal vacuole. Mutants comprising the class B category contain highly fragmented vacuoles with each cell having ~35-40 small vacuoles. These vacuoles retain some vacuolar function but are not recognised as functional targets for the delivery of soluble proteins because these strains are severely defective for the sorting of CPY and other soluble hydrolases (Robinson *et al.*, 1988). The class C *vps* mutants lack any identifiable vacuolar structure, are extremely defective for the sorting of vacuolar proteins, accumulate abnormal membrane structures and exhibit growth defects under conditions of environmental stress (Banta *et al.*, 1988). The class D mutants have normal to enlarged vacuoles, are defective in vacuole segregation at mitosis and do not properly assemble the vacuolar H^+-ATPase. Class E mutants appear to accumulate a novel organelle distinct from the vacuole which contains soluble vacuolar hydrolases. This suggests that the class E compartment may represent an exaggerated prevacuolar endosome-like organelle similar to that identified in wild type cells. Class F *vps* mutants have a large central vacuole surrounded by smaller class B-like fragmented vacuolar structures.
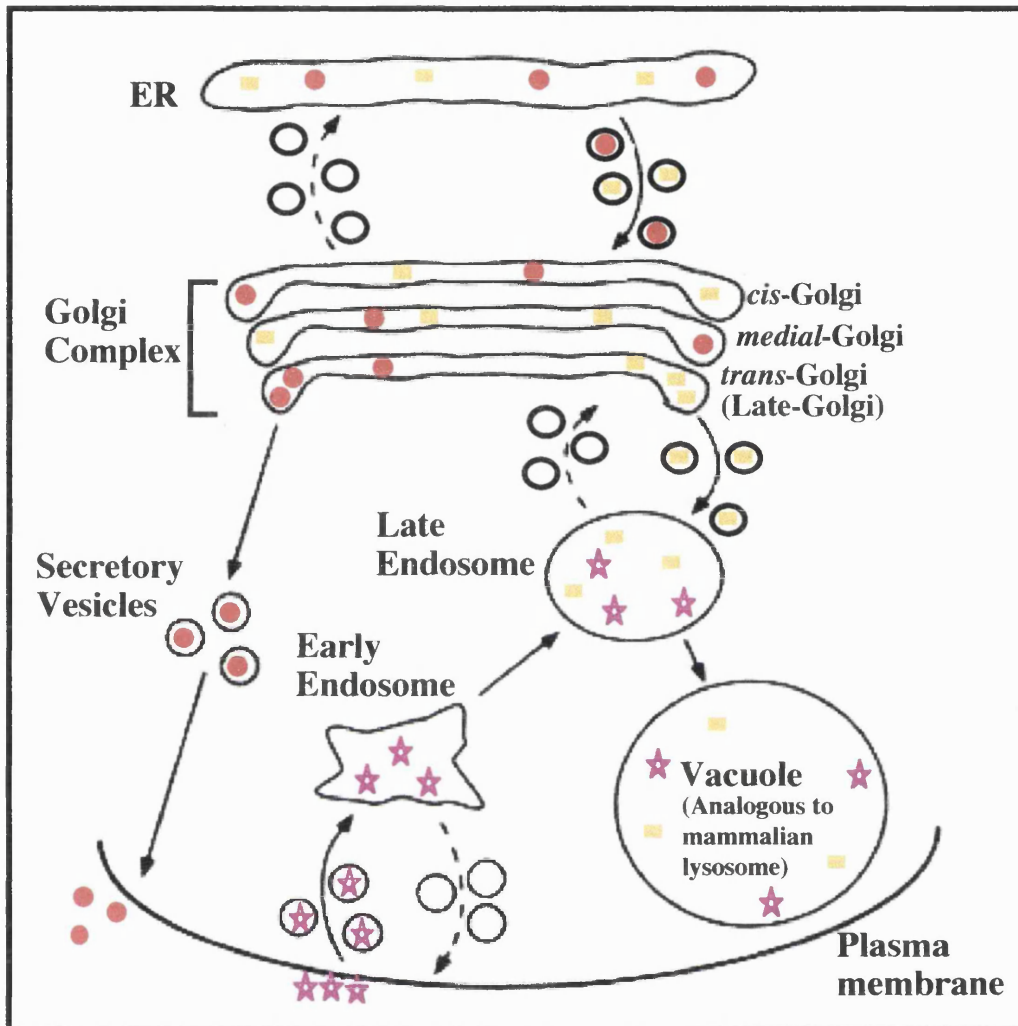
Figure 1.4 The movement of proteins through the yeast biosynthetic, secretory and endosomal pathways.

The endoplasmic reticulum (ER), the Golgi complex, the early and late endosomal compartments, as well as the vacuole, are depicted. Soluble vacuolar proteins (yellow squares) transit the early stages of the secretory pathway together with proteins to be secreted (red circles). In a late Golgi compartment, vacuolar proteins are actively sorted away from secretory proteins and are delivered to a prevacuolar endosome. Vacuolar and endocytosed proteins (purple stars) appear to transit an early endosomal compartment en route to the late endosome. Endocytosed and vacuolar proteins are then transported from the late endosome to the vacuole.

1.5.3 Molecular aspects of vacuolar protein sorting

1.5.3.1 Two *VPS* genes encode a phosphatidylinositol 3-kinase and a protein kinase required for vacuolar protein sorting

Two of the *VPS* genes, *VPS34* and *VPS15*, encode components of a regulatory complex required for the sorting of soluble vacuolar hydrolases (Stack *et al.*, 1995c). Strains deleted for either the *VPS34* or *VPS15* genes exhibit a set of common phenotypes. These observations initially suggested that their protein products act at a similar step in the vacuolar protein sorting pathway and that they may potentially interact with each other. The phenotypes of yeast strains deleted for *VPS34* or *VPS15* include severe defects in the delivery of multiple soluble vacuolar hydrolases. They also have a temperature sensitive growth defect, defects in osmoregulation and in vacuole segregation at mitosis, features which are characteristic of class D *vps* mutants (Herman *et al.*, 1992). Genetic, molecular and biochemical analyses have demonstrated that the *VPS34* and *VPS15* genes encode a phosphatidylinositol 3-kinase and a ser/thr protein kinase, respectively, that are components of a membrane associated complex. These findings indicate that protein and phospholipid phosphorylation events are required to regulate the vesicular delivery of proteins to the vacuole.

1.5.3.2 The Vps34p phosphatidylinositol 3-kinase

The characterisation of the *VPS34* gene revealed that it encoded a 97 kDa protein required for the sorting of vacuolar proteins (Herman and Emr, 1990). A possible biochemical activity for Vps34p was suggested when sequencing of the p110 catalytic subunit (Hiles *et al.*, 1992) of the class I_A mammalian PI 3-kinase found it to be highly homologous (33% identity at the amino acid level) to Vps34p. In mammalian cells the class I_A PI 3-kinase is composed of the 110 kDa catalytic subunit and an 85 kDa targeting subunit (section 1.3.1.1). The p110/85 complex phosphorylates PtdIns and other more highly phosphorylated phosphoinositides, PtdIns4P and PtdIns(4,5)P_2 at the D-3 position of the inositol ring (Cantley *et al.*, 1991; Carpenter *et al.*, 1990). The class I_A PI 3-kinases associate with many signal transducing receptor tyrosine kinases and are postulated to be involved in a wide range of events implicated in regulating cell growth and proliferation (section 1.3.1.4). The possibility that Vps34p may be a lipid kinase suggested that covalent modification of specific membrane phospholipids is involved in regulating the delivery of proteins to the yeast vacuole.

The yeast *S.cerevisiae* has been shown to contain PtdIns 3-kinase activity (Auger *et al.*, 1989a), and strains deleted for the *VPS34* gene are defective in their ability to produce PtdIns3P (Stack *et al.*, 1995c), suggesting that Vps34p is the only PI 3-kinase in yeast.

Alteration of conserved residues in the lipid kinase domain of Vps34p resulted in severe defects in both PtdIns 3-kinase activity and vacuolar protein sorting (Schu *et al.*, 1993). These results demonstrated that Vps34p was a functional PtdIns 3-kinase and that this activity was important for vacuolar protein sorting. In addition, analysis of a temperature-sensitive (*ts*) allele of *VPS34* demonstrated that Vps34p was directly involved in the sorting of soluble vacuolar proteins. A shift of these *vps34-ts* cells to the nonpermissive temperature leads to an immediate block in CPY sorting (Stack *et al.*, 1995a). The vacuolar membrane protein alkaline phosphatase is matured normally in the *vps34-ts* strain, which indicates that inactivation of Vps34p leads to a selective block in the sorting of soluble as opposed to membrane vacuolar proteins. The rapid block in protein sorting observed in *vps34-ts* cells appears to result from the loss of PtdIns 3-kinase activity, since the *vps34-ts* strain shifted to the nonpermissive temperature is defective for this activity (Stack *et al.*, 1995a). These results indicated that the *vps34-ts* strain is temperature sensitive for both CPY sorting and PtdIns 3-kinase activity and directly implicated a Vps34p-mediated PtdIns 3-kinase activity in the delivery of proteins to the vacuole. Furthermore, the screening of ethanemethanesulfonate mutagenised yeast cells for their ability to accumulate the fluid phase endocytic marker lucifer yellow in the vacuole, identified a number of *end* (endocytosis) mutants. A gene identified from this screen, *END 12*, was shown to be the same as *VPS34*, thereby implicating the Vps34p PtdIns 3-kinase in an additional endocytic function (Munn and Riezman, 1994).

Biochemical characterisation has shown that, unlike the class I_A p110 PI 3-kinase, Vps34p is only able to utilise PtdIns as a substrate (and is hence a PtdIns 3-kinase) and is inactive toward PtdIns4P and PtdIns (4,5)P_2 (Stack and Emr, 1994). Vps34p therefore, has been classified as a type III PI 3-kinase (section 1.3.3). Also, unlike the class I_A PI 3-kinases, Vps34p is relatively insensitve to the PI 3-kinase inhibitor, wortmannin (IC_{50}: 3μM). The idea that the products of the class I and class II PI 3-kinases [PtdIns3P, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3] have different effects *in vivo* is suggested by the fact that formation of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 is stimulated by growth factor addition, whereas PtdIns3P levels remain relatively constant (Auger *et al.*, 1989b). Since the 3'- phosphorylated lipids are not hydrolysed by any known eukaryotic phospholipase C, it has been suggested that PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 formed by PI 3-kinases act as intracellular second messengers to signal various events in response to growth factor stimulation (section 1.2.2). The possibility that the different 3'-phosphorylated phosphoinositides may have distinct functional properties in mammalian cells is supported by the identification of multiple PI 3-kinase activities (section 1.3) with different substrate specificities and structural features (Domin and Waterfield, 1997; Vanhaesebroeck *et al.*, 1997a; Zvelebil *et al.*, 1996). The role for Vps34p in vacuolar protein sorting and its characterisation as a PtdIns-specific 3-kinase, has led to the

proposal that production of PtdIns3P is specifically involved in regulating intracellular protein sorting pathways (Stack and Emr, 1994). Recent data have also shown that when osmotically stressed *S.cerevisiae* produce the novel lipid PtdIns(3,5)P_2 (Dove *et al.*, 1997). It was demonstrated that PtdIns3P produced by the *VPS34*-encoded PtdIns 3-kinase is required for the basal or osmotically induced synthesis of PtdIns(3,5)P_2. The PtdIns3P could be phosphorylated by a PI 5-kinase to generate PtdIns(3,5)P_2. These results have been used to suggest that hyperosmotic stress produces elevated levels of PtdIns(3,5)P_2 and that this could be the lipid that is actually implicated in vesicle trafficking. PtdIns(3,5)P_2-dependent vesicle trafficking may somehow be involved in a response to osmotic adaptation which may require the intensification of Golgi-to-vacuole transport or some other vesicle trafficking event (Dove *et al.*, 1997).

The biochemical characteristics of Vps34p suggest that this enzyme may be similar to a PtdIns -specific 3-kinase activity characterised from mammalian cells which also has a similar insensitivity to wortmannin (Stephens *et al.*, 1994a). However, a human PtdIns specific 3-kinase has also been cloned (Volinia *et al.*, 1995) that has extensive amino acid homology with Vps34p (37% identity and 58% similarity) but is sensitive to wortmannin (IC$_{50}$: 2.5nM). This may indicate that this human PtdIns 3-kinase might be a functional homologue of Vps34p or that perhaps, it is one of several Vps34-like mammalian PtdIns 3-kinases. To date, human PtdIns 3-kinase is the only cloned mammalian class III PI 3-kinase.

1.5.3.3 The Vps15p protein kinase

The *VPS15* gene encodes a 170 kDa protein that contains an N-terminal 300 amino acid region which exhibits significant sequence similarity to the ser/thr family of protein kinases (Herman *et al.*, 1991a; Herman *et al.*, 1991b). The functional relevance of this sequence conservation was assessed using mutational analysis of the Vps15p protein. Alterations in several kinase domain motifs were made and the phenotypic consequences were analysed *in vivo* by examining the CPY-sorting defects resulting from each change. Mutations altering Vps15p amino acids that are highly conserved among protein kinases resulted in the biological inactivation of Vps15p, as strains containing these mutant alleles missorted and secreted p2CPY (Herman *et al.*, 1991a; Herman *et al.*, 1991b). The correlation between Vps15p catalytic activity and CPY sorting indicated that Vps15p protein kinase activity plays a role in the sorting of soluble vacuolar proteins. Vps15p also appears to be an active protein kinase; *in vivo* and *in vitro* labelling experiments have demonstrated that it is able to autophosphorylate in a reaction that requires an intact protein kinase domain (Herman *et al.*, 1991a; Stack and Emr, 1994). *In vivo* labelling studies using [^{3}H]myristic acid have also shown that Vps15p is N-terminally myristoylated. This lipid modification may be required for an active protein kinase

activity since its deletion results in a non-myristoylated Vps15 protein that is partially defective for autophosphorylation (Herman *et al.*, 1991b).

Analysis of yeast strains with a temperature sensitive *vps15* allele has also provided evidence for a direct role of Vps15p in the sorting of soluble vacuolar hydrolases (Herman *et al.*, 1991b). This allele (*vps15ΔC30*) encodes a truncated Vps15 protein that lacks 30 amino acid residues from its C-terminus. Yeast *vps15ΔC30* mutants exhibit a temperature-sensitive defect in vacuolar delivery of the soluble hydrolases CPY and PrA, while protein secretion is unaffected. Why deletion of these amino acids should effect the ability of Vps15p to correctly target hydrolases is at present unknown (see section 3.2.3.4). The defect in vacuolar protein sorting is apparently specific for soluble proteins as maturation of the vacuolar membrane protein alkaline phosphatase proceeds with essentially wild type kinetics at the restrictive temperature. The very rapid onset and relative specificity of the *ts* sorting defects observed in the *vps15ΔC30* mutant suggest that the primary role of Vps15p in yeast cells is to mediate the delivery of soluble vacuolar proteins. Interestingly, CPY is not secreted from *vps15ΔC30* cells immediately after shifting to the restrictive temperature. Instead, CPY accumulates as the p2 precursor within a compartment distinct from the vacuole (Herman *et al.*, 1991b). Upon reversal of the temperature block, the intracellular p2CPY was found to be efficiently processed to its mature form, which suggests that it had been delivered to the vacuole. Sucrose gradient analysis of this accumulated intracellular p2CPY indicated that it co-fractionates with Kex2p, a resident late Golgi membrane protein (Vida *et al.*, 1993). These results suggested that the loss of Vps15p function leads to an immediate block in vacuolar protein sorting at the level of the Golgi. Since the late Golgi is the site where the sorting of vacuolar proteins occurs (Graham and Emr, 1991), Vps15p appears to be directly involved in regulating one of the earliest events in the vacuolar protein sorting pathway.

1.5.3.4 Interactions between Vps15p and Vps34p

Vps15p and Vps34p have been shown by genetic and biochemical criteria to interact as a complex that is associated with the cytoplasmic face of an intracellular membrane fraction most likely corresponding to a late Golgi compartment (Herman *et al.*, 1991a; Stack *et al.*, 1993). The CPY sorting defects associated with *vps15* kinase domain mutations are suppressed by the overproduction of Vps34p, which suggests that there is a functional interaction between Vps15p and Vps34p (Stack *et al.*, 1993). Native immunoprecipitation and chemical cross-linking experiments have been used to demonstrate that Vps15p and Vps34p physically interact *in vivo* as a complex. Subcellular fractionation experiments have further shown that Vps15p is responsible for the membrane association of Vps34p. In these experiments, Vps34p was found equally distributed between a membrane pellet fraction (P100) and a supernatant fraction (S100) when yeast spheroplasts were

osmotically lysed and centrifuged at 100,000x*g*. However, when the distribution of Vps34p was examined in a Δ*vps15* strain, the absence of Vps15p resulted in a dramatic shift of Vps34p into the S100 fraction indicating that Vps15p was required for the presence of Vps34p in the P100 fraction (Stack *et al.*, 1993). In addition to recruiting Vps34p to the membrane, Vps15p also serves to activate Vps34p because PtdIns 3-kinase activity is defective in *vps15* mutant strains (Stack *et al.*, 1995a; Stack *et al.*, 1993). The regulatory relationship between the two proteins is emphasised by the analysis of catalytically inactive forms of Vps15p and Vps34p. Kinase-defective forms of Vps34p result in a dominant-negative phenotype when overproduced in a wild type yeast strain. These strains have been shown to missort p2CPY and exhibit defects in PtdIns 3-kinase activity (Stack *et al.*, 1995a). This mutant phenotype appears to be the result of the sequestration of Vps15p into nonfunctional complexes by the Vps34 mutant proteins. In contrast, overproduction of kinase-defective Vps15p mutants does not appear to result in a dominant-negative phenotype. Vps15p kinase domain mutants are defective in their association with Vps34p, suggesting that overproduction of a kinase-defective form of Vps15p is unable to titrate Vps34p and consequently does not result in dominant interference (Stack *et al.*, 1995a). These observations indicate that an intact Vps15p protein kinase domain is required for association with Vps34p and subsequent stimulation of its PtdIns 3-kinase activity. Therefore, the functionally active form of Vps34p is in complex with Vps15p which suggests that the formation of a stable complex between Vps15p and Vps34p is absolutely required for the efficient localisation of soluble vacuolar proteins.

A number of models incorporating a membrane-associated complex of Vps15p and Vps34p in the vesicular delivery of proteins to the vacuole have been suggested. Subcellular fractionation of Vps15p and Vps34p (Herman and Emr, 1990; Herman *et al.*, 1991a) and the localisation of precursor vacuolar proteins in yeast cells lacking Vps15p function (Vida *et al.*, 1993) suggest that Vps15p and Vps34p act at the level of the late Golgi, most likely at the sorting compartment for vacuolar proteins. One model suggests that the Vps15p/Vps34p complex might associate with the cytoplasmic tails of sorting receptors for vacuolar proteins (figure 1.5) (Herman *et al.*, 1992; Stack *et al.*, 1993). Therefore, in a manner analogous to cell surface receptor proteins, it is proposed that ligand binding to vacuolar protein receptors in the sorting compartment may transduce a signal that promotes receptor association with and/or activation of the Vps15p protein kinase. Activation of Vps15p results in the stimulation of Vps34p PtdIns 3-kinase activity. Phosphorylation of membrane PtdIns by Vps34p would then trigger a cascade of events ultimately resulting in the vesicular delivery of receptor-ligand complexes to the vacuole via an endosomal intermediate. In this model, Vps15p and Vps34p effectively act as components of an intracellular signal transduction complex that translates the signal
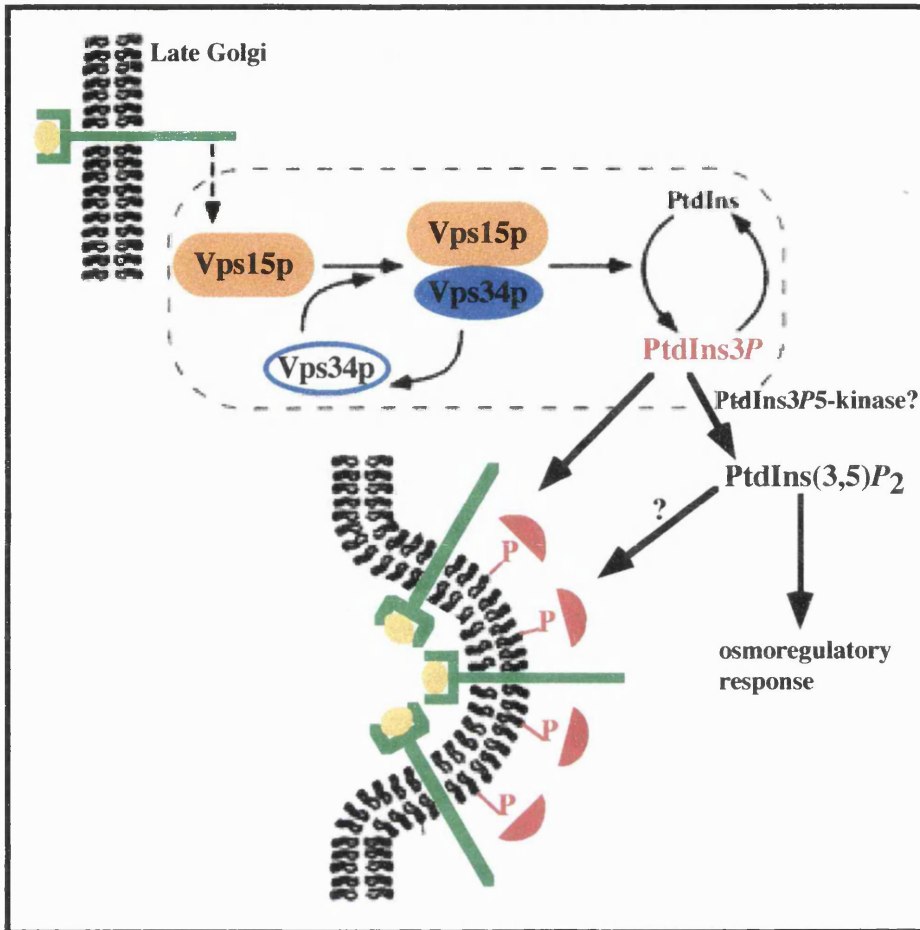
Figure 1.5 A model for Vps15p/Vps34p complex function in vacuolar protein sorting.

The binding of vacuolar protein precursors (in yellow) to their receptor molecules (green), may lead to the activation of Vps15p. Vps15p then recruits Vps34p to the membrane and stimulates its PtdIns 3-kinase activity. Activated Vps34p phosphorylates phosphatidylinositol (PtdIns), possibly resulting in the recriutment and/or stabilisation of coat proteins or other molecules needed for transport vesicle formation. PtdIns3P, generated by Vps15p/Vps34 has also been demonstrated to be required for PtdIns(3,5)P_2 formation (Dove *et al*, 1997) in yeast under hyperosmotic conditions. PtdIns(3,5)P_2 is postulated to play a role in vesicle trafficking, possibly in an acute osmotic adaptation response.

received by specific transmembrane receptor proteins into a second messenger molecule [PtdIns3P which can be used to generate PtdIns(3,5)P_2] that regulates the function of as yet unidentified effector molecules (Herman *et al.*, 1992; Stack *et al.*, 1993).

1.5.4   Receptor-mediated sorting of soluble vacuolar proteins

Similar to the mannose-6-phosphate receptor (MPR) system in mammalian cells (section 1.6), delivery of soluble proteins to the yeast vacuole appears to be mediated by transmembrane receptors. A number of early observations suggested that receptor molecules were involved in this protein sorting pathway. It was noted that overproduction of CPY resulted in the missorting and secretion of a substantial proportion of these proteins (Bankaitis *et al.*, 1986; Johnson *et al.*, 1987; Stevens *et al.*, 1986). When PrA was overproduced, much of the overexpressed protein was also secreted from the cell (Rothman *et al.*, 1986). One possible explanation for these results is that the overproduction of CPY or PrA leads to the saturation of a limiting component, such as a receptor molecule, which normally facilitates their recognition and/or sorting to the vacuole.

The sorting receptor responsible for the delivery of CPY and PrA to the vacuole has been isolated and characterised (Marcusson *et al.*, 1994). This was achieved by the screening of *vps* mutants for a CPY sorting defect. Mutations in the *VPS10* gene were found to have a CPY sorting defect whereby *vps10* mutants missort and secrete more than 90% of p2CPY. Characterisation of the *VPS10* gene product revealed that it encoded a 178 kDa protein which contained all the structural and functional properties of a transmembrane receptor protein. Vps10p is predicted to be a type I transmembrane receptor protein in that it contains an N-terminal signal sequence and a single transmembrane domain near the C-terminus. Subcellular fractionation studies have been used to demonstrate that Vps10p is tightly associated with cellular membranes and is primarily localised to a late Golgi compartment (Marcusson *et al.*, 1994), the site where sorting of vacuolar proteins appears to take place (Graham and Emr, 1991). Vps10p is modified by mannosyl residues added by glycosyltransferases present in the most distal compartment of the Golgi complex, further indicating a late Golgi location for this sorting receptor. Direct biochemical evidence which supports the observation that Vps10p is the sorting receptor for CPY came from cross-linking studies showing that Vps10p specifically associates with the p2 precursor form of CPY.

It is likely that Vps10p functions in a manner similar to the MPR in mammalian cells (section 1.6.1). In this model (figure 1.6), Vps10p binds p2CPY in the late Golgi sorting compartment and the receptor-ligand complex is delivered to a prevacuolar endosomal compartment via vesicular carriers. A pH difference between the yeast TGN and the
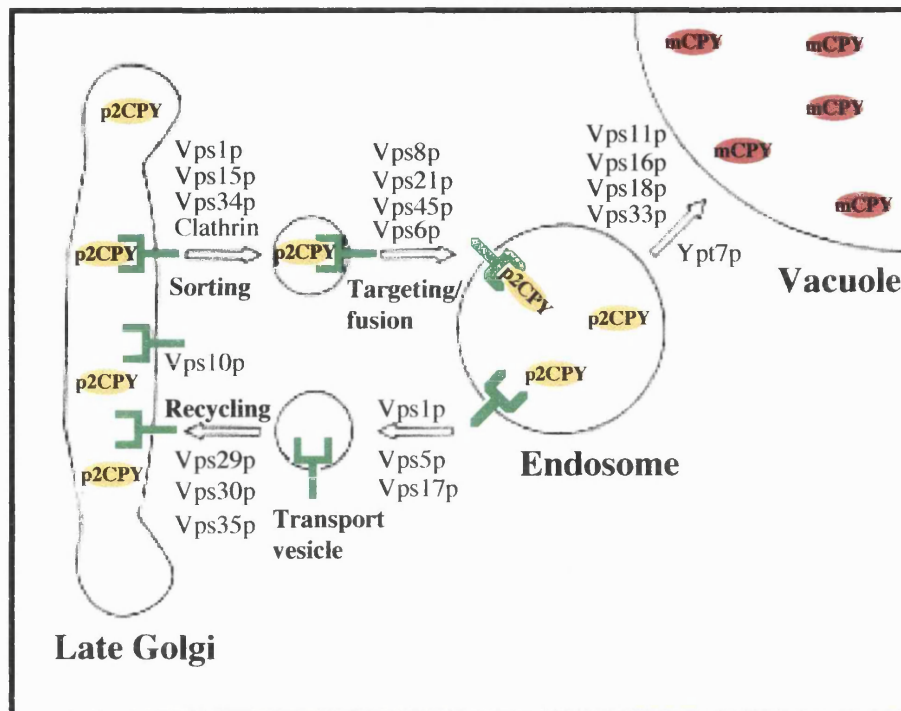
Figure 1.6 A model for vacuolar protein sorting.

After transit through the early stages of the yeast secretory pathway, precursor vacuolar proteins (e.g. p2CPY) are sorted away from secretory proteins by receptor molecules (e.g. Vps10p) in a late- Golgi compartment. Receptor-ligand complexes are packaged into transport vesicles and delivered to a prevacuolar endosomal compartment. This leg of the pathway appears to depend on the functions of Vps1p, Vps15p Vps34p and clathrin. Vps21p, Vps45p, Vps6p and Vps8p appear to be required for vesicle targeting/or fusion. Once at the endosome, the receptor-ligand complexes dissociate and the receptor recycles back to the Golgi for another round of sorting. Vps1p may be required for this recycling event. Transport of precursor vacuolar proteins from the endosome to the vacuole probably requires the function of Ypt7p, Vps33p, Vps16p, Vps18p and Vps11p. After arrival in the vacuole, the precursors are converted to their mature vacuolar form (e.g. mCPY).

acidified endosomal compartment is responsible for the dissociation of CPY from its receptor. The receptor is recycled back to the sorting compartment for another round of protein sorting. p2CPY then moves on to the vacuole where it is processed to its mature form. It has been suggested that Vps10p may be the receptor with which the Vps15p/Vps34p complex interacts to facilitate the sorting of soluble hydrolases. However, so far there has been no genetic or biochemical evidence to link Vps15p/Vps34p function with Vps10p or vice versa (Stack *et al.*, 1995c).

In addition to the interactions discussed above, the anterograde and retrograde trafficking of Vps10p appears to require the function of other class D *VPS* gene products. Mutations in three other genes, *VPS29*, *VPS30* and *VPS35* lead to a selective vacuolar missorting phenotype observed in Vps10p mutants (Paravicini *et al.*, 1992). Subsequent studies have shown that the recycling of Vps10p from the prevacuolar endosome to the late Golgi is dependent on *VPS29*, *VPS30* and *VPS35*. When any of these genes are mutated, Vps10p is not able to recycle back to the TGN and instead is mislocalised to the vacuole, presumably via the default flow of membrane proteins in the yeast secretory pathway which leads to the vacuole (Seaman *et al.*, 1997). Vps35p is a peripheral membrane protein which co-fractionates with membranes enriched in Vps10p, suggesting that Vps35p may directly interact with Vps10p. *VPS29* and *VPS30*, are predicted to encode hydrophilic proteins. The precise role of Vps35p, Vps30p and Vps29p in Vps10p retrieval is unknown, homologues have been identified from the human genome which suggests that these proteins may have a conserved function in evolution (Seaman *et al.*, 1997). The 164-amino acid C-terminal cytoplasmic domain of Vps10p may be involved in interactions with accessory transport factors including Vps29p, Vps30p, Vps35p, GTP-binding proteins and vesicle coat proteins (e.g. clathrin and clathrin adaptor proteins). This concept is supported by the observation that deletion of the Vps10p cytoplasmic tail results in a CPY sorting defect, indicating that an intact Vps10p cytoplasmic domain is essential for receptor function (Stack *et al.*, 1995c).

1.5.4.1 Involvement of GTP-binding proteins

1.5.4.1.1 GTPases in membrane trafficking

The movement of proteins through the secretory and endocytic pathways of eukaryotic cells is mediated by carrier vesicles that bud from a donor organelle and are targeted to and fuse with the appropriate acceptor organelle. The use of genetic approaches in yeast and *in vitro* reconstitution assays using mammalian cell extracts have revealed a large number of components required for the formation, targeting and fusion of these vesicles as they move between compartments (Pryer *et al.*, 1992; Rothman and Orci, 1992). One group of proteins shown to be required in this process are GTPases. By alternating

between conformational states in a fashion controlled by guanine nucleotide exchange and GTP hydrolysis, GTPases act as molecular switches whereby GTPases in their GTP bound form are regarded as active. The GTPases involved in vesicular traffic can be divided into two groups: i) the small GTPases of the Ras superfamily (this includes members of the Rab and Arf families amongst others) which are 20-30 kDa and ii) the dynamin family which are considerably larger, normally in the 60-80 kDa range (Nuoffer and Balch, 1994). These proteins share sequence motifs that are essential for guanine nucleotide binding and GTP hydrolysis. The members of different families are related to each other by variable degrees of homology in regions outside the highly conserved GTP-binding motifs. Such divergent sequences probably determine functionality by conferring specificity to interactions with upstream/downstream effectors and accessory factors which include GEFs (guanine nucleotide exchange factors), GAPs (GTPase activating proteins) and GDIs (guanine nucleotide dissociation inhibitors) (Boguski and McCormick, 1993; Lowy and Willumsen, 1993).

1.5.4.1.2   Vps21p and Rab proteins

The Rab family of small GTPases, comprise at least 30 GTP-binding proteins that are known to be involved in the regulation of specific targeting and/or fusion events in the endocytic and exocytic pathways (Novick and Zerial, 1997). Vesicle mediated movement of proteins from the Golgi to the yeast vacuole also involves small GTP-proteins of the Rab family. The cloning and sequencing of the *VPS21* gene revealed that it encodes a small GTP-binding protein. Sequence comparisons indicate that Vps21p is most similar to the Rab5 protein found in mammalian organisms (Horazdovsky *et al.*, 1994). Rab5 is required for the process of fusion between coated vesicles and early endosomes and for homotypic fusion which takes place between early endosomes (Riezman *et al.*, 1997). In yeast, *vps21* mutant cells exhibit severe defects in the sorting of CPY, PrA and PrB. A detailed mutational analysis has demonstrated that conserved regions in Vps21p, including GTP binding and isoprenylation domains are required for function (Horazdovsky *et al.*, 1994). In addition, Δ*vps21* strains have been shown to accumulate 40-50nm vesicles, indicating a role for Vps21p in the targeting and/or fusion of transport vesicles. The accumulation of membrane vesicles is also seen in mutants of another yeast Rab homologue, Sec4p, which acts at the Golgi to plasma membrane step of the secretory pathway (Novick and Brennwald, 1993). Although the vesicles that accumulate in *sec4* mutant cells are larger (80-100nm) than those seen in *vps21* mutant cells, this phenotype indicates that Vps21p and Sec4p perform similar functions at different sites within the cell. In mammalian cells, Rab5 functions in the early stages of the endocytic pathway and is localised to the early endosome (Bucci *et al.*, 1992). In contrast, Vps21p does not appear to act in the early stages of the yeast endocytic pathway as Δ*vps21* cells internalise the mating pheromone α-factor at nearly wild type levels (Horazdovsky *et al.*,

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1994). Vps21p most likely functions at the Golgi to late endosome or late endosome to vacuole transport steps; however, a role in early to late endosome traffic has not been ruled out. In addition, the accumulation of vesicles in *vps21* mutant cells indicates that Vps21p is specifically involved in the targeting and/or fusion of transport vesicles with the target endosomal or vacuolar membrane. Although the mammalian Vps21p homologue, Rab5, functions at the early stages of the endocytic pathway, this does not preclude the possible existence of mammalian Rab proteins with functional homology to Vps21p acting at mammalian late endosome/lysosome transport steps. Candidates for such Vps21p functional homologues include Rab 9 which is implicated in vesicle trafficking from late endosomes to the TGN and Rab 11 which facilitates transport through recycling endosomes (Novick and Zerial, 1997).

Given that Rab proteins exhibit low intrinsic rates of guanine nucleotide exchange and GTP hydrolysis (Nuoffer and Balch, 1994), the transport machinery is likely to require a set of accessory factors that modulate these reactions. Vps9p is a 52 kDa cytosolic protein that is related to the human protein Rin 1 (Burd *et al.*, 1996). Rin 1 binds Ras and negatively regulates Ras-mediated signalling (Han and Colicelli, 1995). The related region of Vps9p and the Ras binding protein Rin 1 may represent a GTPase binding homology domain. Vps9p therefore, may bind and serve as an effector of a Rab GTPase, such as Vps21p required for vacuolar protein sorting. Vps8p is a 134 kDa protein that peripherally associates with membranes and is required for sorting of vacuolar proteins (Horazdovsky *et al.*, 1996). Interestingly Vps21p and Vps8p have a similar subcellular fractionation pattern. Vps8p membrane association is found to be dependent on Vps21p function since in *vps21* null mutant cells Vps8p is found in the cytosol. These results have been used to show that Vps8p may directly interact with Vps21p and possibly modulate Vps21p function. Vps8p contains a C-terminal cysteine rich Zn^{2+} binding motif and truncation of this C-terminal region partially compromises Vps8p function. Vps21p may recruit Vps8p to the membrane in a manner analogous to the recruitment of Rabaptin-5 by Rab 5 or Rabphilin 3A by Rab 3A (Stahl *et al.*, 1996; Stenmark *et al.*, 1995). Rab 3A is localised to synaptic vesicles in mammalian cells where it is involved in secretion and has a regulatory role in exocytosis (Novick and Zerial, 1997). Rabaptin-5 and Rabphilin-3A are downstream Rab effector proteins which bind GTP-bound Rab 5 and Rab 3A respectively and promote membrane trafficking events (Novick and Zerial, 1997).Though sequence comparisons fail to reveal any extensive homology between Vps8p and Rabaptin-5 or Rabphilin-3A, Rabphilin-3A does contain a cysteine rich amino-terminal domain that is required for its interaction with Rab 3A (Yamaguchi *et al.*, 1993). Similarly, the Vsp8p cysteine rich domain may facilitate its interaction with Vps21p and/or other proteins.

Another small GTP-binding protein, Ypt7p, is a yeast homologue of the mammalian Rab7 protein (Wichmann *et al.*, 1992). Rab 7 has been localised to a late endocytic compartment implicated in controlling late endocytic membrane traffic. Rab 7 may be involved in regulating vesicle traffic from early to late endosomes or late endosomal to lysosomal transport. Rab 7 may achieve these functions by regulating membrane fusion events. Mutations in *YPT7* perturb vacuolar protein sorting and result in fragmentation of the vacuole itself. In addition, the degradation of internalised mating pheromone α-factor is severely inhibited in *ypt7* mutants, indicating that the endocytic pathway may be affected. A more detailed analysis of α-factor internalisation revealed that it appears to accumulate in a late endosome-like compartment in *ypt7* mutants (Schimmoller and Riezman, 1993). These data are most consistent with a functional role of Ypt7p in the trafficking of proteins from the late endosome to the vacuole.

1.5.4.1.3 ARF GTPases

ARF (ADP-ribosylation factor) was originally discovered as a cofactor required for the ADP-ribosylation of the heterotrimeric G protein G_S (Kahn and Gilman, 1984). The ARF family now includes at least 5 mammalian and two yeast members (Boman and Kahn, 1995). Although a small GTP-binding protein, ARF differs from other members of the Ras superfamily, such as the Rabs, by the absence of a carboxy-terminal prenylation motif. Instead, ARFs undergo post-translational myristoylation on an amino-terminal glycine residue, a modification which is essential for membrane association and function (Kahn *et al.*, 1992). Even though ARF proteins are not involved in vacuolar protein transport in yeast, they have been localised to the cytoplasmic face of Golgi cisternae and Golgi derived carrier vesicles (Orci *et al.*, 1993; Stearns *et al.*, 1990). In yeast and mammalian cells ARF proteins are required for maintaining the structural integrity of the Golgi, mediating ER to Golgi transport and transport between Golgi compartments (Nuoffer and Balch, 1994). In addition, ARF is required for endosome and late Golgi trafficking and as a component for the formation of coated transport vesicles (section 1.5.4.2.1).

1.5.4.1.4 Vps1p and Dynamin

The *VPS1* gene encodes a large GTP-binding protein of the dynamin family (Collins, 1991). Vps1p contains a GTP-binding domain which is conserved in a number of known GTP-binding proteins and binds and hydrolyses GTP *in vitro*. In yeast, mutations in *VPS1* result in defects in the sorting of soluble vacuolar proteins (Rothman *et al.*, 1990). Mutational analysis of *VPS1* was used to show that the mutations produce phenotypes which fall into two categories: a) dominant-negative mutations that cause a protein sorting defect in wild type cells and b) recessive loss-of-function mutations (Vater *et al.*, 1992). Mutations resulting in a dominant-negative phenotype all mapped to the N-terminal region

of Vps1p, whereas recessive loss-of-function mutations gave rise to unstable or truncated protein products. In addition, the dominant-negative phenotype requires an intact Vps1p C-terminus, which suggests that it may be invoved in interaction with other components of the vacuolar protein sorting machinery. Although its precise functional role is not known, Vps1p appears to be involved in the retention of Golgi membrane proteins. Genetic screens designed to isolate yeast mutants defective in the retention of the Golgi membrane protein Kex2p, identified a mutant strain (lam1) that was allelic to vps1 (Wilsbach and Payne, 1993). A model has been proposed in which Vps1p functions in recycling membrane proteins from the post-Golgi endosomal compartment to the Golgi. Since the CPY sorting receptor (Vps10p) would also be expected to use this pathway, blocking receptor recycling back to the late Golgi sorting compartment would result in a CPY sorting defect. Alternatively, Vps1p may function at the late Golgi to facilitate the anterograde movement of vacuolar and Golgi membrane proteins. It has been reported that Vps1p is required for the movement of membrane proteins form the Golgi to the early endosome rather than in their recycling back to the Golgi (Nothwehr et al., 1995). In the absence of Vps1p function, Golgi and vacuolar membrane proteins are mislocalised to the cell surface and subsequently endocytosed to the vacuole. These results have led to the proposal that Vps1p is required at the late Golgi for the formation of vesicles carrying vacuolar and Golgi membrane proteins to the endosome, although it is unclear whether Vps1p acts in the anterograde or retrograde movement of proteins (figure 1.6).

Vps1p and dynamin differ strikingly from Ras-like GTPases by their higher (~10 to 1000-fold, respectively) intrinsic hydrolysis rates (Nuoffer and Balch, 1994). Based on sequence homology, Vps1p has been found to be closely related to dynamin. Given the structural characteristics shared by Vps1p and dyanmin, it seems likely that both proteins will participate in similar protein-protein interactions. However, distinct factors may direct their recruitment to different membranes by binding divergent regions of the two proteins. For example, the C-terminal region of dynamin contains two inserts not found in Vps1p, one a PH domain and the other a region rich in proline and basic residues. It is possible that conserved motifs found in both dynamin and Vps1p are more likely to mediate interactions with conserved elements of the budding machinery. Further genetic screens in yeast, as well as a more extensive characterisation of the existing VPS gene collection, may well lead to the discovery of other factors that regulate the formation of Golgi vesicles (Conibear and Stevens, 1995).

Initial evidence for a role of dynamin in the endocytic pathway, came from the recognition that the shibrie locus in Drosophila melanogaster encodes a homologue of mammalian dynamin (Chen et al., 1991). Flies with temperature-sensitive alleles of

shibře exhibit rapid paralysis at elevated temperatures (Grigliatti *et al.*, 1973). This correlates with a depletion of synaptic vesicles from nerve terminals and the accumulation of membrane invaginations and coated pits on the cell surface. Mammalian dynamin was further implicated in endocytic trafficking when it was demonstrated to assemble into rings that form collars around invaginated pits, suggesting a direct role for dynamin in endocytic vesiculation (Hinshaw and Schmid, 1995).

Three distinct dynamin genes have recently been identified in mammals: dynamin I (*Dyn 1*) is expressed exclusively in neurons (Cook *et al.*, 1994; Obar *et al.*, 1990); dynamin II (*Dyn2*) is found in all tissues (Cook *et al.*, 1994); and dynamin III (*Dyn3*) is restricted to the testis, brain and lung (Nakata *et al.*, 1993). Each dynamin gene encodes at least four alternatively spliced isoforms (Cook *et al.*, 1996). Recently, a form of dynamin (Dyn2) has been localised to the Golgi complex of mammalian cells through the use of biochemical, immunological and morphological techniques (Henley and McNiven, 1996). Further studies have also shown that Dyn2 may function in vesicle budding events at the TGN, indicating that the function of dynamin is not restricted to endocytic vesiculation events (Jones *et al.*, 1998c). It is possible that the Dyn2 dynamin isoform may have a similar function to that of Vps1p which is postulated to have a role in vesicle trafficking between TGN and endosomal compartments in yeast.

1.5.4.2 Clathrin and coat proteins

1.5.4.2.1 Clathrin and adaptor proteins

The first step in the trafficking of proteins from a donor to an acceptor membrane compartment is the formation of a transport vesicle. This process is mediated, in both yeast and mammalian cells, by coat proteins which are recruited from the cytosol onto the donor membrane. Here they form a scaffold that drives vesicle budding while at the same time the vesicle cargo is selected through interactions which occur between the cytoplasmic domains of selected transmembrane proteins (Schekman and Orci, 1996). The first coated vesicles to be described were the clathrin-coated vesicles. Clathrin is a trimeric scaffold protein which organises itself into cagelike lattices (Kirchhausen *et al.*, 1997). Clathrin has the shape of a triskelion, where each one of the three legs is made of a heavy and a light chain. The extended conformation of a clathrin leg allows it to pack along a lattice edge, forming the characteristic open hexagonal and pentagonal facets of the coat. The assembly of a clathrin lattice on the cytosolic side of the plasma membrane or TGN membrane occurs during the formation of a coated pit and as a consequence, a section of membrane is ultimately captured into a coated vesicle. Clathrin therefore, is an organising framework for the proteins that carry out receptor sorting, membrane budding and other steps in the cycle of vesicle assembly uncoating and fusion.

The major proteins that promote clathrin coat formation are the 'clathrin AP (adaptor protein) complexes' or 'clathrin adaptors' which have largely been identified in mammalian cells. Clathrin AP complexes have been shown to bud from the TGN and plasma membrane where they concentrate cargo proteins for delivery to endosomal compartments (Robinson, 1997). The clathrin associated with these two membranes is the same, but the adaptors are different. The AP-1 complex is associated with the TGN and facilitates delivery of proteins from the TGN to the late endosome. The AP-2 complex is associated with the plasma membrane and plays a role in the receptor mediated endocytosis of proteins from the plasma membrane to the early endosome (Kirchhausen et al., 1997). AP-1 contains two large chains or 'adaptins' (γ and $\beta 1$) together with the medium ($\mu 1$) and small ($\sigma 1$) chains. AP-2 contains the large chains α and $\beta 1$ or $\beta 2$ and the $\mu 2$ and $\sigma 2$ subunits (Robinson, 1994). The most recent addition to the AP family is AP-3. The AP-3 complex is involved in the biosynthetic pathway and is localised to the TGN and a post-TGN compartment that is not necessarily a conventional endosome (Simpson et al., 1997). AP-3 contains the δ and $\beta 3$ chains together with the smaller $\mu 3$ and $\sigma 3$ chains. However, unlike AP-1 and AP-2, AP-3 is not thought to interact with clathrin and represents a component of a novel, nonclathrin coat.

1.5.4.2.2   COPI and COPII coatomers

Other coated vesicles have been identified which do not require clathrin, these are the COPI and COPII-coated vesicles which mediate transport in the early secretory pathway. COPI-coated vesicles bud from the Golgi stack and are required for intra-Golgi transport. COPII-coated vesicles bud from the ER and are implicated in ER to Golgi trafficking (Aridor and Balch, 1996b; Schekman, 1996). The COPI 'coatomer' is a heptameric cytosolic protein complex composed of α, β, β', γ, δ, and ζ subunits (Kreis et al., 1995). Yeast COPI homologues include Sec21p (γ), Sec26p (β), Sec27p (β'), Ret1 (α), Ret2p (δ) and Ret3p (ζ) (Aridor and Balch, 1996b). COPII coats involved in ER export were first identified in yeast through genetic screens and are composed of proteins using Sec13p, Sec31p, Sec23p and Sec24p as subunits (Rothman, 1994). Mammalian homologues have also been identified (Shaywitz et al., 1995). Although clathrin, COPI and COPII coats are assembled from different components, there are certain biochemical features that are shared by all coated vesicles. In all cases the membrane needs to be primed by the binding of a small GTPase such as ARF, before the coat proteins (in particular AP-1 COPI and COPII) can be recruited (Robinson, 1997). In the case of the AP-1 and AP-2 adaptors the GTPase dynamin is also required at a later stage to enable the coated pit to pinch off as a coated vesicle (Damke, 1996; Henley and McNiven, 1996).

1.5.4.2.3 Formation of coated vesicles in the vacuolar protein sorting pathway

Initial characterisation of the yeast clathrin heavy chain gene *CHC1* indicated that clathrin is not required for cell growth, protein secretion, or delivery of proteins to the vacuole (Payne *et al.*, 1987). The latter observation was quite surprising given the well-characterised association of clathrin with vesicles which are thought to deliver lysosomal proteins from the TGN to the lysosome. Resolution of this apparent paradox came with the generation of a temperature-conditional allele of the yeast clathrin heavy chain gene (*chc1-ts*). In contrast to Δ*chc1* strains, which show no defect in maturation of CPY, one of the immediate consequences of shifting such *chc1-ts* cells to the nonpermissive temperature is the missorting of CPY and PrA (Seeger and Payne, 1992). Extended incubation at the nonpermissive temperature prior to labelling allowed *chc1-ts* cells to recover the ability to sort CPY. This unusual adaptation phenomenon suggested that a non-clathrin-mediated mechanism may functionally substitute for clathrin in the sorting of soluble vacuolar proteins. Delivery of CPY to the vacuole in Δ*chc1* cells does not appear to involve transport to the cell surface and subsequent endocytosis to the vacuole. The extremely rapid onset of the sorting defect in *chc1-ts* cells shifted to the nonpermissive temperature further argues for a direct role for clathrin in vacuolar proteins sorting. By analogy to lysosomal protein sorting in mammalian cells, it has been suggested that clathrin functions to concentrate receptors for soluble vacuolar proteins (e.g. Vps10p) into coated pits. Clathrin-coated vesicles then bud off the late Golgi and receptor-ligand complexes are delivered to an endosomal compartment where ligand dissociates from receptors and receptors then recycle back to the Golgi for additional rounds of transport (figure 1.6). A yeast γ-adaptin homologue has been identified and the Vps1p dynamin homologue has been demonstrated to function together with clathrin-coated vesicles (Conibear and Stevens, 1995). Taken together, these data suggest that Vps1p, adaptins and clathrin all participate in the formation of Golgi vesicles that sort CPY. This is consistent with the view where dynamin, AP-1/AP-2 and clathrin function in mammalian cells and suggests a conservation of function in eukaryotic vesicular trafficking pathways.

The class B *vps5* and *vps17* mutants result in the missorting of CPY. The sorting defects observed for the *vps5* and *vps17* mutants appear to result in part, from the mislocalisation of the vacuolar protein-sorting receptor Vps10p (Horazdovsky *et al.*, 1997). This suggests that Vps5p together with Vps17p may participate in the intracellular trafficking of the Vps10p receptor. A role for Vps5p in CPY-sorting, involving receptor trafficking is further supported by the observation that Vps5p and the mammalian protein, sorting nexin 1 (SNX1) share amino acid sequence homology (Kurten *et al.*, 1996). SNX1 has been shown to interact with a domain of the epidermal growth factor (EGF) receptor that

includes the predicted lysosome targeting signal (YLVI). Overexpression of SNX1 was shown to result in a decrease in the amount of EGF receptor at the cell surface of CV-1 cells and an overall decrease in the amount of receptor found inside the cells, consistent with its degradation in the lysosome. These results indicate that SNX1 participates in EGF receptor trafficking to the lysosome. Vps5p may play a similar role in the vacuolar protein-sorting system by regulating the trafficking of Vps10p CPY-sorting receptor. However, a direct physical interaction between Vps5p and Vps10p has yet to be demonstrated. Vps5p and Vps17p have however, been shown to form a heterodimeric complex which is peripherally associated with a dense membrane fraction that is distinct from that of Golgi, endosomal and vacuolar membranes (Horazdovsky *et al.*, 1997). The association of Vps5p with this membrane fraction is dependent on Vps17p, although it is presently unclear if Vps17p recruits Vps5p to the membrane or if only the Vps5p/Vps17p complex is capable of mediating membrane association. Although these two proteins lack significant sequence homology with other proteins, the density of the Vps15p/Vps17p containing membranes are strikingly similar to that observed for COPI ($1.18g/cm^3$) and COPII ($1.19g/cm^3$) coated vesicles (Serafini *et al.*, 1991). It is possible therefore, that the Vps5p/Vps17p complexes may function as coat proteins which are implicated in the formation of coated vesicles that participate in the endosome to Golgi recycling pathway, returning Vps10p back to the Golgi.

The δ adaptin subunit of the AP-3 complex is the mammalian homologue of the *Drosophila garnet* gene product (Simpson *et al.*, 1997). The *garnet* mutant alleles affect pigmentation in the fly eye. The most likely mechanism which generates the *garnet* phenotype involves a defect in the delivery of proteins to pigment granules (Simpson *et al.*, 1997). In mammalian cells, pigment granules have been shown to be similar to modified lysosomes. Patients with Chediak Higashi syndrome (who suffer from hypopigmentation and immunodeficiency) not only have giant lysosomes but also giant melanosomes (Burkhardt *et al.*, 1993) (section 3.2.3.5). Less is known about the formation of pigment granules in flies but it seems likely that they too are lysosome-like in origin. This possibility is supported by the discovery that *VPS18* (a class C *VPS* gene), involved in the sorting of CPY to the yeast vacuole (Robinson *et al.*, 1991), is homologous to another fly eye colour gene *deep orange*. Vps18p physically and functionally interacts with Vps11p, Vps16p and Vps33p. Mammalian homologues of these proteins have been identified as expressed sequence tags (ESTs). This suggests that the function of Vps18p, Vps11p, Vps16p and Vps33p in protein transport to the vacuole/lysosome (or modified lysosome such as a melanosome or pigment granule), is likely to be conserved throughout eukaryotic organisms (Rieder and Emr, 1997).

1.5.4.3 Homologues of synaptic vesicle proteins and SNAREs

1.5.4.3.1 The 'SNARE' hypothesis

Studies of synaptic vesicle exocytosis and intra-Golgi vesicular transport in mammalian cells and vesicular traffic in the yeast secretory pathway have revealed that similar mechanisms appear to regulate the formation, docking and fusion of vesicles with their target membranes. Such observations have led to the formulation of a generalised hypothesis for the regulation of transport vesicle docking and fusion with target membranes (Rothman, 1994; Rothman and Warren, 1994). In this model, each type of transport vesicle contains a unique membrane protein v-SNARE [soluble NSF (NEM-sensitve factor) attachment protein receptor] that specifically interacts with a complementary t-SNARE on the target membrane. The SNARE hypothesis predicts that pairing of v- and t-SNAREs is regulated by the Rab GTPases (section 1.5.4.1.2). After the v- and t-SNAREs have paired, the soluble ATPase, N-ethylmaleimide-sensitive factor (NSF), binds the SNARE complex through soluble NSF-attachment protein (α-SNAP) and hydrolyses ATP. This results in reorganisation of the complex and induces membrane fusion (Hay *et al.*, 1997; Jahn and Hanson, 1998; Pfeffer, 1996).

1.5.4.3.2 Synaptic vesicle exocytosis as a v-SNARE/t-SNARE paradigm

The SNARE hypothesis was initially derived from the results of studies made through molecular dissection of the proteins involved in synaptic vesicle transport in neurons (Bock and Scheller, 1997; Sudhof, 1995). Studies of neurotransmitter-filled vesicles from the brain uncovered a prototype v-SNARE known as VAMP (vesicle associated membrane protein) or synaptobrevin (Trimble *et al.*, 1988). The prototype t-SNARE called syntaxin 1, was characterised as a nerve terminal protein that associates with proteins on the synaptic vesicle. This interaction between proteins on opposing membranes was proposed to mediate docking of the vesicle at the plasma membrane (Bennett *et al.*, 1992). For exocytosis to occur, the synaptic vesicle docks and then proceeds through a partial fusion reaction known as priming to make it competent for the final Ca^{2+}-triggered step (summerised in figure 1.7). During priming, a complex called the core complex is assembled by three abundant synaptic proteins, two from the plasma membrane [syntaxin and SNAP-25 (synaptosome associated protein)] and one from the synaptic vesicle (synaptobrevin). The core complex forms the anchor for a cascade of protein-protein interactions required for exocytosis to occur. Before exocytosis can occur however, synaptic vesicles are predocked at a specialised area of the presynaptic plasma membrane called the active zone. When docking occurs synaptic vesicle synaptobrevin is bound to a protein called synaptophysin, whilst syntaxin on the target plasma membrane
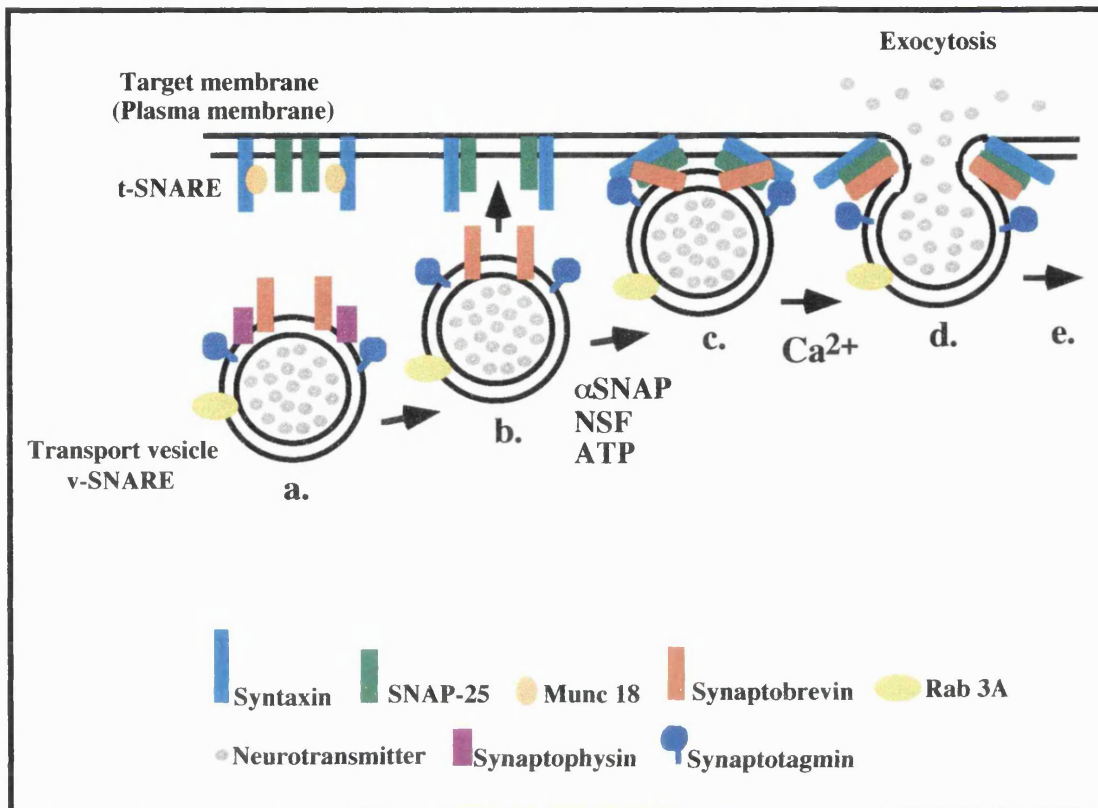
Figure 1.7 Synaptic vesicle exocytosis as a
v-SNARE/t-SNARE paradigm.

a. Before exocytosis synaptic vesicles are predocked at a specialised
area of the presynaptic membrane called the active zone. To inhibit
spontaneous synaptic core complex formation, syntaxin is bound to
munc 18 and synaptobbrevin is bound to synaptophysin.
b. munc 18 and synaptophysin dissociate by an unknown mechanism
to allow the formation of the synaptic core complex. The GTPase Rab 3A
is functionally but not physically associated with this complex and is
required for vesicle docking and fusion events.
c. Syntaxin and SNAP-25 interact to form a high affinity binding
site for synaptic vesicle synaptobrevin which results in the synaptic
core complex. This binds αSNAP and NSF. ATP hydrolysis by NSF
disrupts the core complex and leads to fusion.

d. Synaptotagmin acts as a Ca^{2+} sensor and completes fusion by a Ca^{2+}
triggered interaction with syntaxin (as shown in c.) allowing exocytosis
to occur.
e. Immediately after fusion synaptotagmin acts as a nucleus for clathrin coat
assembly facilitating endocytosis allowing the synaptic vesicle to be recycled.

is bound to a protein called munc18 (Sudhof, 1995). The synaptophysin/synaptobrevin and munc18/syntaxin interactions are thought to inhibit the formation of the synaptic core complex which would otherwise assemble spontaneously and indiscriminately at the active zone (Hayashi *et al.*, 1994). After docking, munc18 and synaptophysin dissociate by an unknown mechanism, allowing SNAP-25 to form a complex with syntaxin thereby creating a high affinity binding site for synaptic vesicle synaptobrevin, which results in the synaptic core complex. The synaptic vesicle membrane protein synaptotagmin also interacts with the synaptobrevin/syntaxin complex (Sudhof, 1995; Yoshida *et al.*, 1992). The GTPase Rab3A is functionally but not physically associated with this complex and is required for vesicle docking and fusion. The synaptic core complex serves as a receptor for $\alpha/\beta/\gamma$-SNAPs which in turn bind NSF. ATP hydrolysis by NSF disrupts the core complex and leads to fusion. Recent studies have also indicated that NSF and α-SNAP may be required prior to SNARE docking complex assembly, although the universality of a predocking function for these factors remains to be established (Bock and Scheller, 1997; Nichols *et al.*, 1997). Synaptotagmin, which acts as a Ca^{2+} sensor, completes the fusion reaction by a Ca^{2+}-triggered interaction with syntaxin, allowing exocytosis to occur (Sudhof, 1995). Synaptotagmin contains two C2 domains, C2A and C2B. C2A mediates the Ca^{2+} dependent binding of synaptotagmin to phospholipid vesicles, which may allow a remodelling of membrane phospholipids facilitating membrane fusion during exocytosis (Davletov and Sudhof, 1993; Perin *et al.*, 1990). Immediately after fusion, synaptotagmin acts as a nucleus for clathrin coat assembly by serving as a receptor for the AP-2 adaptor complex (section 1.5.4.2.1). The C2B synaptotagmin domain acts as a high affinity binding site for AP-2 (Zhang *et al.*, 1994). Clathrin together with dynamin binding to AP-2 then drives endocytosis allowing the synaptic vesicle to be recycled for another round of neurotransmitter exocytosis.

1.5.4.3.3 SNARE homologues in the yeast secretory pathway

Biochemical and genetic studies using different cell types indicated that the pairing between v- and t-SNAREs defined a model that was not restricted to exocytosis but encompassed many different vesicle trafficking steps throughout the secretory pathway, in cells as evolutionarily distant as yeast and neurons (Bennett and Scheller, 1993; Ferro Novick and Jahn, 1994). Studies in yeast identified a set of v- and t-SNAREs that mediate transport between the ER and Golgi apparatus. A different set of SNAREs was found to underlie shuttling between the Golgi and the vacuole (Becherer *et al.*, 1996). This suggested that specific pairs of vesicle and target membrane proteins (of the synaptobrevin and syntaxin families respectively) could mediate the fidelity of vesicle trafficking with SNAPs, NSF and small GTPases (such as Rabs) mediating membrane fusion.

In yeast some of the key regulatory proteins that appear to modulate the interaction between v-SNARE and t-SNARE proteins are members of the sec1p-like protein family (Novick and Brennwald, 1993). Within the family of sec1p-like proteins, each member appears to interact with a specific subset of t-SNARE proteins. Several isoforms of sec1p-like proteins have been described, one of which is mammalian munc18. Two sec1 protein family members have been identified in the vacuolar protein sorting pathway; Vps33p (Banta *et al.*, 1990) and Vps45p (Cowles *et al.*, 1994). Mutations in *VPS33* or *VPS45* result in vacuolar protein sorting defects, yet have strikingly different effects on vacuole morphology. Large vacuoles are seen in *Δvps45* mutant cells (Cowles *et al.*, 1994), however, *Δvps33* cells completely lack a vacuole (Banta *et al.*, 1990). This suggested that Vps45p and Vps33p may act at different stages in the pathway and that distinct, probably sequential, vesicle transport steps mediate protein delivery to the vacuole (i.e. Golgi to endosome and endosome to vacuole). A mammalian homologue to the yeast Vps45p has been identified (mVps45p), which is found expressed as a peripheral membrane protein that is localised to perinuclear Golgi-like and TGN compartments (Tellam *et al.*, 1997).

A t-SNARE homologue, Vps6p has also been found in the yeast vacuolar protein sorting pathway. Vps6p is the structural homologue of the t-SNARE syntaxin (Becherer *et al.*, 1996). Vps6p has been demonstrated to bind Vps45p and Sec18p (a yeast NSF homologue) and is required for Golgi to endosome trafficking. The interactions of Vps45p and Sec18p with Vps6, suggest they function directly in the docking and fusion of Golgi derived transport vesicles with the prevacuolar endosome (Burd *et al.*, 1997). As a syntaxin homologue, Vps6p (present on the prevacuolar endosome) is predicted to bind a v-SNARE protein, homologous to synaptobrevin (that would be found on a Golgi to endosome transport vesicle). All known class D *VPS* genes (of which Vps6p and Vps45p are members) have been cloned and characterised, but no candidate synaptobrevin homologues have been found. Nevertheless, a small number of open reading frames encoding putative SNARE molecules have been identified from the yeast genome which may be candidate v-SNAREs (Burd *et al.*, 1997). The function of Sec1p family proteins such as Vps45p may be regulated by rab GTPases. In the *VPS* pathway, the class D *VPS* gene product Vps21p is a Rab GTPase required for Golgi to endosome transport (Horazdovsky *et al.*, 1994); section 1.5.4.1.2). Vps21p may regulate the function of Vps45p and/or Vps6p. Consequently, Vps6p functions with Sec18p at a prevacuolar endosomal compartment for the docking and/or fusion of Golgi-derived vesicles containing p2CPY bound to the CPY receptor Vps10p. Vps45p is required for Vps6p function and may mediate a conformational change in Vps6p or other associated proteins. The GTP cycle of Rab proteins is thought to constitute a timer that temporally constrains membrane fusion (Aridor and Balch, 1996a; Rybin *et al.*, 1996), therefore in

the VPS pathway, Vps21p is predicted to monitor the protein-protein interactions required for membrane fusion at the endosome (Burd *et al.*, 1997). The fact that mammalian homologues of Vps6p, Vps45p, Vps21p and Sec18p have been identified suggests that protein delivery to the vacuole (mammalian lysosome) involves evolutionarily conserved mechanisms.

1.6 Lysosomal protein sorting in mammalian cells

Soluble and membrane lysosomal proteins are synthesised on ER-bound ribosomes and translocated into the lumen of the ER where they are modified by N-linked carbohydrate addition. Lysosomal proteins are then delivered via transport vesicles to the Golgi complex before being sorted in a late Golgi compartment (the TGN) away from proteins to be secreted (Kornfeld and Mellman, 1989). This general mechanism as established in mammalian cells, is highly analogous to the lysosomal protein sorting system observed in yeast (Stack *et al.*, 1995c). The majority of soluble (lumenal) enzymes are delivered to the mammalian lysosome through the acquisition of a specific carbohydrate moiety, mannose-6-phosphate. This feature, however, is not shared by yeast. Delivery of yeast proteins to the lysosome-like vacuole does not involve a carbohydrate-specific sorting signal, instead, the sorting signal of yeast vacuolar hydrolases resides in their amino acid sequence (section 1.5.1). Nevertheless, just as the delivery of soluble proteins to the yeast vacuole requires the transmembrane receptor Vps10p (section 1.5.4), the mannose-6-phosphate receptor (MPR) system in mammalian cells functions in an analogous manner. Mannose-6-phosphate modified lysosomal enzymes are recognised by MPRs, these ligand-receptor complexes are transported by clathrin-coated vesicles to a late acidic endosomal compartment where ligand dissociates from receptor. The soluble ligand continues to the lysosome, while the MPRs are recycled back to the Golgi for additional rounds of transport or are delivered to the cell surface where they function to internalise external lysosomal enzymes (Kornfeld and Mellman, 1989). In general, endocytosed material from the plasma membrane is initially delivered to an early endosome and then to a late endosome where it meets vesicular traffic from the biosynthetic route. Proteins destined for the lysosome from the biosynthetic and endocytic pathways then move from the late endosome to the lysosome (figure 1.4). A variety of signals have been characterised that direct sorting of the receptors between the Golgi, endosome and plasma membrane.

1.6.1 Mannose-6-phosphate receptors

Lysosomal proteins containing phosphomannosyl residues bind to MPRs in a late Golgi compartment and are transported to an endosomal compartment by clathrin-coated vesicles (Kornfeld, 1992). There are two mannose-6-phosphate receptors MPR46 and MPR300, both are glycoproteins and single transmembrane receptors. However, MPR46

is 46 kDa and cation-dependent whilst MPR300 is a larger (300 kDa) cation-independent receptor that also binds insulin-like growth factor II (IGF-II). Several studies have shown that both MPRs are in rapid equilibrium between different membrane bound compartments connected by vesicular transport, namely the TGN, endosomes and the plasma membrane (Kornfeld, 1992; Stack *et al.*, 1995c). MPR46 and MPR300 exhibit various affinities for different subgroups of lysosomal enzymes *in vivo*. Consequently, these MPRs probably function in concert to sort the ~40-50 different hydrolases required for efficient lysosomal degradation.

The cytoplasmic tail of each MPR contains signals for delivery from the Golgi to the endosome and for endocytic internalisation from the cell surface. Deletion and mutagenesis studies have identified a short sequence at the C-terminus of each of the MPRs consisting of a di-leucine motif that is required for the sorting of proteins from the Golgi to the lysosome (Johnson and Kornfeld, 1992a; Johnson and Kornfeld, 1992b). Upon transport of mannose-6-phosphate containing ligands to the endosome, the MPRs either recycle to the Golgi or are delivered to the cell surface. Endocytic internalisation of MPRs is mediated by a clathrin-dependent process whereby plasma membrane-specific adaptin proteins (section 1.5.4.2.1) link clathrin to MPR cytoplasmic tails by binding to tyrosine-containing motifs (Kornfeld, 1992). Collectively, the work on Golgi sorting and plasma membrane internalisation signals indicates that plasma membrane adaptins (AP-2) associate with tyrosine-containing motifs in the MPRs to facilitate clathrin-dependent endocytosis, whereas Golgi adaptins (AP-1) associate with other determinants in the cytoplasmic tail including di-leucine motifs, to also direct transport to the endosome in a clathrin-dependent manner.

1.6.2 Mannose-6-phosphate independent sorting

In patients with I-cell disease (ICD), lysosomes accumulate undigested material because the phosphotransfer activity responsible for the mannose-6-phosphate modification is eliminated and lysosomal enzymes are missorted and secreted (Kornfeld and Mellman, 1989). However, some cell types in I-cell patients exhibit normal levels of cellular lysosomal enzymes (Owada and Neufeld, 1982). Cathepsin D is a soluble lysosomal enzyme normally delivered to the lysosome by the MPR system (section 7.6.1). In lymphoblasts derived from an I-cell disease patient, more than 50% of newly synthesised cathepsin D was found to be delivered to the lysosome (Glickman and Kornfeld, 1993). In addition, a mutant form of cathepsin D lacking its N-linked glycosylation sites was found to be transported to the lysosome in ICD lymphoblasts in a manner nearly identical to the transport of wild type cathepsin D in the same cells. These results indicated that the sorting of cathepsin D to the lysosome in ICD lymphoblasts occurs by a pathway independent of mannose-6-phosphate recognition. Overproduction decreases the

efficiency of cathepsin D sorting in ICD lymphoblasts, which suggests a saturable delivery system, possibly including a receptor that recognises a peptide determinant in lysosomal enzymes (Glickman and Kornfeld, 1993). Further characterisation of this mannose-6-phosphate-independent sorting system may reveal the existence of sorting receptors that recognise peptide determinants and are analogous to the yeast VPS10p CPY sorting receptor (section 1.5.4).

1.7 Phospholipids in membrane trafficking

1.7.1 Roles for specific lipids in vesicular transport

Membrane traffic involves the movement of lipid membranes and associated proteins among intracellular organelles and the plasma membrane. Much of this movement is thought to occur through the transport of membrane vesicles between donor and target membranes. The generation of transport vesicles through budding processes and the fusion of vesicles with acceptor membranes is thought to require significant alterations in membrane structure and it is likely that transformations of lipid components either accompany or help to induce these processes (De Camilli et al., 1996).

It is possible that several steps in vesicle trafficking could be facilitated by specific lipids. A first step would be the definition of the locations where vesicles should form. Charged lipids such as phosphoinositides generated in a limited local area of the membrane bilayer by PI kinases may act as a site for vesicle formation. As a consequence small GTP-binding proteins or adaptors that link vesicle cargo to coat proteins could become activated by binding to the modulatory lipids. A transient local concentration of high-affinity binding sites for additional coat components might be created. This assembly would dissociate unless bound by additional coat components. Once nascent protein complexes are stabilised by protein-protein interactions, the coat and even its growth might become self-sustaining. An example of such a process would be the activation of the clathrin adaptor AP-2 by PtdIns3P, (Roth and Sternweis, 1997) which is followed by the binding of AP-2 by clathrin, which stabilises the high-affinity binding state of AP-2. As the coat grows the lipid activator could be metabolised, perhaps limiting the growth of the coat and preparing the way for vesicle fission (Rapoport et al., 1997).

A second step in vesicular transport that might be regulated by lipids, is the rearrangement of the lipid bilayer that must occur during vesicle fission and fusion events. During both fission and fusion, a neck and a pore between the vesicle and the donor/acceptor membrane must exist that would subject the joined bilayers to extreme curvature. One way to reduce the energy barrier imposed by this topology would be to generate lipids that favour positive curvature on the luminal side of the pore and lipids

5 0

favouring negative curvature on the cytosolic side of the pore. Phosphoinositides with their large charged headgroups may have a biophysical function in promoting membrane curvature. Lipids with a small head groups such as phosphatidic acid (PtdOH) and DAG might be generated locally at the site of vesicle stalk formation to facilitate progression of the membrane through the intermediates to vesicle fission or fusion (Roth and Sternweis, 1997)

A third role for lipid regulators of vesicular transport might be one of coordinating various steps of vesicle formation or to relate this process to other aspects of cellular metabolism. For example, the same lipid that might bind and activate exchange proteins for ARF or Rab small GTPases which promote vesicular transport, may also activate exchange factors for proteins like Rho or Rac that stimulate rearrangements of the cytoskeleton. Although there is no direct evidence yet for this type of signal integration, a number of reports have documented that reagents that affect the activity of Rho proteins also have effects on vesicular transport (Lamaze *et al.*, 1996; Sugai *et al.*, 1992).

1.7.1.1 Potential targets for PtdIns(4,5)P_2

Several different families of GTPases play important regulatory roles as switches in membrane traffic (section 1.5.4.1). Members of the ARF Ras GTPase superfamily participate in vesicle coat recruitment and are required for the formation of carrier vesicles, whereas members of the Rab branch of the superfamily are required for a later event, perhaps to facilitate the docking of vesicles with the target membrane (Novick and Zerial, 1997; Nuoffer and Balch, 1994). ARF1 is a myristoylated GTPase that is known to play a critical role in the assembly of the COPI coat as well as the clathrin coat at the TGN (section 1.5.4.1.3). Although ARF1 is soluble in its GDP-bound form it has been shown to associate with membranes in its GTP-bound form, indicating that the attachment reaction is coupled to nucleotide exchange. PtdIns(4,5)P_2 stimulates guanine nucleotide exchange and activates ARF (Terui *et al.*, 1994). PtdIns(4,5)P_2 could therefore play a role as a cofactor in coat assembly, because the GTP-bound form of ARF1 triggers the attachment of coat proteins (Donaldson *et al.*, 1992; Palmer *et al.*, 1993). A way in which PtdIns(4,5)P_2 can activate ARF is through acceleration of its rate of GTP binding. This may be achieved by ARNO (ARF nucleotide-binding site opener) which contains a PH domain that mediates stimulation of its exchange activity by PtdIns(4,5)P_2 (Chardin *et al.*, 1996). However, acidic lipids such as PtdIns(4,5)P_2 have also been demonstrated to stimulate the hydrolysis of GTP on ARF that is mediated by GAPs (Makler *et al.*, 1995; Randazzo and Kahn, 1994), a reaction that inactivates ARF. The site of action for this effect has been proposed to be on ARF itself and to result from the direct effects of PtdIns(4,5)P_2 on ARF1 (Randazzo, 1997; Terui *et al.*, 1994). GTP hydrolysis is necessary for vesicle uncoating, which in turn is a requirement for fusion

with the target membrane. Nevertheless, at present, it is not clear how the apparently opposing effects of PtdIns(4,5)P_2 on ARF activity would be integrated.

During coat assembly, activated ARF appears to function in concert with PtdIns(4,5)P_2 to activate an isoform of phospholipase D (PLD). ARF regulation of PLD activity has been demonstrated to play an important role in the release of nascent secretory vesicles from the TGN of endocrine cells (Chen *et al.*, 1997b). PLD generates PtdOH primarily from PtdCho; PtdOH then activates a PtdIns4P 5-kinase to generate additional PtdIns(4,5)P_2 (Brown *et al.*, 1993). Such a mechanism could set up a local feedback amplification loop in the generation of PtdIns(4,5)P_2 and PtdOH at the expense of PtdCho, which could result in locally elevated amounts of PtdIns(4,5)P_2 and PtdOH in a membrane bud or vesicular carrier generated by an ARF1 dependent mechanism (Liscovitch and Cantley, 1995). In this model, a putative key function of ARF, which is required for the assembly of both COPI and clathrin coats at the TGN, is the generation of a high local concentration of PtdIns(4,5)P_2. This possibility is supported by the observation that in a cell-free assay, the requirement for exogenous ARF in COPI binding can be replaced by overexpression of PLD (Ktistakis *et al.*, 1995). ARF-stimulated PLD activity enhances the binding of the β-COP subunit of coatomer to isolated Golgi membranes, suggesting that changes in membrane lipid composition influences coat recruitment (Ktistakis *et al.*, 1996).

The modulation of PLD by PtdIns(4,5)P_2, places PLD as an enzyme implicated with phosphoinositides in intracellular protein traffic or secretory events at the plasma membrane. In the latter case a phosphoinositide transfer protein (PI-TP) and a PtdIns4P 5-kinase have been implicated as factors in the regulated secretory pathways of HL60 and PC12 cells (Fensome *et al.*, 1996; Hay *et al.*, 1995). One of the first indications that inositol phospholipids are directly involved in Ca^{2+} regulated exocytosis, came from studies of permeabilised adrenal chromaffin cells. The observed requirement of ATP for secretion could be partially explained by the need to generate PtdIns(4,5)P_2 (Eberhard *et al.*, 1990). These observations were strongly corroborated by the identification and characterisation of cytosolic factors from the brain, PEPs (priming of exocytosis proteins) which are required for ATP-dependent priming of secretory granule exocytosis from PC12 cells (Hay *et al.*, 1995). Two of the three protein factors isolated, PEP1 and PEP3, are enzymes involved in the synthesis of PtdIns(4,5)P_2. PEP1 is a PtdIns4P 5-kinase and PEP3 is the mammalian PI-TP. PI-TP was originally identified as the factor that exchanges PtdIns and PtdCho between lipid bilayers and can transfer PtdIns from its site of synthesis (in the ER and Golgi complex) to other membranes. PI-TP has also been shown to participate in the synthesis of PIs by presenting PtdIns to PtdIns 4 -kinase and to PtdIns4P 5-kinase (Kauffmann Zeh *et al.*, 1995; Liscovitch and Cantley, 1995)

(section 6.4.1). Evidence suggests that PtdIns 4-kinase is also involved in exocytosis (Del Vecchio and Pilch, 1991; Wiedemann et al., 1996), as a result, PI-TP, in concert with a membrane-bound PtdIns 4-kinase and a cytosolic PtdIns4P 5-kinase, appears to function in the generation of PtdIns(4,5)P_2, which is needed for a critical step in exocytosis.

Two other proteins known to be important for vesicular traffic, namely dynamin and the clathrin-associated complex AP-2, also interact with PIs. The 100 kDa GTPase dynamin I is required for the terminal steps in forming clathrin-coated endocytic vesicles at presynaptic terminals (section 1.5.4.1.4) (Damke, 1996). Dynamin I has a high affinity binding site for the AP-2 α-adaptin subunit. Dynamin can also bind PtdIns(4,5)P_2 through its PH domain (Lin and Gilman, 1996). PtdIns(4,5)P_2 can stimulate the GTPase activity of dynamin more than fivefold. It is possible that binding of PtdIns(4,5)P_2 by dynamin, induces a conformational change which would favour GTP turnover. The activity of the dynamin GTPase might subsequently be limited through removal of its PtdIns(4,5)P_2 lipid activator. PtdIns4P does not stimulate the GTPase activity of dynamin and synaptic vesicles contain a phosphatidylinositol 5-phosphatase, synaptojanin, that is positioned to decrease the concentration of PtdIns(4,5)P_2 in the coated vesicle (McPherson et al., 1996). A dynamin isoform has been identified by immunocytochemistry on the Golgi complex (section 1.5.4.1.4), (Henley and McNiven, 1996) and the OCRL-1 (oculocerebrorenal-1) protein, which is a phosphatidylinositol 5-phosphatase that is similar to synaptojanin, also appears to be located on the Golgi complex (Zhang et al., 1995b). This would support a possible role for PtdIns(4,5)P_2 in mechanisms that regulate vesicle budding at the Golgi complex as well as at the plasma membrane.

1.7.1.2 Potential targets of 3'-phosphoinositides

As well as PtdIns(4,5)P_2, 3'-phosphoinositides have also been shown to bind to proteins implicated in vesicular trafficking. The AP-2 adaptor complex links clathrin and cargo proteins in coated pits at the plasma membrane (section 1.5.4.2.1). The 50 kDa μ subunit of AP-2 binds to tyrosine-dependent internalisation signals in transmbrane proteins (Ohno et al., 1995) and is responsible for sorting many cell surface receptors into clathrin-coated vesicles. As in the case of dynamin, the activity of clathrin-associated AP-2 complex is modulated by lipid. The 3'-phosphoinositides, PtdIns3P, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 increase the affinity of AP-2 for receptor internalisation signals by sevenfold to tenfold (Rapoport et al., 1997). Incorporation of AP-2 into clathrin coats also increases the affinity of AP-2 for internalisation signals by a comparable magnitude and renders binding independent of the phosphoinositides. Polymerisation of clathrin and its increased association with AP-2 would serve to maintain the high-affinity interaction

of AP-2 with receptors and allow further efficient incorporation of receptors into the coated pit without the presence of the lipid activator. For both AP-2 and dynamin, activation by a specific lipid may initiate subsequent events that are facilitated by other interactions.

The protein GRP1 (general receptor for phosphoinositides 1) and the closely related protein cytohesin-1 have been found to specifically bind $PtdIns(3,4,5)P_3$ through their respective PH domains. Cytohesin-1 catalyses guanine nucleotide exchange of ARF1 and has functional and amino acid sequence similarity to ARNO (section 1.7.1.1). Studies using the PI 3-kinase inhibitor wortmannin have shown that PI 3-kinase activity may be required in maintaining the vesicular morphoplogy of endosomes (section 1.7.1.3.2), (Shpetner *et al.*, 1996). Since the maintenance of vesicular organelle morphology requires cytosolic coat proteins and the activity of the small GTP-binding protein ARF1 (Rothman and Wieland, 1996), this suggests that ARF proteins could be regulated by a PI 3-kinase mediated pathway. The observation that the cytohesin-1 PH domain is a target for $PtdIns(3,4,5)P_3$, suggests a model in which a class I PI 3-kinase (section 1.3.1) generates $PtdIns(3,4,5)P_3$ to localise cytohesin at a membrane site, which in turn can regulate guanine nucleotide exchange of ARF1 (Klarlund *et al.*, 1997). The PH domains of GRP1, cytohesin-1 and ARNO exhibit very high sequence similarity. Therefore, the PH domain of ARNO may also bind $PtdIns(3,4,5)P_3$. Insulin stimulation of 3T3 L1 adipocytes causes a PH-domain-dependent translocation of ARNO to the plasma membrane (Venkateswarlu *et al.*, 1998). This translocation is inhibited by wortmannin, LY294002 and through co-expression with a dominant-negative p85 mutant, suggesting that insulin dependent translocation also requires PI 3-kinase activity. In addition, ARNO has been shown to have the binding characteristics of a $PtdIns(3,4,5)P_3$ receptor. Collectively these results have been used to suggest that ARNO might bind $PtdIns(3,4,5)P_3$ via its PH domain allowing its translocation to the plasma membrane where it might regulate ARF6 (an ARF isoform that functions at the cell surface) and subsequent plasma membrane cycling events. This may represent a mechanism by which the GRP1, cytohesin 1 and ARNO proteins may mediate the regulation of protein sorting and membrane trafficking through changes in $PtdIns(3,4,5)P_3$ levels.

In addition to its role in binding to GRP1 and cytohesin-1, $PtdIns(3,4,5)P_3$ has been demonstrated to bind the Golgi coatamer protein α-COPI (Chaudhary *et al.*, 1998). The phosphoinositide binding selectivity of Golgi coatamer COPI polypeptides was examined using photoaffinity analogues of the soluble polyphosphates $Ins(1,4,5)P_3$, $Ins(1,3,4,5)P_4$ and $InsP_6$ and of the polyphosphoinositides $PtdIns(3,4,5)P_3$, $PtdIns(4,5)P_2$ and $PtdIns(3,4)P_2$. The highly phosphorylated $InsP_6$ probe labelled six of

the seven COP subunits, however, of the polyphosphoinositides, only the PtdIns(3,4,5)P_3 probe showed specific labelling of only one of the COP subunits, α-COPI. Labelling by PtdIns(4,5)P_2 and PtdIns(3,4)P_2 photoaffinity probes was less intense indicating that PtdIns(3,4,5)P_3 may play an important role in COPI vesicle trafficking.

The EEA1 (early endosomal antigen 1) protein has been shown to bind specifically to liposomes containing PtdIns3P *in vitro*. In addition, EEA1 has been demonstrated to be rapidly released from cell membranes in response to wortmannin, suggesting that the effect of wortmannin is due to the inhibition of PtdIns3P production (Patki *et al.*, 1997). The data indicating that PI 3-kinase inhibition results in the loss of EEA1 binding to endosomes, implicates a role for this protein in mediating the effects of a PI 3-kinase or PtdIns 3-kinase on membrane trafficking. The specific function of EEA1 is presently not known. However, it is interesting that a Zn^{2+} finger motif at the C-terminus of EEA1 is highly homologous to that found in the yeast protein Vps27p. Vps27p belongs to a familiy of proteins involved in the sorting of soluble hydrolases to the yeast vacuole (section 1.5). Vps27p has been implicated in the control of traffic through an endocytic prevacuolar compartment (Piper *et al.*, 1995), a site at which the yeast class III PtdIns 3-kinase, Vps34p may also function. It is possible therefore, that EEA1 is a downstream target of a mammalian PtdIns 3-kinase and may mediate vesicle transport at the early endosome.

1.7.1.3 PI 3-kinases and membrane traffic

As described in section 1.7.1.2, 3'-phosphoinositides have been shown to directly interact with proteins implicated in vesicle trafficking. However, genetic studies in yeast and experiments using the PI 3-kinase inhibitors wortmannin (Arcaro and Wymann, 1993) and LY294002 (Vlahos *et al.*, 1994) have also suggested the action of PI 3-kinases (and hence 3'-phosphoinositides) in membrane trafficking events.

1.7.1.3.1 Involvement of PI 3-kinase in lysosomal targeting

The first indication of a direct role for PI 3-kinase activity in membrane trafficking followed the cloning of a cDNA encoding the class I_A bovine PI 3-kinase, p110α (Hiles *et al.*, 1992) and the discovery of its extensive amino acid sequence homology with Vps34p. Vps34p is required for the receptor-mediated transport of soluble hydrolases between the late Golgi and the vacuole in *Saccharomyces cerevisiae* (section 1.5.3.2), (Stack *et al.*, 1995c). Vps34p is a class III PI 3-kinase (section 1.3.3) which uses PtdIns as its only lipid substrate. Specific point mutations in the Vps34p lipid kinase domain that abolish activity cause mislocalisation of the soluble vacuolar hydrolase CPY, suggesting that the catalytic activity of Vps34p is required for vesicle transport of soluble hydrolases

to the vacuole (Schu *et al.*, 1993). Vps34p is a soluble protein and its function is dependent on its targeting to the appropriate membrane and hence its substrate, through an adaptor molecule Vps15p. Vps15p contains an autocatalytic protein ser/thr activity that is essential for the recruitment and activation of Vps34p (section 1.5.3.4), (Stack *et al.*, 1995a; Stack and Emr, 1993).

The molecular machinery underlying membrane trafficking events appears to be highly conserved among all eukaryotes (Ferro Novick and Jahn, 1994), there being several instances in which proteins derived from yeast and mammals are directly exchangeable. For example, yeast Sec18p is homologous to mammalian NSF protein (section 1.5.4.3.3) and can functionally replace NSF in a mammalian cell free assay for inter-Golgi compartment protein transport (Wilson *et al.*, 1989). The yeast vacuole is the major degradative organelle of the cell and is therefore functionally equivalent to the mammalian lysosome. For both yeast and mammalian cells, transport of newly synthesised soluble proteins to vacuoles or lysosomes is receptor mediated and involves clathrin-coated vesicles. Such mechanistic similarities have led to the prediction that the various *VPS* proteins, and specifically that a Vps34p-like protein, might be required for transport of newly synthesised mammalian lysosomal hydrolases between the TGN and endosomal/lysosomal compartments.

Studies based upon the use of selective PI 3-kinase inhibitors have been use to show that the treatment of rat and human cell lines with wortmannin and LY294002, caused newly synthesised lysosomal hydrolases such as cathepsin D, to be secreted rather than targeted accurately (Brown *et al.*, 1995b; Davidson, 1995). In each case, the enzymes were secreted in their precursor forms (such as procathepsin D), analogous to the aberrant secretion of CPY in yeast cells expressing mutant Vps34p (section 1.5.3.2), (Stack *et al.*, 1995c). In contrast to yeast, mammalian cells contain several PI 3-kinase isoforms and at present, the PI 3-kinase involved in lysosomal enzyme transport remains to be established. A PI 3-kinase activity has been described in mammalian cells that like Vps34p has a substrate specificity for PtdIns and is insensitive to wortmannin (Stephens *et al.*, 1994a). A human cDNA encoding an enzyme that has a substrate specificity for PtdIns and has amino acid homology (37% identity and 58% similarity) with Vps34p has been identified and is known as PtdIns 3-kinase (Volinia *et al.*, 1995). PtdIns 3-kinase is found in complex with a 150 kDa protein that is postulated to be the human homologue of the Vps34 associated protein, Vps15p (Volinia *et al.*, 1995). However, unlike Vps34p and the mammalian lipid kinase identified by Stephens *et al*, human PtdIns 3-kinase is sensitive to wortmannin (IC_{50} 2.5nM) and LY294002. The dose sensitivity of this inhibition nevertheless, correlates well with the effects of these drugs on lysosomal

targeting in mammalian cells, suggesting that the human PtdIns 3-kinase may be the lipid kinase implicated in lysosomal protein trafficking.

The pre-lysosomal compartment, rich in MPRs (section 1.6.1), functions as a junction-point within the endocytic system, receiving input from earlier compartments on the endocytic pathway as well as from the TGN. In addition to causing the secretion of newly synthesised cathepsin D into the media, both wortmannin and LY294002 cause a concentration of MPR300 in a swollen endocytic/pre-lysosomal compartment (Brown *et al.*, 1995b; Reaves *et al.*, 1996). Inhibition of PI 3-kinase also results in the redistribution of lysosomal glycoproteins (lgp120 and lgp110) to swollen structures that are both distinct from the swollen, MPR300 enriched compartment and are hydrolase negative (Reaves *et al*, 1996). This suggests that retrograde trafficking from dense lysosomes proceeds in the presence of this drug while movement of proteins from late endosomes to lysosomes is inhibited.

The reason for the swelling of these compartments is unlikely to be due to dissipation of a pH gradient since uptake of acridine orange, which accumulates in acidic organelles, was unaffected by wortmannin or LY294002 (Reaves *et al.*, 1996). The morphology of both lysosomes and the TGN appears unchanged in wortmannin-treated cells, suggesting a perturbation in membrane flow that is specific to the late endocytic/pre-lysosomal compartments. These data are supported by studies investigating the effect of wortmannin on the trafficking of MPR300 in murine L cells and normal rat kidney cells (Kundra and Kornfeld, 1998). In this case wortmannin induced a 90% decrease in the steady-state level of MPR300 at the plasma membrane without affecting the rate of internalisation, indicating that the return of receptor from endosomes to the plasma membrane was retarded. Wortmannin also slowed the movement of receptor from endosomes to the TGN by about 60%. Such a kinetic block would dramatically reduce the number of MPR300 in the TGN but cause an increase in endosomes. This has also been observed in the previously described morphological studies where wortmannin caused MPR300 to accumulate in swollen late endosomes (Reaves *et al.*, 1996).

The kinetic effects on the trafficking of MPR300 also help to explain the hypersecretion of procathepsin D that occurs in response to wortmannin. By slowing the return of MPR300 to the TGN, wortmannin treatment would reduce the number of receptor molecules in that compartment available for binding newly synthesised acid hydrolases, including procathepsin D. When the number of available receptor molecules becomes insufficient to bind the newly synthesised acid hydrolases, hypersecretion will result. These data indicate that wortmannin inhibits membrane trafficking out of multiple

compartments of the endosomal system and suggest a role for a Vps34p like PtdIns 3-kinase in regulating these processes.

1.7.1.3.2 PI 3-kinase and the endocytic pathway

In addition to a role for the Vps34p PtdIns 3-kinase in targeting CPY to the yeast vacuole, there is evidence to suggest that it also plays a role in sorting events that follow the initial stages of endocytosis. A screen designed to identify yeast mutants defective in endocytosis resulted in the isolation of an allele of *VPS34*, designated *end12*, that is important in targeting endocytosed α-factor for degradation (section 1.5.3.2), (Munn and Riezman, 1994). In *end12* mutants, internalisation of α-factor proceeds normally, but proteolysis is inhibited. Although the defect in degradation of α-factor could be due to a lack of delivery of hydrolases to the vacuole, an inhibition of accumulation of endocytosed lucifer yellow in the vacuole of the *end12* mutants supports the hypothesis that there is a block in transport at some point within the endocytic pathway. This finding suggests that delivery to the vacuole from both the TGN, and the endocytic system may require PI 3-kinase activity. Mutants of *Dictyostelium discoideum* have been constructed that lack *Dictyostelium discoidium* PI 3-kinase PIK1 and PIK2, two kinases more closely related to the mammalian class I_A p110 PI 3-kinase than to Vps34p. These mutant cells are impaired in the delivery of fluorescent dextran in the fluid phase to lysosomes and post-lysosomal vesicles (Buczynski *et al.*, 1997).

Although the late endosome has been implicated as a target of wortmannin action, this PI 3-kinase inhibitor has been shown to act at additional sites within the endosomal system. Transferrin receptor recycling which involves early endosomal compartments, is inhibited by wortmannin while endocytosis of this receptor has been reported to be either increased or decreased (Martys *et al.*, 1996; Shpetner *et al.*, 1996; Spiro *et al.*, 1996). In the presence of wortmannin and PDGF, PDGF receptors were internalised and sorted from transferrin receptors, but were not properly delivered to lysosomes (Shpetner *et al.*, 1996). In one set of experiments, tyrosines in the cytoplasmic tail of the PDGF receptor that are phosphorylated in response to PDGF binding were systematically mutated and the effect of these mutations on PDGF receptor trafficking was assessed (Joly *et al.*, 1995). These mutations were shown to interfere with the binding of the SH2 domain containing regulatory subunit of the p110 PI 3-kinase, p85α (section 1.3.1.1). Specific changes in the tyrosine residues known to bind PI 3-kinase blocked delivery of PDGF receptor lysosomes and stabilised the receptor, whereas other mutant receptors trafficked normally (Joly *et al.*, 1995). This correlated with the observation that inhibition of PI 3-kinase activity by wortmannin blocks lysosomal trafficking of PDGF receptor and resulted in recycling of the receptor to the plasma membrane (Joly *et al.*, 1995; Shpetner

et al., 1996). These data suggest that the class I_A p85/p110 PI 3-kinase complex is required to sort activated PDGF receptor to the lysosmal delivery pathway.

The PI 3-kinase inhibitor wortmannin has also been found to inhibit transcytosis of the polyimmunoglobulin receptor across epithelial cells and to inhibit endocytosis of bulk fluid (Clague *et al.*, 1995; Hansen *et al.*, 1995). In addition wortmannin has been shown to cause a dramatic alteration in the morphology of endosomal elements containing transferrin and a fluid phase marker (Shpetner *et al.*, 1996). In these studies, low concentrations of wortmannin caused a rapid decrease in endogenous PtdIns3P under conditions of exponential growth. A similar decrease in PtdIns3P occurred under conditions of serum starvation. The effects of wortmannin on organelle structure are evident both in exponentially growing and in serum-deprived mammalian cells. PtdIns3P was the only lipid detectable under both of these conditions which decreased in response to wortmannin with a time course that closely paralleled the appearance of alterations in endosome morphology (Shpetner *et al.*, 1996). This suggested that the 3'-phosphoinositide PtdIns3P and consequently a PtdIns 3-kinase or PI 3-kinase activity was involved in the control of organelle structure. The alteration on the morphology of endosomal compartments by wortmannin can be blocked by pre-treatment of cells with AlF_4^-. AlF_4^- inhibits the hydrolysis of GTP bound to ARF proteins (Shpetner *et al.*, 1996). This result suggests the hypothesis that AlF_4^- sensitive GTP-binding proteins may operate downstream of PI 3-kinase (section 1.7.1.2).

After initial internalisation events, a common sorting site within the cell is the early endosome. A number of factors including the small GTP-binding protein Rab5 (section 1.5.4.1.2) which is the mammalian homologue of the VPS protein Vps21p, are involved in the homotypic fusion events governing delivery of endocytosed material to this compartment (Li *et al.*, 1994). Evidence for the involvement of a PI 3-kinase in this membrane traffic step come from reports using *in vitro* assay systems in which endosome fusion was inhibited by wortmannin and LY294002 (Jones and Clague, 1995; Jones *et al.*, 1998a; Li *et al.*, 1995b). Addition of a constitutively active p110α subunit of mammalian class I_A PI 3-kinase was found to stimulate fusion almost threefold, whereas addition of p110 lacking the kinase domain failed to support fusion (Li *et al.*, 1995b). Although wortmannin inhibited endosome fusion in the presence of additional wild type Rab5, this inhibition could be reversed by a constitutively activated GTP-bound Rab5 mutant, suggesting that PI 3-kinase activity is involved in a step upstream of Rab5 function. These experiments therefore implicate PI 3-kinase involvement in this fusion event and suggest that PI 3-kinase may affect the state of guanine-nucleotide exchange of the Rab family of small GTP-binding proteins.

PI 3-kinase activity has also been implicated in exocytosis events. Wortmannin has been reported to inhibit the insulin induced exocytosis of the GLUT-4 glucose transporter (James *et al.*, 1994; Shepherd *et al.*, 1996) and there is an inhibition of histamine secretion by wortmannin in RBL-2H3 cells (Yano *et al.*, 1993). These and other effects mentioned in this section can be elicited by low nanomolar concentrations of wortmannin (summarised in figure 1.8). However, some studies report that micromolar concentrations of wortmannin are required, for example, to induce lysosomal enzyme hypersecretion in Chinese hamster ovary cells (Martys *et al.*, 1996), suggesting that one or more PI 3-kinase isoforms with differing wortmannin sensitivities (section 1.3) may be involved in producing this effect. Therefore, in comparing the concentration of wortmannin required to achieve an effect, it should be taken into consideration that the drug is metabolised and that the rate of metabolism may vary among cell types. Consequently, it can be difficult to distinguish which of the many PI 3-kinase isoforms is truely responsible for a particular membrane trafficking event inhibited by wortmannin. In addition the interpretation that PI 3-kinases are involved in all the pathways studied with inhibitors is limited by the observations that wortmannin also inhibits (albeit at a high nM-μM ranges) PtdIns 4-kinase (Nakanishi *et al.*, 1995), phospholipase A2 (Cross *et al.*, 1995b) and myosin light chain kinase activities (Nakanishi *et al.*, 1992).

However, taken together, the studies using low nanomolar concentrations of wortmannin and data investigating the potential targets of 3'-phosphoinositides (section 1.7.1.2), indicate that membrane trafficking events requiring modulation by PI 3-kinase activity may be localised specifically to the endosomal/pre-lysosomal system. Whether the mechanism of action is the same at each step remains to be determined. From studies using PI 3-kinase inhibitors it is difficult to ascertain which lipid kinase is involved in membrane trafficking as a number of PI 3-kinase isoforms are inhibited by wortmannin at low nanomolar concentrations (Shepherd *et al.*, 1996). Nevertheless, the identification of a human Vps34p homologue, PtdIns 3-kinase and the involvement of the p85/p110 PI 3-kinase in receptor mediated trafficking events strongly suggests that these two enzymes are most likely to be implicated in vesicle trafficking roles. Nevertheless, PI 3-kinase activity has been shown to act at a variety of sites within the endosomal system and which PtdIns 3-kinase or PI 3-kinase functions at what point is still unclear.
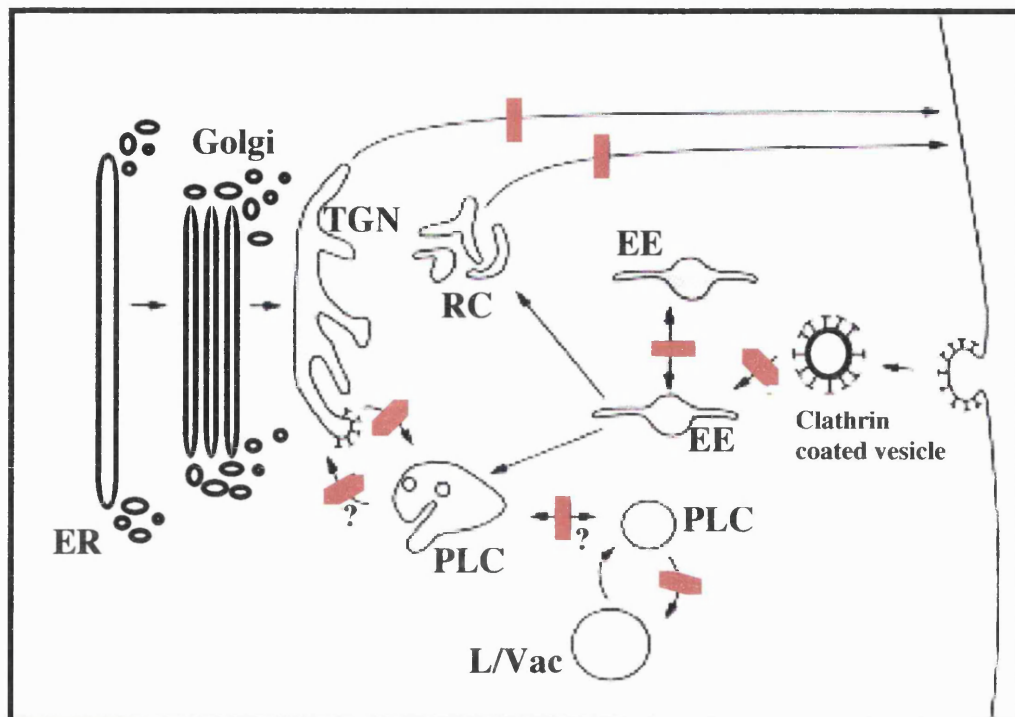
Figure 1.8 Potential sites of action of PI 3-kinase in membrane traffic pathways.

Blocks in delivery to various compartments within the endocytic/recycling and pre-lysosomal pathways as discussed in section 1.7.1.3.2, are indicated by a red bar. Speculative blocks are indicated by a question mark. Pathways indicated are based on findings from both yeast systems and higher eukaryotes (early endosome fusion, traffic from RC to cell surface and traffic from either the TGN or early endosomes through the late endocytic compartments to the lysosome). ER, endoplasmic reticulum; EE, early endosome; L, lysosome; RC, recycling compartment; PLC, pre-lysosomal compartment; TGN, *trans-*Golgi network; Vac, yeast vacuole.

1.8 Aim of thesis

This study was initiated to investigate the function of the human Vps34p homologue, PtdIns 3-kinase. Human PtdIns 3-kinase is found in complex with a 150 kDa protein which is predicted to be the human homologue of the yeast Vps34p adaptor protein Vps15p (Volinia *et al.*, 1995). Vps15p has a regulatory role in facilitating Vps34p to correctly target soluble hydrolases to the yeast vacuole (section 1.5.3.4). The identification of a human Vps15p homologue and its subsequent biochemical characterisation would greatly contribute to the investigation and understanding of PtdIns 3-kinase function in intracellular signalling.

The aim of the work presented this thesis therefore, was a) to clone the 150 kDa, PtdIns 3-kinase associated protein (p150) and to investigate whether p150 is the human homologue of Vps15p, b) to perform a biochemical analysis of the role of p150 on PtdIns 3-kinase function, including site directed mutageneis and domain deletion studies to investigate any functional domains present in p150 and c) to use immunofluorescence techniques and confocal microscopy to examine the subcellular localisation of p150 and PtdIns 3-kinase in mammalian cells with a view to gaining further insight into the possible involvement of these proteins in vesicle trafficking events.

CHAPTER 2

Experimental Procedures

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Experimental Procedures

2.1 Nucleic acid manipulations

2.1.1 Preparation of DNA fragments for subcloning

2.1.1.1. Preparation and annealing of oligonucleotide primers

Oligonucleotide primers were designed using the known DNA sequence of the template and restriction enzyme sites created at the ends of primers to facilitate subsequent subcloning of fragments. Oligonucleotides were purchased from Genosys. Annealing temperatures (T_m) for each primer were calculated using the equation: $T_m(^{\circ}C)=2(A+T) + 4(G+C)$.

2.1.1.2 DNA amplification by the polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) was used to amplify required regions of DNA from various templates and to clone cDNAs from libraries (Section 2.1.5.3).

PCR was performed in a 50 µl volume containing 8 µl 1.25 mM dNTPs, 40 pmoles of each primer and either 1 U of Taq polymerase (Gibco BRL) in 1 X Taq buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl, 5 mM $MgCl_2$) or 1 U Vent polymerase (New England Biolabs) in 1 X reaction buffer (10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl pH 8.8, 0.1% Triton X-100, supplemented with 2-6 mM $MgSO_4$) and covered with two drops of mineral oil (Sigma). Template DNA was either 10 ng plasmid DNA, or 2 µl cDNA library phage supernatant, and amplification was performed on a Perkin Elmer Cetus thermocycler. Samples were denatured at $94^{\circ}C$ for 30 s, annealed at a temperature appropriate for the length and sequence of the oligonucleotide primers ($45-55^{\circ}C$ - see above equation) for 30 s and extended at $72^{\circ}C$ for 30 s per 500 bp length amplified for 30 cycles. The mineral oil was removed by careful pipetting and the PCR products were resolved by TAE/agarose gel electrophoresis. The required DNA band was excised and purified from the agarose using a Qiaex kit (QIAGEN), according to the manufacturer's instructions. In the case of weak amplification, 2 µl were removed from the reaction tube and used as a template in an additional round of amplification.

2.1.1.3   DNA digestion with restriction endonucleases

Restriction enzymes were obtained from Gibco BRl or MBI Fermentas and digests were performed in the appropriate accompanying digestion buffer, as directed. Different restriction enzymes require different salt concentrations for optimal activity and this was taken into account when performing double digests. 0.5-5 µg DNA was digested with 5-10 U restriction enzyme in a volume of 30 µl, and reactions were incubated at 37°C for 1-2 h.

2.1.1.4   "Blunt-ending" digested fragments

To fill in digested 5' overhangs for subsequent blunt-end ligations, the digestion reaction was first heated at 65°C for 10 min to heat inactivate the restriction enzyme. 1.5 µl of 5 U *E.coli* DNA polymerase I Klenow fragment (New England Biolabs) was added to a 25 µl reaction (1 U/µg DNA) together with 1 µl of 1.25 mM dNTPs, in a final volume of 30 µl and incubated at room temperature for 15 min. The enzyme was heat-inactivated at 75°C for 10 min, removed by a phenol/chloroform extraction and then the DNA precipitated with ethanol (Section 2.1.3) or resolved by TAE/agarose gel electrophoresis and gel purified using a Qiaex kit (QIAGEN).

2.1.1.5   Phosphatase treatment of restriction fragments

To prevent self-ligation of digested vector in a ligation reaction without inserts, the vector was first dephosphorylated. The restriction enzyme was heat inactivated at 65°C for 10 min, the salt concentration adjusted to 1 mM MgCl$_2$ and 1 U of calf intestinal alkaline phosphatase (Boehringer Mannheim Ltd.), were added. The mixture was incubated at 37°C for 45 min. The reaction was terminated by heat-inactivation at 65°C for 20 min and the DNA was resolved by TAE/agarose gel electrophoresis and gel purified using a Qiaex kit (QIAGEN).

2.1.1.6   Site directed mutagenesis

In vitro site-directed mutagenesis is an invaluable technique for studying protein structure-function relationships, identifying intramolecular regions or amino acids, which may mediate these functions. The QuikChange™ site-directed mutagenesis kit (Stratagene) allows site-specific mutation in virtually any double-stranded plasmid, therefore eliminating the need for subcloning into M13-based bacteriophage vectors and for single strand DNA rescue. The QuikChange™ site-directed mutagenesis method was performed according to the manufacturer's instructions. This mutagenesis method was performed using *Pfu* DNA polymerase which replicates both plasmid strands with high fidelity and without displacing the mutant oligonucleotide primers. The basic procedure utilises a supercoiled, double-stranded DNA vector with an insert of interest and two synthetic oligonucleotide primers containing the desired mutation. The oligonucleotide

primers, each complementary to opposite strands of the vector, extend during PCR cycling by means of *Pfu* DNA polymerase. On incorporation of the oligonucleotide primers, a mutated plasmid containing staggered nicks is generated. Following PCR cycling, the product is treated with *Dpn* I. The *Dpn* I endonuclease (target sequence: 5'-G^{m6}ATC-3') is specific for methylated and hemimethylated DNA and is used to digest the parental DNA template and to select for mutation-containing synthesised DNA. DNA isolated from almost all *Escherichia coli* strains is *dam* methylated and therefore susceptible to *Dpn* I digestion. The nicked vector DNA incorporating the desired mutations is then transformed into *E.coli.* (section 2.1.2.7), the presence of the desired mutation in selected clones was checked by DNA sequencing (section 2.1.4.1).

2.1.1.7   Electrophoresis of DNA fragments

DNA molecules migrate according to their size when electrophoresed on an agarose gel. 1% (w/v) gels were generally used, although 1.5% (w/v) gels were employed for analysis of DNA fragments less than 500 bp in size, and 0.8% (w/v) gels were used for DNA fragments larger than 4 kb. The appropriate weight of agarose was added to TAE buffer (40 mM Tris-Acetate, 1 mM EDTA) and heated to allow the agarose to dissolve. The solution was cooled to approximately 60°C, ethidium bromide added to a final concentration of 1 μg/ml and the mixture added to a gel mould and allowed to set. Samples were mixed with 6 X loading buffer (0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF, 30% (v/v) glycerol in water), loaded onto the gel and fragments separated by electrophoresis in TAE at 100 V. Standard molecular weight markers (Gibco, 1 kb DNA ladder) were electrophoresed alongside the samples. Gels were viewed and photographed under a long-wave UV lamp.

2.1.1.8   Purification of DNA fragments

The band corresponding to the DNA fragment of interest was localised using a long-wave UV lamp and excised with a scalpel. The DNA was then purified from the gel slice using the QIAEX DNA Gel Extraction kit (QIAGEN), which uses silicagel particles to bind DNA in the presence of high salt, according to the manufacturer's directions.

2.1.2   DNA ligation and transformation

2.1.2.1   Bacterial strains

Escherichia coli XL-1 Blue cells. Genotype: supE44, hsdR17, recA1, endA1, gyrA46, thi relA1 lac-F[proAB^{+} lacI^{q} lac ZΔM15, Tn10(tet^{r})].

2.1.2.2 Plasmids

Plasmids used were, Bluescript SK (Stratagene), pAcGEX-2T (Cambridge Bioscience), pVL1393 (Invitrogen) and pMT2SM (Kaufman *et al.*, 1989).

2.1.2.3 Growth media

Luria Bertani (LB) media [1% (w/v) bactotryptone, 0.5% (w/v) yeast extract and 1% (w/v) NaCl] was prepared using pre-prepared LB powder (25 g/litre ddH_2O; Gibco BRL).

Terrific broth (TB) [1.2% (w/v) bactotryptone, 2.4% (w/v) yeast extract, 0.4% (v/v) glycerol, 0.23% (w/v) KH_2PO_4 and 1.25% (w/v) K_2HPO_4] was prepared using pre-prepared TB powder (47 g/litre ddH_2O; Gibco BRL).

NZY media [0.5% (w/v) NaCl, 0.2% (w/v) $MgSO_4.7H_2O$, 0.5% (w/v) yeast extract and 1% (w/v) NZ amine] was prepared using pre-prepared NZY powder (21.1 g/litre ddH_2O; Gibco BRL).

TYM media was prepared using 2% (w/v) bactotryptone, 0.5% (w/v) yeast extract, 0.1 M NaCl, and 10 mM $MgSO_4$.

For LB agar, 1.5% of bacto-agar (Gibco BRL) was added to LB media, similarly for NZY agar, 1.5% bacto-agar was added to NZY media. For NZY top agarose, 0.7% (w/v) agarose was added to NZY media. Liquid media was sterilised by auto claving and stored at room temperature.

2.1.2.4 Antibiotics

Ampicillin and kanamycin were prepared as 100 mg/ml and 10 mg/ml solutions in ddH_2O respectively, filter sterilised and stored in 1 ml aliquots at -20°C. Tetracycline was prepared as a 12.5 mg/ml solution in ethanol/ddH_2O (50% v/v), filter sterilised and stored in the dark at -20°C.

For bacterial agar plates, melted LB agar media was allowed to cool to 48°C before adding ampicillin to a final concentration of 100 µg/ml. Plates are poured, left to set and dry for 10 min and stored at 4°C. Alternatively, LB plates can be made without the addition of antibiotic. Antibiotic (e.g. tetracycline or kanamycin) can then be spotted onto the plate and left to dry.

2.1.2.5 Ligation of DNA fragments

To drive ligation reactions and minimise self-ligation of plasmids without inserts, ligation reactions contained a molar excess of insert DNA (insert:vector = 5:1). DNA was quantitated approximately by running a small aliquot of the purified sample on an agarose gel against known standards. Most ligations were performed in 10 µl final volume with 2 µl of 5 X ligation buffer [250 mM Tris-HCl pH 7.6, 50 mM $MgCl_2$, 5 mM ATP, 5 mM dithiothrietol and 25% (w/v) polyethylene glycol-8000 (PEG)] and 5 U of T4 DNA ligase

(Gibco BRL). The reaction was incubated either at 16°C overnight or at room temperature for 1-4 h. A control ligation containing plasmid DNA with ligase but no insert DNA was always carried out in parallel to assess background self-ligation of plasmids. Blunt end ligations were performed at 16°C overnight.

2.1.2.6 Preparation of competent *E.coli*.

2 ml LB or TB media was inoculated with a single XL-1 Blue *E.coli* colony and grown at 37°C overnight with shaking. This was then used to inoculate 50 ml of LB supplemented with 12.5 µg/ml tetracycline at a dilution of 1/50-1/100. After growth at 37°C to $OD_{600}=0.5$, cells were cooled on ice, pelleted by centrifugation at 4°C for 10 min at 2000 g and resuspended in 1/3 culture volume in a filter sterilised buffer containing 100 mM KCl, 50 mM $CaCl_2$, 10% (v/v) redistilled glycerol and 10 mM potassium acetate. The mixture was incubated on ice for 10 min after which the cells were centrifuged at 1000 g at 4°C for 15 min and then resuspended in 1/12.5 of the original culture volume. Cells were stored on ice until used or 200 µl aliquots of the cell suspension were 'flash-frozen' in a dry-ice/ethanol bath for 5 min and then stored at -70°C.

Highly competent cells were prepared by the following procedure: 20 ml of TYM media was inoculated with a single XL-1 Blue *E.coli* colony (from a tetracycline plate) and grown shaking at 37°C in a 250 ml flask, until the OD_{600} was between 0.2 and 0.8. The culture was transferred to a 2 litre flask containing 100 ml TYM broth and agitated at 37°C, until OD_{600} reached 0.5-0.9. A further 500 ml TYM broth was added and the incubation continued until the OD_{600} reached 0.6. The culture was cooled rapidly by swirling the flask in an ice bath and the bacteria were then pelleted (5000 rpm, 15 min) and resuspended in 100 ml ice-cold TfBI (30 mM KOAc, 50 mM $MnCl_2$, 100 mM KCl, 10 mM $CaCl_2$, 15% (v/v) glycerol) by gentle shaking/pipetting. The bacteria were re-pelleted ($5000xg$, 8 min) and resuspended in 20 ml TfBII (10 mM NaMOPs pH 7.0, 75 mM $CaCl_2$, 10 mM KCl, 15% (v/v) glycerol). Finally, 0.6 ml aliquots were 'snap-frozen' in pre-chilled screw-cap microcentrifuge tubes in liquid nitrogen and stored -70°C.

2.1.2.7 Transformation of *E.coli*.

Plasmid DNA (50 ng) or ligation mix was added to 100 µl of carefully thawed competent cells, mixed very gently and incubated on ice for 10 min to allow the DNA to be absorbed on to the cells. The cells were induced to take up the DNA by heat-shock at 42°C for 2 min kept on ice for 2 min and allowed to recover in 1 ml of SOC media (2% (w/v) bactotryptone, 0.5% (w/v) yeast extract and 0.05% (w/v) NaCl) for 45min at 37°C with shaking. The bacterial cells were then briefly centrifuged and 900µl of the SOC medium removed. The cells were resuspended in the remaining 100µl and then pipetted on to dry,

warm agar plates, gently spread with a flamed glass pipette and incubated overnight at 37°C. LB agar plates containing 100 µg/ml ampicillin were used for antibiotic selection of the plasmid. To analyse the bacterial colonies for recombinants, 10 ml cultures were grown up in TB and their plasmid DNA isolated by DNA 'Mini-preps' (Section 2.1.3.3) and analysed by restriction enzyme digestion (Section 2.1.1.3).

2.1.2.8 Freezing bacterial cell stocks (glycerol stocks)

After identification of recombinant clones, a colony was streaked on to a LB-amp plate and left at 37°C overnight. A single colony was then grown overnight in 5 ml LB media. 750 µl of this cell culture were then mixed with 250 µl of 50% sterile glycerol at -20°C, 'flash-freezed' in a dry ice/ethanol bath and stored at -70°C.

2.1.3 Preparation of DNA

2.1.3.1 Ethanol precipitation of nucleic acids

Nucleic acids were precipitated by the addition of 0.1 volumes of 3 M potassium acetate pH 5.2 and 2 volumes of ethanol that had been stored at -20°C. Small volumes were made up to 100 µl before addition of salt and ethanol. The samples were mixed thoroughly and then incubated on dry ice for 15 min or on ice for 20 min, followed by centrifugation at 14,000xg for 20 min at 4°C. The nucleic acid pellets were washed with 70% (v/v) ethanol, dried under vacuum and resuspended in distilled water to the required concentration.

2.1.3.2 Phenol/chloroform extraction

TE saturated phenol was made according to Sambrook *et al.*, and mixed 1:1 with chloroform:isoamylalcohol (24:1) (Sambrook *et al.*, 1989). The volume of the DNA was adjusted to 200 µl and an equal volume of phenol and/or chloroform:isoamylalcohol added. The phases were mixed well by vortexing for several minutes and separated by centrifugation for 5 min. The upper aqueous phase was transferred to a fresh tube, leaving behind the protein impurities at the interphase. A phenol extraction was always followed by a chloroform extraction to remove any residual phenol and the DNA precipitated with ethanol as described above.

2.1.3.3 Small scale purification of plasmid DNA (Miniprep)

The following modified alkaline-lysis/PEG precipitation procedure (ABI) was used to produce plasmid DNA at sufficient purity for DNA sequencing using the *Taq* DyeDeoxy™ terminator cycle sequencing kit or for Sf9/mammalian cell transfections. It was also followed until the PEG precipitation stage for producing DNA for rapid identification purposes.

10 ml TB with an appropriate amount of antibiotic, was inoculated with a bacterial colony or 10 µl glycerol stock, and grown overnight with shaking at 37°C. The culture was pelleted by centrifugation for 10 min at 3000xg at 4°C. The supernatant was removed and the pellet resuspended in 200 µl GTE buffer (50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0). 300 µl of freshly prepared 0.2 N NaOH/1% (w/v) SDS were added, mixed and incubated on ice for 5 min. The solution was neutralised by addition of 5 M potassium acetate, pH 5.2, mixing and incubating on ice for 5 min. Cellular debris was removed by centrifuging for 10 min in a microcentrifuge and the supernatant was transferred to a clean tube containing RNase A (Boehringer Mannheim) to a final concentration of 20 µg/ml. After RNase treatment at 37°C for 1 h, the supernatant was extracted twice (or only once for rapid mini preps) with 400 µl of chloroform, mixing by hand for 30 sec and centrifuging for 1 min to separate the phases. To precipitate the DNA from the aqueous phase, an equal volume of 100% isopropanol was added and immediately microcentrifuged for 10 min at room temperature. The DNA pellet was washed with 70% ethanol, dried under vacuum for 3 min and resuspended in 20µl ddH2O. For sequencing grade purity, the plasmid DNA in 32 µl ddH2O, was precipitated with 8 µl 4 M NaCl and 40 µl autoclaved 13% PEG$_{8000}$. After thoroughly mixing, the sample was incubated on ice for 20 min and the plasmid DNA pelleted by centrifugation for 15 min at 4°C in a fixed-angle rotor. The supernatant was removed, the pellet washed with 70% ethanol, dried under vacuum and resuspended in 20 µl ddH2O (or sterile ddH2O if the DNA is to be used in Sf9/mammalian cell transfections) and stored at -20°C.

2.1.3.4 Large scale purification of plasmid DNA (Maxiprep)

This purification procedure by caesium chloride gradient centrifugation was used to produce large amounts of high purity plasmid DNA (Sambrook *et al.*, 1989). 500 ml TB media supplemented with ampicillin, were inoculated with 10 µl bacterial glycerol stock or a bacterial colony and grown overnight at 37°C. Bacterial cells were pelleted by centrifugation at 5000xg for 20 min at 4°C, drained well and resuspended in 20 ml of GTE (50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl pH 8.0). 40 ml of a fresh solution containing 0.2 M NaOH and 1% (w/v) SDS were added and the tube was inverted several times and incubated on ice for 5 min. The DNA was purified from proteins by precipitation using 20 ml of 5 M KAc pH 4.8 on ice for 10 min and then centrifugation at 8000xg for 10 min at 4 °C. The supernatant was transferred to a clean GSA tube and nucleic acids precipitated by the addition of 0.6 volumes of isopropanol and centrifugation at 8000xg for 20 min. The DNA pellet was then washed with 20 ml ethanol, left to air dry and resuspended in 4 ml 50 mM Tris-HCl pH 8.0/10 mM EDTA (TE). The pH of the solution was checked and adjusted to pH 7.5-8.0 with 2 M Tris-base if required. The dissolved pellet was made up to 4.5 ml with TE and added to a tube

containing exactly 5g of CsCl. When the CsCl had dissolved, 0.5 ml of 10 mg/ml ethidium bromide was added and the solution subjected to centrifugation at 10 000xg for 10 min to remove any cellular debris. The supernatant was then transferred to Beckman polyallomer quick seal ultracentrifuge tubes. The tubes were topped up with the TE/CsCl solution (ideal weight 9.3-9.9 g to ensure the correct density gradient is achieved) and centrifuged overnight at 200,000xg at room temperature. Closed circular plasmid DNA was isolated by drawing out the lower of the two florescent bands visible on UV illumination (the upper band contained chromosomal DNA). To remove the plasmid DNA, 2 ml of water-saturated butanol was added, vortexed and the top layer removed. This was repeated until all the ethidium bromide had been removed (5-6 times). The volume was made up to 3 ml with ddH$_2$O, 7 ml cold 100% ethanol were added and the mixture was left on dry ice for 15 min. The precipitated DNA was spun down at 10,000xg for 20 minutes at 4°C. The supernatant was removed, the pellet washed in cold 70% ethanol (to remove precipitated salt), left to air dry and then redissolved in 1 ml of TE buffer.

Alternatively, large amounts of purified plasmid DNA were prepared using the Wizard^{TM} Maxiprep DNA purification system from Promega and following the protocol supplied.

2.1.3.5 DNA quantification

To determine the concentration of plasmid DNA in ddH$_2$O, an OD$_{260nm}$ was taken at a 1/1000 dilution. An OD$_{260nm}$ reading of 1 was taken as being equal to a double stranded DNA concentration of 50 µg/ml. Quantity and quality of DNA samples were also checked by gel electrophoresis (Section 2.1.1.7).

2.1.4 Automated DNA sequencing

2.1.4.1 Principle

All DNA sequences of constructs containing PCR products or clones derived from cDNA library screens were checked by sequencing using the *Taq* DyeDeoxy^{TM} Terminator Cycle Sequencing kit for use with the Automated Fluorescent Laser ABI Model 373A DNA sequencer^{TM} (Applied Biosystems). This kit relies on four dye-labelled dideoxy nucleotides: G, A, T and C DyeDeoxy terminators. When these terminators replace standard dideoxy nucleotides in enzymatic sequencing, a dye label is incorporated into the DNA along with the terminating base. All four termination reactions can be performed in one tube.

2.1.4.2 Cycle Sequencing

The following reagents were mixed in a 0.6 ml microcentrifuge tube: 8.0 µl of reaction premix (contains DyeDeoxy^{TM} dNTPs and Ampli*Taq* DNA polymerase), 0.5 µg of double-stranded template DNA (prepared using the alkaline lysis/PEG precipitation protocol section 2.1.3.3) and 3.2 p mol (approximately 20 ng) of primer. The final reaction volume was made up to 20 µl with ddH$_2$O and overlaid with a drop of mineral oil. The PCR cycle sequencing reaction was performed using a Perkin-Elmer Cetus DNA Thermal Cycler under the following conditions: denaturation cycle at 96°C for 30 sec, annealing at 50°C for 15 seconds and polymerisation at 60°C for 4 minutes. After 25 cycles the mineral oil was removed, and the extension products were precipitated by adding 2 µl of 3 M sodium acetate, pH 4.6 and 50 µl of 95% ethanol. This was vortexed and incubated on ice for 10 min, centrifuged in a microcentrifuge at top speed for 15 min and the pellet washed with 70% ethanol and dried under vacuum for 3 min.

2.1.4.3 Preparing and loading samples

500 ml of sequencing gel loading buffer were made up using a 5:1 ratio of de-ionised formamide:50 mM EDTA, pH 8.0. Blue dextran was added to give the buffer colour and to make loading easier. 3 µl of gel loading buffer was added to each sample, vortexed and then heated at 90°C for 2 minutes and loaded on to the sequencing gel. Reagents from the Sequagel Sequencing System (National Diagnostics) were used to make the sequencing gel: 5 ml Buffer, 33 ml Diluent, 12 ml Concentrate, 400 ml fresh 10% (w/v) ammonium persulphate and 20 ml TEMED. The gel was allowed to set for 2 h, samples loaded into wells made by using a sharks-tooth comb and 1 X Tris Borate EDTA (TBE) buffer, pH 8.3 (from 10 X TBE stock solution supplied by BioWhittaker) added as electrode buffer. The samples were electrophoresed overnight on the Automated Fluorescent Laser DNA Sequencer^{TM} according to the system's manual settings. Using the AUTOASSEMBLER software (Applied Biosystems) sequence data could be edited, re-tracked and analysed before printing out or being entered into databases.

2.1.5 cDNA cloning techniques

2.1.5.1 Plaque assay for library screening

A U937 poly A selected cDNA library was made by Dr Stefano Volinia (LICR) using the Stratagene Lambda Zap I system was plated out on NZY agar plates (10cm) at various dilutions in order to deduce the correct titre for transfer: Serial dilutions of cDNA library were made (1:100, 1:1000, 1:10000 etc). 1 µl of each dilution was taken and added to 100 µl of XL-1 Blue cells (a colony from XL 1 Blue cells streaked onto a tetracycline plate was used to inoculate 10 mls of LB media with 10 mM MgSO$_4$. The cells were grown at 37°C overnight and spun at 3000x*g* for 10 min. at 4°C and resuspended in 5

ml 10 mM MgSO4). The virus and XL-1 Blue cells are incubated at 37°C for 10 min. The virus/library dilution mixture is mixed with 3 mls melted NZY top agarose and poured onto a 10 cm NZY agar plate. The plates are incubated inverted at 37°C overnight.

2.1.5.2 Library plating

From the titre assay the dilution of virus required to give 0.5 million clones per 530 cm^2 square plate can be calculated. The procedure for library plating is essentially the same as that for plaque assays. The required library dilution made up in SM buffer (0.1 M NaCl, 15 mM MgSO4.H2O, 50 mM 1M Tris-HCl pH 7.5 and 0.01% (w/v) gelatin) is added to 1 ml of XL 1 Blue cells (prepared as described in section 2.1.5.1) and incubated at 37°C for 15 min. This is then mixed with 30 mls of melted NZY top agarose and poured onto a square 530 cm^2 NZY agar plate. The plates are incubated inverted at 37°C overnight.

2.1.5.3 Generation of cDNA probe

A partial cDNA sequence from the unidentified human gene HFBEP44 was observed to encode amino acids that are homologous to peptide kV^F/$_T$AIQDPXLP from p150 (Chapter 3). Primers encompassing the partial cDNA were designed (5'-CCAGATCCTTTCTGTAG and 5'-GAATGGACGGGTACTGATGCGATC) and PCR was performed using U937 cDNA library as template (Lambda Zap cDNA^{TM} Stratagene) using the following conditions: Denaturation, 94°C for 30 seconds, Annealing: 55°C for 15 seconds and Polymerisation, 72°C for 30 seconds (30 cycles). The resulting PCR product was run on a 1.5% LMP gel, excised gel purified and radioactively labelled using random sequence hexanucleotides to prime DNA synthesis.

2.1.5.4 Random priming of cDNA probes

Feinberg and Vogelstein, introduced the use of random sequence hexanucleotides to prime DNA synthesis on denatured template DNA at numerous sites along its length (Feinberg and Vogelstein, 1983). The ability of the Klenow fragment of DNA polymerase I to prime DNA synthesis in the 3'-5' direction without 5'-3' exonuclease activity ensures that labelled nucleotides incorporated by the polymerase are not subsequently removed as monophosphates. This approach therefore, generates a stable probe of high specific activity. A multiprime DNA labelling kit (Amersham) was used to label DNA fragments, often generated by PCR, by this method.

The DNA sample was first denatured by heating to 100°C for 2 min in a boiling water bath and then chilled on ice. The following reaction was then set up on ice in an Eppendorf tube: 200 ng DNA, 10 μl soln 1 (dATP, dGTP and dTTP in Tris-HCl pH 7.8, MgCl2 and β-mercaptoethanol), 5 μl primer (hexanucleotides), 5 μl dCTP (α-^{32}P

0.5 mCi; Amersham), 2 units DNA polymerase I Klenow fragment and water to 50 µl. The contents were gently mixed by pipetting and then incubated at 37°C for 30 min. Unincorporated [α-^{32}P]dCTP was removed by passing through NucTrap^{TM} push columns (Stratagene) as described below.

2.1.5.5 Purification of labelled probes

NucTrap^{TM} push columns (Stratagene Cloning Systems) were used to separate unincorporated nucleotides from radiolabelled DNA probes. The column was first pre-wet with 70 ml of 1 X STE (100 mM NaCl, 20 mM Tris-HCl pH 7.5, 10 mM EDTA). A syringe was attached to the column, inserted into the push column device (Stratagene) and the plunger pushed down until drops came out of the end of the column. The radiolabelled sample was then loaded in a volume of 70 ml, on to the top of the column and the column pushed down through the shielded device. The eluent was collected in an Eppendorf tube at the base of the device. The column was then washed 3-5 times with 70 ml volumes of STE and collected separately. The unincorporated small molecules remained in the push column resin. Fractions containing the majority of the labelled probe were pooled and used immediately for hybridisation.

2.1.5.6 Screening library filters by DNA hybridisation

Prior to screening, library plates were cooled for 2 h at 4°C. The plaques from the plated library were transferred onto nitrocellulose filters (Hybond-N Amersham) for 1 min. The filters were pricked with a needle through the membrane into the agar for orientation, and duplicate lifts taken for each plate (2 min). After transfer, the filters were denatured by submerging in denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 2 mins; neutralised in solution (1.5 M NaCl, 0.5 M Tris-HCl pH 8.0) for 5 mins and then rinsed in 0.2 M Tris-HCl pH 7.5 and 2x SSC (300 mM NaCl, 30 mM Na3Citrate) for 2 min. The filters were baked in a vacuum oven at 80°C for 2 hours. The filters were then pre-wet in ddH2O and pre-hybridised (to prevent non-specific binding of labelled probe) in Church hybridisation mix (0.5 M Sodium phosphate pH 7.2, 7% SDS, 1 mM EDTA, pH 8.0) for 2 hours at 65°C. On addition of radiolabelled probe the filters are hybridised overnight at 65°C. Filters were washed using 0.5x SSC, 0.1% SDS twice for 20 min at 60°C, wrapped in Saran wrap and autoradiographed at -70°C. Plates were aligned on the developed films and putative positives were picked, the agar plugs dissolved and diluted in SM buffer (1/1000) and the phage re-plated (on 10 cm NZY plates) and re-screened as before for secondary and tertiary screens. Once single positive plaques had been isolated, *in vivo* excision of the pBluescript phagemid from the Lambda Zap II vector was carried out using the ExAssist/SOLR^{TM} system (as described by Stratagene) for subsequent Southern blot analysis and sequencing.

2.1.5.7 Southern blotting

PCR products or restriction digest analysis of positive rescued clones, were separated by agarose gel electrophoresis as described in section 2.1.1.7. The DNA fragments were denatured by incubating the gel in 1.5 M NaCl/0.5 M NaOH for 30 min and then neutralised in 1.5 M NaCl/0.5 M Tris-HCl, pH 8.0 for a further 30 min with gentle agitation. The gel was washed in 2 X SSC. and the DNA was transferred from the gel to a nitrocellulose filter by capillary blotting in 10 X SSC. The filter paper was placed on top of the gel with 4 pieces of 3 MM Whatmann paper and a stack of paper towels. A weight was placed on the top and transfer was allowed for overnight. Complete transfer was checked under a hand-held UV lamp and the filter was pre-hybridised using Church hybridisation buffer at 65°C and then hybridised with a radiolabelled probe overnight at the same temperature. The filter was washed and autoradiographed described as in section 2.1.5.6.

2.2 Cell culture methodology

2.2.1 Mammalian cells

2.2.1.1 Cell lines

The table below lists the cell lines cultured and used for biochemical or immunofluorescence analysis.

Table 2.1. Description of cell lines cultured

Name	Cell description
U937	Human monoblastic leukaemia
Jurkat	Human T-cell leukaemia
COS-7	African green monkey kidney fibroblasts (SV40 transformed).
MDCK	Madin Darby canine kidney epithelial cells.

2.2.1.2 Tissue culture media and general cell culture techniques

Subculturing procedures for all cell lines were carried out in a laminar flow hood in a sterile environment using media/reagents, that were all prewarmed to 37°C. All cells were kept at 37°C in a humidified atmosphere containing 10% CO_2. Cell lines in suspension (U937 and Jurkat) were maintained routinely in stationary suspension culture in RPMI-1640 medium supplemented with 5-10% (w/v) foetal calf serum (FCS), L-glutamine, 25 mM HEPES and 100 µg/ml penicillin/streptomycin (Gibco BRL). Cells in suspension were grown in 40 ml media in 75 cm^2 T-flasks and subcultured (twice weekly) at a 1/5 split at a cell density of 5-9x10^5 cells/ml. Adherent monolayer cells (COS-7, MDCK) maintained in Dulbecco's modified Eagle's medium (DMEM),

supplemented with 10% (w/v) FCS and 100IU/ml of antibiotic-antimycotic (Gibco BRL). Monolayer cells were grown in 10 ml media in 10 cm dishes and subcultured (twice weekly) at a 1/10 split when cells were 70-80% confluent. Medium was removed from confluent cells, cells were rinsed twice with versene (Gibco BRL) and 0.5 ml trypsin (trypsin; Gibco BRL 25050-014, diluted 1/5 with versene) added. The tissue culture dish was tapped to dislodge cells from the surface and the cells were suspended in DMEM media and split into 10cm dishes.

2.2.1.3 *In vivo* labelling with [^{35}S]-methionine or [^{32}P]-orthophosphate

To label cells with ^{35}S methionine, U937 or Jurkat (1-5x10^7 cells) were spun down at 1000x$g$for 5 min and the pellet resuspended in 10 ml methionine-free RPMI medium supplemented with Di-sodium cystine, glucose, L-glutamine, L-arginine, i-inositol and L-leucine (100 X from Gibco BRL). The cells were incubated in a 50 ml falcon tube at 37°C for 1 h, after which they were re-pelleted as before. The pellet was resuspended in 5 ml methionine-free RPMI medium with the supplements and in addition, 10% dialysed FCS. 30 µl of [^{35}S]-methionine (300 µCi; Amersham) were added and the cells incubated at 37°C, 10% CO$_2$ in a small flask, overnight. Cells were harvested the next day and washed once with cold PBS.

To label cells with ^{32}P-orthophosphate, U937 or Jurkat cells were spun down (1000xg), washed twice with phosphate-free DMEM supplemented with the above amino acids and resuspended at 2x10^7 cells/ml in the same phosphate-free DMEM with an additional 1% dialysed FCS. Cells were then labelled with 1 mCi/ml [^{32}P]-orthophosphate (acid and carrier free; Amersham) for 4 h at 37°C. After this time, radiolabelled cells were incubated in the presence or absence of PMA (Phorbol 12-Myristate 13-Acid; 10 ng/ml) for 20 min, harvested and washed once with cold PBS.

Labelled cells were lysed, immunoprecipitationperformed using the supernatants and the radiolabelled proteins separated by SDS-PAGE analysis (section 2.3.5) and visualised by autoradiography. Prior to drying gels for autoradiography, gels with [^{35}S]-labelled samples were processed with fluorographic reagent (Amplify; Amersham) according to the manufacturer's instructions, for signal enhancement.

2.2.1.4 DNA transfection of mammalian cells

Quality plasmid DNA constructs for transfection were prepared form minipreps using the alkaline lysis/PEG precipitation procedure (section 2.1.3.3), alternatively the Maxiprep procedure from Promega, was employed and the DNA re-precipitated under sterile conditions. For transient transfections electroporation was used, electroporation allows

the reversible breakdown of cell membranes caused by a high-voltage discharge which enables a rapid, simple and efficient method for introducing DNA into mammalian cells (Knutson and Yee, 1987). Routinely, COS-7 cells were used for electroporation of the plasmid DNA. COS-7 cells (70-80% confluent) in 10cm dishes were split into 15 cm dishes 24 h before transfection. For each electroporation event $1\text{-}2\text{x}10^7$ cells in log phase are required. For transfection, cells were trypsinised and counted (usually, a 70-80% confluent 15 cm dish provided enough cells for one electroporation event). The cells were spun at 700xg for 5 min at 4°C, washed once in HEPES Buffered Saline (HBS; 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM glucose and 21 mM HEPES, pH 7.1, this is filter sterised and kept as 30 ml aliquots at -20°C) and then re-pelleted at 700xg for 5 min at 4°C. The pellet was resuspended in HBS such that there were $1\text{-}2\text{x}10^7$ cells in 0.5 ml per electroporation. 1-20 μg of sterile DNA was added to 0.5 ml cells in an electroporation cuvette (Bio-Rad, 0.4 cm), agitated slightly, and left for 10 min at room temperature before pulsing in a Genepulser (Bio-Rad), set at 260 V, 960 mF. The time constant was noted for each pulse. A sample was pulsed with empty vector as a control ('mock' transfection). Cuvettes were then agitated slightly and left at room temperature for 10 min, 1 ml of medium was then added to each cuvette and the cells transferred to 10 ml of media in a sterile tube for each transfection. At this stage 100 μl of the transfected cell suspension can be seeded onto a glass coverslip for immunostaining (after 36 h). The remaining cells are made up to 15 ml with DMEM and plated onto a 15cm tissue culture dish and incubated at 37°C, 10% CO_2. Transfected cells were analysed after 36 h.

2.2.1.5 Freezing cells

On acquiring a new cell line or on the production of a novel transfected cell line, multiple stock back-up samples were always made. $1\text{-}5\text{x}10^6$ cells/ml were pelleted at 700x g for 5 min. The medium was aspirated in the tissue culture hood and the cells carefully resuspended in fetal bovine serum with 10% dimethylsulphoxide (DMSO). 1 ml of cells was transferred to labelled cryotubes (Nunc, Denmark) and placed in a slow-cooling container (Nalgene) at -70°C overnight. The next day, the tubes were 'snap-frozen' in liquid nitrogen and stored in the liquid nitrogen vessels.

2.2.2 Insect cells

2.2.2.1 Tissue culture media and general cell culture techniques

Spodoptera frugiperda (Sf9) cells were maintained as described by Summers and Smith, (Summers and Smith, 1987). All culture medium was incubated at 27°C prior to use and the cells routinely incubated at 27°C. The culture medium used throughout was IPL4-1 (Gibco BRL) supplemented with 10% (v/v) FCS, 2% (v/v) yeastolate, 1% (v/v) lipid

concentrate, 1% (v/v) Fungizone and 0.1% (v/v) Gentamycin (all available from Gibco BRL). Sf9 cells have a doubling time of 18-24 hours in supplemented IPL4-1 and were therefore subcultured at least twice a week. Confluent cells were routinely subcultured in a 175cm^2 flask by tapping the side of the flask gently with minimal foaming to dislodge cells and 2 ml of culture transferred to another flask containing 18 ml of fresh IPL4-1 media. The flask was rocked to evenly distribute the cells and incubated at 27°C.

2.2.2.2. Transfection of Sf9 cells

Plasmids containing a foreign gene were transferred to a modified version of the baculoviral *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) genome using Lipofectin (Gibco BRL). The modified AcMNPV (Baculogold, Pharmingen) contains a lethal deletion, so the baculovirus does not survive in tissue culture. Only co-transfection of the viral DNA with a complementing plasmid construct reconstitutes viable virus particles inside insect cells. Consequently, 99% of the virus particles derived from plasmid-rescued viruses using Baculogold are recombinant viral particles expressing the foreign gene cloned. For transfection 3x10^6 Sf9 cells were seeded into a 25 cm^2 flask and allowed to attach for 15 min, after which the medium was carefully aspirated off and the cells washed twice in IPL4-1 media without FCS. 1.5 ml of IPL4-1 media without FCS was added to the flask and left at room temperature. 1 μg of Baculogold DNA, 2 μg of recombinant transfer plasmid (containing the protein cDNA sequence), 8 μl of Lipofectin (Gibco BRL) and sterile ddH$_2$O to a final volume of 24 μl were mixed in a sterile tube. After 15 min, the DNA solution was added to the cells dropwise, the flask swirled and incubated at 27°C overnight. The transfection media was then replaced with 5 ml of fresh IPL4-1 containing FCS and left at 27°C for 4 days. After this time, the virus created by the transfection was harvested and amplified (as described in 2.2.2.4) to a passage number suitable for testing protein production.

2.2.2.3 Plaque assay

This procedure was performed to ensure the production of a single recombinant virus clone. 3x10^6 viable Sf9 cells were seeded on to 5 cm gridded dishes in fresh complete IPL4-1 medium. The cells were allowed to attach for 30 min while serial dilutions of virus inoculum in 5 ml were prepared (10^{-1} to 10^{-6}). Once the cells had adhered to the surface of the dish, the medium was aspirated off and 2 ml of the diluted inoculum were gently added to each plate. Infection was allowed to occur for 1 h at 27°C and in addition, a negative control of uninfected cells was set up. Meanwhile, a final 1% agarose overlay was prepared from a 2% stock, heated and mixed with pre-warmed 2 X Grace's medium with a double concentration of additives (to 100 ml 2 X Graces: 20 ml FCS, 2 ml fungizone, 200 ml gentamicin) in a 38°C waterbath. After the 1 h incubation period, the virus was aspirated off from the cells and 4 ml of the overlay were carefully added to

the edge of the dish so as not to disturb the cells. The dishes were left undisturbed for 1 h to allow the agarose to solidify, after which they were placed into a sandwich box containing damp tissues, the box sealed and incubated for 7 days at 27°C. Recombinant plaques were identified visually and examined under a light microscope. Plaques were picked with a pasteur pipette, reamplified and analysed for protein production.

2.2.2.4 Infection of Sf9 cells

Cells were counted and seeded into flasks at the appropriate density:

Table 2.2: Appropriate seeding densities for typical vessel sizes.

Vessel	Cell density	max. virus vol.	Final vol.
25cm^2 flask	3x10^6	1ml	5ml
75cm^2 flask	9x10^6	2ml	10ml
175cm^{2}flask	2x10^7	4ml	20ml
450cm^2 (3 layered)	1.4x10^8	8ml	60ml

Cells were allowed to attach for 15 min at room temperature. For virus amplification, cells were allowed to attach in a 25 cm^2 flask and were incubated with 0.5 ml of the original plaque purified virus for 4 days. The virus was harvested by centrifugation at 2000xg and then 1 ml was used to infect cells in a 75 cm^2 flask. After harvesting 4 days post-infection, 1 ml of this virus stock was used to infect cells in a 175 cm^2 flask. Virus from this amplification was then used to build up a passage-4 virus stock which could then be titred for optimum protein production. To optimise protein production a number of infections were set up using varying dilutions of the virus stock in 75 cm^2 flasks, i.e. from 10 µl to 500 µl. The infected cells were harvested 2.5 days post infection by gently hitting the flask against a surface, to dislodge the cells. The cells were spun at 900xg for 5 min, the supernatant decanted and the cells washed once in PBS. These cells were then lysed as described in section 2.3.1.2 and virus titred by infecting cells at different dilutions of virus and protein production examined by means described in section 2.3.3.

2.2.2.5 In vivo labelling of Sf9 cells with [^{3}H]myristic acid

To analyse the post-translational modification of proteins by N-terminal myristoylation, Sf9 cells (3x10^6) in 25 cm^2 flasks were infected with recombinant baculovirus. 24 h postinfection, infected cell monolayers were washed once with labelling medium (IPL41 which has 1% yeastolate as its only supplement). 1 mCi of [9,10-^{3}H]myristic acid (Amersham) in 1ml of ethanol was dried under nitrogen to 100 µl. Sf9 monolayers were labelled for 16 h with 160 µCi/ml of [9,10-^{3}H]myristic acid in a 1 ml volume. Cells were dislodged from the surface of the tissue culture flask by gentle tapping, harvested by

centrifugation at 700x*g* for 10 min at 4°C and washed once with cold PBS. Labelled cells were lysed, immunoprecipitates performed using the supernatants and radiolabelled proteins separated by SDS-PAGE analysis (sections 2.3.3 and 2.3.5). The gel was treated for fluorography using Amplify (Amersham), dried and autoradiographed.

2.3   Analysis of cellular proteins

2.3.1   Cell Lysis

2.3.1.1   Preparation of whole cell lysates

Cells in suspension were washed twice with PBS and resuspended in an equal volume of TE (10 mM Tris pH 8.0, 1 mM EDTA) and 2x sample buffer (250 mM 1 M Tris-HCl pH 6.7, 20% (v/v) glycerol, 4% (w/v) SDS, 3% (w/v) DTT, 0.01% (w/v) bromophenol blue). Monolayer COS-7 cells (70-80% confluent 15cm dishes) were washed twice in PBS and scraped in 200 μl of 2x sample buffer. The samples were passed through an 18 gauge syringe needle to shear chromosomal DNA and then heated to 100°C for 5 min and analysed by SDS-PAGE.

2.3.1.2   Triton X-100 lysis of cells

Cells in suspension were washed twice in ice-cold phosphate-buffered saline (PBS) and resuspended in 10 times the pellet size volume, with ice-cold Extraction Buffer (EB; 10 mM Tris HCl pH 7.5, 150 mM NaCl, 50 mM NaF, 5 mM EDTA, 1% (v/v) Triton X-100, 0.1 mM sodium orthovanadate, 1 mM aminoethyl-benzenesulphonyl fluoride (ABSF; Melford Labs.), 5 mM Benzamidine 100 kallikrein inhibitor units/ml of aprotinin, and 20 μM leupeptin). For monolayer COS-7, cells were washed twice with ice-cold PBS and then scraped in 200 μl of 2x EB. The cell lysate was passed through a 25 gauge syringe needle 5 times to aid cell lysis and incubated on ice for 15 min, after which it was centrifuged at 14,000x*g* for 20 min at 4°C to pellet the Triton X-100 insoluble cell debris. The supernatant "S" fraction was then removed to a clean tube and used for immunoprecipitates or with the addition of 5x sample buffer [0.5M Tris-HCl, pH 6.7, 50% (v/v) glycerol, 10% (w/v) SDS, 5% (w/v) DTT and 0.01% (w/v) bromophenol blue] resolved directly by SDS-PAGE. 5x sample buffer was also added to the remaining Triton X-100 insoluble pellet "P" fraction and used for biochemical analyses.

2.3.2   Estimating protein concentration

To estimate protein concentration in solution, the Pierce Coomassie Protein Reagent was employed. This is based on the absorbance shift from 465 to 595 nm which occurs when Coomassie brilliant blue G-250 binds to proteins in an acidic solution 0.5 ml of reagent

was mixed with protein (1-10 ml) in 0.5 ml of ddH$_2$0 and the absorbance monitored at OD$_{595}$ and compared with a blank (no protein). The protein concentration was then determined by comparison with a bovine serum albumin standard curve.

2.3.3 Immunoprecipitation and affinty purification of proteins

Lysates were prepared as described in Section 2.3.1.2 and incubated with an appropriate antibody on a rotating wheel for 2 h at 4°C. Affinity purified rabbit polyclonal antibody (5 μl) or IgG$_1$ EEmAb (5-10 μl) was used. Protein A- and protein G-Sepharose was then added to bind rabbit polyclonal and mouse monoclonal antisera respectively and the incubation continued for a further 1 h. The immune complexes were then collected by low speed centrifugation (1000xg) and washed 4 x in cold lysis buffer. The samples were boiled in 2 x sample buffer and analysed by SDS-PAGE. Cell lysates containing recombinant GST fusion proteins were incubated with glutathione Sepharose beads (Pharmcia) for 1 h on a rotating wheel at 4°C. After 1 h immobilised proteins were collected by low speed centrifugation, washed and analysed by SDS-PAGE as described above.

2.3.4 *In vitro* protein kinase assays

Protein kinase activity was detected using *in vitro* protein kinase assays on immobilised recombinant or endogenous cellular proteins. After immunoprecipitation or affinity purification protein complexes were washed 3 times in EB (extraction buffer, section 2.3.1.2) and washed once in kinase buffer (50 mM HEPES pH 7.5, with the addition of divalent cation, 10 mM MnCl$_2$ or 10 mM MgCl$_2$, or 10 mM CaCl$_2$) and then resuspended in 30 μl kinase buffer containing 10 μCi [γ-^{32}P] ATP (Amersham). Samples were incubated at 30°C for 30 min, and then the reaction terminated by boiling in 5 x sample buffer. Phosphorylated proteins were then analysed by autoradiography of SDS-PAGE gels. Protein kinase assays were also performed using the above kinase buffer with the addition of 10% (v/v) of glycerol or 15 μM ATP, alternatively other kinase buffers were used which contained 30 mM Tris-HCl pH 8.0 with 10 mM divalent cation (as above) in the absence or presence of 10% (v/v) glycerol, 1 mM DTT or 15 μM ATP.

2.3.4.1 Phosphatase treatment

Proteins can be dephosphorylated by incubating in the presence of phosphatases. Alkaline phosphatase (Boehringer Mannheim) is a non-specific phosphatase. This phosphatase was used to examine the phosphorylation state of recombinant proteins phosphorylated *in vitro*.

Protein complexes were immuoprecipitated or affinity purified from Sf9 cells. Immobilised proteins were washed twice in EB, twice in protein kinase buffer and then

phosphorylated for 30 min at 30°C in the presence of 10 μCi [γ^{32}P]ATP. Radiolabelled proteins were washed extensively with EB and then washed twice in alkaline phosphatase buffer (50 mM NaCl, 10 mM Tris HCl [pH 7.4], 10 mM MgCl$_2$, 1 mM DTT). Samples were incubated for 20 min at 30°C with 10 units of alkaline phosphatase. After several washes samples were resolved using SDS-PAGE and analysed by autoradiography.

2.3.4.2 *In vitro* protein kinase assays using exogenous peptide substrates

The protein kinase activity of immobilised recombinant proteins was additionally determined by incubating the immobilised protein with 30 μl of a phosphorylation mixture containing 0.1 μCi [γ-^{32}P]ATP, 30 mM Tris-HCl pH 8.0, 20 mM MgCl$_2$ or MnCl$_2$ and 3.0 mg/ml of protein or peptide substrate. Substrates used (all from Sigma) were: Protein kinase C substrate (PLSRTLSVATAKK), Myelin basic protein (MBP), Kemptide (LRRASLG), Tyrosine protein kinase substrate (RRLIGDAGYAARG), p34^{cdc2} protein kinase substrate (ADAQHATPPKKKRKEDPKDF), Histone and Syntide 2 (PKTPKKAKKL). After 30 min of incubation at 30°C the kinase reaction was stopped by spotting 25 μl of the supernatant on P-81 phosphocellulose paper. The [γ–^{32}P]ATP was separated from the labelled substrate by washing the P-81 filter papers 5x for 5 min in 75 mM H$_3$PO$_4$. The papers were dried and the radioactivity incorporated into the protein or peptide substrates determined by Cerenkov counting.

2.3.5 SDS-Polyacylamide gel electrophoresis (PAGE) analysis of proteins

Proteins were fractionated by size using the discontinuous electrophoresis system as described by Laemmli, (Laemmli, 1970). SDS-PAGE uses an upper stacking gel and a lower resolving gel. The stacking gel is at a lower percentage of acrylamide (pH 6.7) than the resolving gel (pH 8.8). This ensures that the proteins run through the stacking gel as tight bands and are only separated when they begin to migrate through the higher pH of the resolving gel. The percentage of acrylamide used depends on the size of the protein of interest. 12.5% - 7.5% resolving gels were used depending on the size of the proteins of interest; lower percentage gels for high molecular mass proteins and high percentage resolving gels for low molecular mass proteins. The resolving gel was mixed from a stock solution of 30% acrylamide (acrylamide:bis 30:0.8), and Tris-HCl (pH 8.8) and SDS were added to final concentrations of 375 mM and 0.1% (w/v) respectively. Polymerisation was initiated with the addition 0.05% (w/v) ammonium persulphate and 0.005% (v/v) TEMED. The gel was then quickly poured between the plates to approximately 3/4 of the height of the plates and overlayed with water-saturated butanol. When the gel was set, the butanol was poured off and the surface of the gel washed well with water. The stacking gel (4.5% (w/v) polyacrylamide) was prepared in the same way

but with Tris-HCl pH 6.7, layered over the resolving gel and the comb inserted. Following polymerisation, the comb was removed, the wells flushed out and the samples boiled in 2x or 5x sample buffer, loaded with protein markers (Bio-Rad, broad range molecular weight standards or Sigma, prestained markers). Electrophoresis was then performed in running buffer (200 mM glycine, 25 mM Tris-HCl pH 8.8, 0.1% (w/v) SDS), typically at 40 V overnight, or at 30mA with cooling for 3-5 h during the day.

2.3.5.1 Coomassie Blue stain

Following electrophoresis, some gels were stained for the presence of proteins by soaking in Coomassie Blue stain for 20 min 0.2% (w/v) Coomassie Blue R250, 45% (v/v) methanol and 10% (v/v) acetic acid, followed by destaining in 20% (v/v) methanol (v/v) and 7% (v/v) acetic acid with agitation. The gel was then dried under vacuum at 80°C for 2 h.

2.3.5.2 Silver stain

A more sensitive method for the staining of proteins was used if only nanogram quantities were present. After electrophoresis, the SDS-PAGE gels (0.75 mm) were silver stained in the following way. The gel was incubated for 5 min in a solution of 50% (v/v) methanol, 12% (w/v) TCA and 2% (w/v) $CuCl_2$, 5 min in solution A [10% (v/v) ethanol , 5% (v/v) acetic acid , 5 min in 0.01% (w/v) $KMnO_4$], 1 min solution A, 5 min 10% (v/v) ethanol, 5 min ddH_2O, 5 min 0.1% (w/v) $AgNO_3$, 20 sec in developer [2% (w/v) K_2CO_3, 0.01% (v/v) formaldehyde], a further 2-3 min in fresh developer until bands are seen and then 2 min in Solution A. After this point the gel was left in water and finally dried as above.

2.3.5.3 Detection of radiolabelled proteins in gels

To detect radiolabelled proteins from *in vitro* kinase assays (section 2.3.4) or cell metabolic labelling experiments (section 2.2.1.3 and 2.2.2.5), gels were first placed in Coomassie blue stain and then destained as described above to reveal protein bands/molecular weight markers, gels with [^{35}S]- or [^{3}H]-labelled samples were then flurographically processed by incubation for 30min at room temperature in Amplify (Amersham), to enhance radioactive signal. All gels were then dried under vacuum on 3 MM Whatmann paper under cling-film, for 1 h-2 h at 80°C. Gels were either exposed to X-ray film (Fuji medical X-ray film) at -70°C or analysed using a Phosphoimager (Molecular Dynamics).

2.3.6 Immunoblotting procedures

Proteins separated by SDS-PAGE can be selectively identified by specific antisera using the process known as immunoblotting or Western blotting.

2.3.6.1 Transfer of proteins

Following SDS-PAGE of samples with prestained markers (Sigma) the gel was immersed in transfer buffer [192 mMglycine, 25 mM Tris Base, 0.1% (w/v) SDS and 20% (v/v) methanol] for approximately 5 min. The gel was then placed on top of a piece of nitrocellulose paper (Schleicher & Schuell) or methanol-soaked Immobilon polyvinylidene difluoride (PVDF) membrane (Millipore Corporation), both of which were pre-soaked in transfer buffer, and sandwiched between several layers of pre-soaked Whatmann 3 MM on the transfer apparatus (semi-dry blotter, Bio-Rad). Bubbles were removed and with the nitrocellulose between the anode and the gel, the proteins were transferred from the gel on to the nitrocellulose/PVDF by applying a current dependent on the dimensions of the gel for 90min; mA= area cm^2 x 0.8. Alternatively, gels were "wet-blotted" onto nitrocellulose/PVDF using the following transfer buffer, [386 mM glycine, 50 mM Tris Base, 0.025% (w/v) SDS, and 10% (v/v) methanol] and the Trans-Blot^{TM} electrophoretic transfer cell, according to the manufacturer's instructions (Bio-Rad) at 180 mA for 8 h.

2.3.6.2 Enhanced Chemiluminescence (ECL)

This method, developed by Amersham, was subsequently used for all antibody detection because of the speed of the reaction and the exclusion of radioactivity. ECL is a light emitting, non-radioactive method for detection of immobilised specific antigens conjugated with horseradish peroxidase labelled antibodies. The system utilises a chemiluminescent reaction which takes place when the cyclic diacylhydazide luminol is oxidised in the presence of the catalyst hydrogen peroxide (H_2O_2). Following oxidation, the luminol is in an excited state which decays to the ground state via a light emitting pathway.

After transfer, the nitrocellulose/PVDF filter was incubated in blocking buffer (1 X PBS, 5% (w/v) Marvel milk protein, 0.05% (w/v) Tween-20) for 1 h at room temperature to block non-specific binding sites. The filter was then incubated with the primary antibody in a minimal volume of blocking buffer at the appropriate dilution (1/200 - 1/2000). Excess antibody was removed by rinsing the filter in blocking buffer twice and then washed 3 times for 5 min at room temperature. A species-specific horseradish peroxidase-conjugated second antibody (Amersham) was then applied in a minimal volume of blocking buffer for 1 h at room temperature at a dilution of 1:3000. The filter was then rinsed in blocking buffer twice and then washed 3 times for 5 min at room temperature. Immunoreactive proteins were detected by enhanced chemiluminescence. Equal volumes of ECL (Amersham) detection solutions 1 and 2 were mixed and added to the filter. The reaction was allowed to proceed for 1 min, the filter blotted dry, wrapped in saran wrap and exposed on X-ray film for 5 sec-5 min at room temperature.

2.3.6.3 Stripping and re-probing

Immunoblots could be stripped and reprobed, if the blots had been stored wet in Saran wrap at 4°C after immunodetection, using the following protocol. The membrane was immersed in stripping solution (100 mM 2-mercaptoethanol, 2% (w/v) SDS, 62.5 mM Tris-HCl pH 6.7) and incubated at 50°C for 30 min with occasional agitation. The membrane was then washed 2 x 10 min in PBS containing 0.1% (v/v) Tween-20 in a large volume of buffer. The membrane was then blocked and immunoblotted as described above.

2.4 Analysis of ^{32}P-labelled phosphoinositides

2.4.1 Assay of *in vitro* Phosphatidylinositide 3-kinase activity

PI 3-kinase assays were performed essentially as described (Whitman *et al.*, 1985), in a volume of 50 μl containing 20 mM Tris/HCl, pH 7.5, 100 mM NaCl, 3.5 mM MnCl$_2$, 40 μM ATP (0.2 μCi [γ-^{32}P]ATP) and 200 μg/ml phosphoinositide [PtdIns, PtdIns(4)P or PtdIns(4,5)P_2], (Sigma). After pre-incubating the enzyme and lipid for 20 min at 4°C, each reaction was initiated by the addition of [γ-^{32}P]ATP and allowed to proceed for 15 min at room temperature. To examine the effect of PI-TP on lipid kinase activity, PI-TP or Sec14p (0-2.6 μM final) was preincubated with 1 mM PtdIns at 37°C for 5 min, then added to recombinant PtdIns 3-kinase for 20 min on ice before addition of ATP. Assays were terminated with acidified chloroform/methanol (1:1 v/v) the lipids extracted and the lower organic phase spotted on thin layer Silica Gel-60 plates (Whatman) which were pre-treated with 1% (w/v) oxalic acid, 1mM EDTA in ddH$_2$O:methanol (6:4,v/v). Samples were resolved by thin layer chromatography (TLC) using the following solvent systems: for standard assays using PtdIns as substrate, the solvent used was chloroform/methanol/4 M ammonium hydroxide (45:35:10, v/v). For separation of PtdIns3P, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3, propan-1-ol:2M acetic acid (65:35, v/v) was used as the solvent system. For separation of PtdIns3P from PtdIns4P a borate buffer system was used (Walsh *et al.*, 1991). PtdIns 4-kinase assays were performed on A431 cell lysates in a volume of 50 μl containing 10 mM MgCl$_2$, 25 mM Tris/HCl pH 7.5, 70 μM ATP (0.2μCi [γ-^{32}P]ATP) and 1 mM PtdIns. Reactions were carried out as described for PI 3-kinase assays above. Radioactivity was analysed by autoradiography or quantified using a PhosphorImager (Molecular Dynamics).

2.4.2 Analysis of *in vivo* cellular phosphoinositide production

2.4.2.1 [^{32}P]-labelling and extraction of cellular lipids

COS-7 cells were transiently transfected by elctroporation (section 2.2.1.4) with pMT2SM construct or mock transfected and seeded onto 10 cm tissue culture dishes. After 36 h cells were washed twice in labelling media (phosphate free DMEM with Di-sodium cystine, L-methionine, glucose, L-glutamine, L-arginine, i-inositol and L-leucine supplements and 1% dialysed FCS). Cells were incubated in labelling media for 1 h prior to labelling. Cells were labelled with [^{32}P]-orthophosphate (acid and carrier free; Amersham) at 200 µCi/ml for 1 h at 37°C. Labelled lipids were extracted using the following method adapted from Jackson *et al.*, (Jackson *et al.*, 1992). The labelling media was aspirated and the cells from each 10 cm dish scraped into ice-cold 0.8 ml 1 M HCl and put into a screw-cap borosilicate tube. The dishes were rinsed twice with 0.1% (w/v) butylated hydroxy toluene antioxidant (BHT) made up in methanol and added to the sample tubes in addition to 1ml chloroform supplemented with 30 µg/ml of brain phosphoinositides (Sigma P6023). Methanol was also added until the samples were one phase (200 µl-1 ml). The samples were vortexed well and allowed to stand at room temperature for 15 min. 3 ml of chloroform and 0.5 ml of 1 M HCl was added, the samples vortexed for 10 min and then centrifuged at 3000x*g* for 5 min at 4°C (centrifugations after this point were performed at room ttemperature). The lower organic phase was transfered to a fresh tube and the remaining aqueous layer was washed twice with 1 ml chloroform:methanol:2.4 M HCl , 86:14:1 [this can be prepared from the lower phase of chloroform:methanol:2.4 M HCl, 8:4:3 (v/v)] and the resulting organic phases pooled. The pooled organic phase was then washed twice with 2 ml methanol:1M HCl, 25 mM EDTA:chloroform, 48:47:3 [this can be prepared as the upper phase of chloroform:methanol:1M HCl, 25 mM EDTA, 8:4:3 (v/v)]. the washed organic phases were transfered to fresh tubes, and the extracted lipids dried down under nitrogen and subjected to de-acylation (section 2.4.2.3).

2.4.2.2 Extraction of [^{32}P]-labelled phosphoinositides from TLC plates

To obtain PtdIns3*P*, PtdIns(3,4)*P*$_2$ and PtdIns(3,4,5)*P*$_2$ standards for HPLC analysis (section 2.4.2.4), *in vitro* PI 3-kinase assays using immobilised recombinant GST-p110α and PtdIns, PtdIns4*P* and PtdIns(4,5)*P*$_2$ as substrates were performed and resolved by TLC as described in section 2.4.1. Positions of the 3'-phosphorylated lipids were located by autoradiography marked on the TLC plate. The TLC plate was sprayed with ddH$_2$O and the 3'-phophoinositides in the silica gel were scraped into Eppindorf tubes ready for de-acylation.

2.4.2.3 De-acylation of [^{32}P]-labelled phosphoinositides

The following de-acylation procedure was used to generate glycerophosphoinositols (GroPIns) form [^{32}P]-labelled lipids, suitable for HPLC analysis (Clarke and Dawson, 1981). 1ml of methylamine reagent [33% methylamine in ethanol:ddH$_2$O:butan-1-ol, 50:15:5 (v/v)] was added to the 3'-phosphoinositides in silica gel (section 2.4.2.2) or dried cellular lipids (section 2.4.2.1) and incubated at 53°C for 50 min in a fume hood. After this time reaction products were dried down under nitrogen. Cellular lipids were dissolved in 1 ml ddH$_2$O wheras silica residues were washed with 0.5 ml ddH$_2$O followed by 0.35 ml H$_2$O twice and the washes pooled. Dissolved GroPIns were extracted 2x with 0.6 ml butan-1-ol mixture [butan-1-ol:petroleum ether:ethyl formate, 20:4:1 9 (v/v)]. The aqueous phase from these extractions was dried down in a SpeedVac. Extracted de-acylated lipids were resuspended in 0.5ml of HPLC grade H$_2$O together with ATP, GTP and ADP (200 µM final of each) as an internal calibration, ready for HPLC analysis, routinely, 1 µl of the resuspended GroPIns were added to 5 ml scintillation fluid and the radioactivity measured by Cerenkov counting.

2.4.2.4 HPLC analysis of glycerophosphoinositides

Anion exchange HPLC using a Partisphere Sax column (Whatman) was performed as previously described (Auger *et al.*, 1989b). The HPLC system consisted of pumps A and B, a digital controller, a mixing device and a 1ml injection loop. Pump A pumped HPLC grade H$_2$O and pump B pumped 1 M (NH$_4$)$_2$HPO$_4$ adjusted to pH 3.8 with H$_3$PO$_4$. GroPIns-containing samples were filtered through a 0.2 µm membrane (Whatman) and injected. Immediately after injection, samples were eluted using a linear gradient of (NH$_4$)$_2$HPO$_4$ at 1 ml/min. The elution gradient from 0 to 1M (NH$_4$)$_2$HPO$_4$ pH 3.8 was developed in 115 min. Dual pumps were used to establish the gradient (0% B for 10 min, to 25% B with a duration of 60 min then to 70% B for 30 min, reaching 100% B in 5 min for a duration of 10 min). Deacylated [^{32}P]-labelled PtdIns3*P*, PtdIns(3,4)*P*$_2$ and PtdIns(3,4,5)*P*$_2$ were used as standards in all column runs. ATP, GTP and ADP (200 µM each) were used as internal standards for each sample, their elution profiles were observed using an online UV detector. Radiolabelled eluate from the HPLC column flowed into an on-line continuous flow scintillation detector for isotope detection (using a Reeve model 9702 precision mixer and model 9701 radioactivity monitor) and data was transmitted to a Reeve 27K computer for further analysis.

2.5 Production of antibodies

2.5.1 Generation of rabbit antisera against p150

2.5.1.1 Coupling of peptides to keyhole limpet haemocyanin (KLH)

Polyclonal antibodies were raised against two C-terminal peptides of p150. Peptides 1 and 2 [Nt-VTASRDGIVKVWK-Ct (amino acids 1346-1358) and Nt-KQKVGPSDDTPRRGPESL-Ct (amino acids 1307-1324) respectively] were synthesised by Severn Biotech. Ltd. and coupled to activated keyhole limpet hemocyanin by a method modified from Sambrook *et al*, (Sambrook *et al.*, 1989). 20 mg KLH (Calbiochem) was made to 1 ml with PBS adjusted to pH 6.0 with HCl, dialysed against PBS pH 6.0 for 20 h at 4°C, tranferred to a flat-bottomed 5 ml polystyrene tube (Bibby Sterilin Ltd.) and made to 2 ml with PBS, pH 6.0. The KLH solution was stirred vigorously on a magnetic stirrer and 3 mg *m*-maleimidobenzoic acid N-hydroxysuccinimide ester (MBS, Sigma) in 100 µl dimethyl sulphoxide (DMSO) was added, under the surface of the KLH solution. The mixture was stirred at room temperature for 30 min and became blue/grey as coupling occurred, then the KLH-MBS was separated from unconjugated MBS and dimethylsulphoxide on a 20 ml bed volume Sephadex G-25 (Sigma) column. The column was equilibrated in PBS, pH 7.4, the sample was applied, then eluted in PBSA and 1 ml fractions were collected. After the void volume, two cloudy/grey fractions containing coupled KLH-MBS eluted and were pooled. 5 mg peptide 1 and 2 was dissolved in 400 µl PBS, pH 7.4 (5 µl 5 M NaOH aliquots were added to aid solubilisation), 1 ml (~10 mg) KLH-MBS was added to each peptide solution, then the mixtures were vortexed vigorously and stirred for 1.5 h. Each peptide-KLH solution was made to 3 ml with PBS pH 7.4 and stored at -20°C in 1 ml aliquots. Each aliquot contained approximately 3.3 mg KLH coupled to 1.7 mg peptide – enough antigen for one immunisation of two rabbits.

2.5.1.2 Immunisation schedule

Peptides 1 and 2 coupled to KLH were used for the immunisation of 4 rabbits (2 rabbits/peptide, performed by Eurogentec). The animals were boosted every month and the serum tested by immunoprecipitation and Western blotting (section 2.3.6). Four bleeds were taken and the third affinity purified on a carboxy-terminal peptide-Actigel affinity matrix as described below.

2.5.1.3 Affinity purification

The peptides used for immunisation were first coupled to an Actigel ALD-Superflow resin from Sterogene, whose monoaldehyde coupling chemistry couples to primary

amines, for affinity purification of the rabbit sera. 6 mg of the synthetic peptide was weighed out and dissolved in 500 μl of coupling buffer (100 mM phosphate buffer. pH 7.8) (Sambrook *et al.*, 1989). 2 X 1 ml bed volume of Actigel beads (6% agarose) in two separate 2 ml Eppendorf tubes, were washed several times with coupling buffer and then 250 μl of the solubilised peptide added to each tube in a final volume of 1.8 ml. Coupling of the peptide to the gel was initiated by adding 200 μl (1/10 of final volume) of ALD Coupling solution (1 M NaCNBH$_3$ supplied by Sterogene) resulting in a 0.1 M final concentration of the reducing agent. The tubes were incubated on a wheel for 6 h at 4°C and for 2 h at room temperature. The beads were then washed twice in 100 mM Tris, pH 8.0/500 mM NaCl and once in 100 mM Tris, pH 8.0. Beads were finally incubated with 100 mM Tris, pH 8.0 for 2-4 h to block uncoupled sites and stored in 20 mM Tris, pH 8.0 with 0.02% (w/v) sodium azide at 4°C. The coupled resin was then ready for affinity purification of the p150 anti-peptide antibodies and a 2 ml column was prepared and washed with PBS. 8 ml sera were centrifuged at 15,000xg, added to the beads and incubated in the closed column (Bio-Rad) on a rotating wheel at 4°C for 2 h. The sera were collected and the column washed extensively with PBS. Bound antibodies were eluted with 0.1 M glycine pH 3.0 and collected as 1 ml fractions in tubes containing 100 ml of 1 M Tris-HCl pH 8.0 (total 10 tubes) under gravity. Protein concentration was measured using 10 μl from each fraction, as described in section 2.3.2. Peak protein fractions were combined, dialysed twice against PBS and once against 50% glycerol/PBS and stored at -20°C.

2.5.2 Crosslinking antibodies

A method adapted from Harlow and Lane (Harlow and Lane, 1988) was used to cross-link purified polyclonal antibodies covalently to either Protein A- or Protein G-Sepharose. Crosslinking eliminates the need for separate incubations of antibody and Sepharose beads and, in addition, immunoglobulins remain on the beads after boiling and hence, are not visualised in subsequent immunoblotting analysis where they may mask immunoreactive proteins of a similar size.

Protein A- or G-Sepharose beads in an Eppendorf tube, were washed in 1 X PBS. Affinity purified antibodies (0.5 ml beads/ 200 μg of IgG) were added and incubated for 2 h on a wheel at room temperature. The beads were then washed three times in PBS and then twice in 0.2 M Borate pH 9.0 (warmed to 30-35°C in preparation to dissolve). Beads were then resuspended in 1 ml 0.2 M sodium borate pH 9.0 and antibodies cross-linked by the addition of 5.2 mg/ml of dimethylpimelimidate (20 mM final) and incubation for 30 min on a rotating wheel at room temperature. Beads were then washed once in 0.2 M ethanolamine pH 8.0 and the incubation continued with 0.2 M ethanolamine pH 8.0 for 2 h at room temperature. Finally, the Sepharose-bound

antibodies were washed twice in 0.1 M glycine pH 3.0, 3 X in PBS and stored in PBS/0.02% (w/v) sodium azide at 4°C.

2.6 Investigation of subcellular localisation using immunofluorescence

2.6.1 Primary antibodies

Affinity purified rabbit anti-PtdIns 3-kinase antisera (Volinia *et al.*, 1995) was used at a 1/200 dilution, as a control, pre-immune serum were used at a 1/100 dilution. Rabbit polyclonal antibodies raised against the following proteins were kind gifts: GM130 used at 1/200 (Dr Graham Warren, ICRF; (Nakamura *et al.*, 1995)), LAMP-1 used at 1/300 (Dr Mark Marsh, LMCB UCL), cathepsin D used at 1/200 (Dr Howard Davidson, Addenbrooke's Hospital Cambridge). The mouse IgG_1 monoclonal anti-EE('EFMPME')-tag antibody (EEmAb) was used at a 1/200 dilution (Frank McCormick, ONYX). The rabbit polyclonal and mouse IgG_1monoclonal anti-α-tubulin antibodies were purchased from Sigma and were diluted 1/100 for immunocytochemistry. Similarly mouse IgM monoclonal anti-vimentin antibody and rhodamine conjugated phallodin were purchased from Sigma and used at 1/200 and 1/500 dilutions respectively. A rabbit polyclonal anti-biotin antibody was used at a 1/200 dilution (Southern Biotechnology Associates, Inc).

2.6.2 Secondary antibodies

The primary antibodies were visualised with the commercially available secondary anti-antibodies, conjugated to fluorophores, FITC [(Fluorescein Isothiocyanate), green fluorescence] and TRITC [(Tetramethylrhodamine B Isothiocyanate), red fluorescence]. The following second layer antibodies were used: a donkey anti-rabbit TRITC (Southern Biotech.); goat anti-mouse FITC antibody (Jackson-Immuno Research); and goat anti-mouse IgG_1 (Southern Biotechnology Associates, Inc.). All secondary antibodies were used at a 1/200 dilution. AMCA [(7-Amino-4-methylcoumarin) blue fluorescence)] conjugated to streptavidin (Southern Biotechnology Associates Inc) was used at a dilution of 1/50 for triple staining experiments. A DNA-specific, Bis Benzimide stain (Hoechst No.33342, Sigma) was also used at 50 mg/ml in order to visualise the cell chromosomes and hence locate mitotic cells.

2.6.3 Immunostaining

Cells in suspension were washed in PBS by centrifugation ($1000xg$, 5 min) and then resuspended to a final concentration of approximately 10^6 cells/ml. 13 mm diameter No. 1 coverslips (BDH) (acid washed in nitric acid, oven baked and flamed in 70% ethanol before use) were coated with poly-L-lysine solution (Sigma), by placing in a 0.01%

poly-L-lysine solution for 5 min and then dried in a 60°C oven for 1 h. Cells were immobilised by settling on to the pre-coated poly L-lysine coverslips for 30 min and then washed in PBS. Adherent monolayer cells were trypsinised and seeded onto sterile coverslips in a 24 well tissue culture dish or seeded directly after electroporation (section 2.2.1.4). When required for immunostaining, growing media was aspirated and the coverslips were washed once in PBS. All cells were fixed and permeabilised in 100% methanol at -20°C for 20 min. If examining actin staining cells were fixed for 30 min in 3.7% (v/v) formaldehyde, washed 6x with PBS and permeabilised for 5min with 0.2% (v/v) Triton X-100 in PBA. All coverslips were then washed 2x in blocking solution (0.5% (w/v) Marvel milk protein in PBS) After washing coverslips were left in blocking solution for 30min at room temperature. 150 μl of the primary antibody or a mixture of 2 or 3, diluted in blocking solution, were pipetted on to the cell side of the coverslips. Cells were incubated for one hour in a perspex box and then washed five times with blocking solution. 150 μl of the second stage, fluorescently labelled antibody or a mixture, diluted in the blocking solution, was then pipetted on to the cells and incubated for 1 h. Cells were finally washed five times in blocking solution and then mounted upside down onto a glass slide in glycerol containing 2.5% DABCO (Sigma), an anti-fading agent (Johnson et al., 1982). The coverslips were sealed with nail varnish to prevent movement.

2.6.4 Drug treatments prior to immunostaining or cell lysis

Prior to harvesting transfected (or mock transfected) COS-7 for lysis, or processing coverslips for immunofluorescence, selected COS-7 samples were subjected to various drug treatments. Wortmannin (Sigma) was made up as a 10mM stock in DMSO and used at a final concentration of 50 nM for 30 min. Cytochalasin D (Sigma) was made up as a 1 mM stock in DMSO and used at a final concentration of 1.2 μM for 2 h. Nocodazole (Sigma) was made up as a 1 M stock in DMSO and used at a final concentration of 10 μM for 2 h and Brefeldin A (BFA) was made up as a 18 mM stock in methanol and used at a final concentration of 36 μM for 2 h. For drug treatment, COS-7 growing media was aspirated and complete DMEM containing the required concentration of drug, in the case of wortmannin, this was added to cells in FCS free DMEM. The cells were incubated for the times stated for each drug above and then harvested for lysis or coverslips were processed for immunostaining.

2.6.5 Microscopy and image preparation

Confocal laser scanning microscopy was carried out with an MRC 500 confocal visualisation system (Bio-Rad, Hemel Hempstead, U.K.) mounted over an infinity corrected Axioplan microscope fitted with a x10 eyepiece and a x63 NA 1.4 oil immersion objective (Zeiss, Jena, Germany) aligned and used as described previously (Entwistle and Noble, 1994). Filter sets B & E (Entwistle and Noble, 1992) were used

for the detection of the emissions from fluorescein and rhodamine derivatives respectively and any contribution from one fluorophore into the data intended to describe the emission from the other was eliminated by a process of cross-talk correction. Image files, a matrix of 768 x 512 pixels describing the average of 96-128 frames scanned at 1 Hz were generated by collecting at least 12 bits of data per pixel and saving the 8 most significant bits. Images were generated by making montages of single image files using Photoshop (Adobe inc., California, USA) and printing them on a Phaser IISDX dye sublimation printer (Tektronix, Oregon, USA). Wide-field fluorescence microscopy was carried out using the Axioplan microscope fitted with fluorescence filter sets supplied by the manufacturer. Routinely cells were examined using a Zeiss Axiophot microscope and pictures of immunofluorescence images were shot onto Kodak Ektachrome 400ASA slide film.

CHAPTER 3

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cDNA cloning of p150

CHAPTER 3

cDNA cloning of p150

3.1 Introduction

The yeast PtdIns 3-kinase Vps34p was originally identified as part of the molecular complex responsible for the control of intracellular protein trafficking (Herman and Emr, 1990; Schu *et al.*, 1993). Wild type *VPS* yeast genes are responsible for the active diversion of proteins from the secretory pathway to the vacuole. In mammalian cells a similar sorting mechanism exists. Lysosomal enzymes, analogous to the soluble yeast hydrolase carboxypeptidase Y, are transported with secreted proteins through the early stages of the secretory pathway. Then, in the late Golgi compartment these proteins are segregated away from those destined for secretion (Stack *et al.*, 1995c).

The lipid kinase activity of Vps34p was demonstrated to be of physiological relevance when point mutations altering residues in the Vps34p kinase domain resulted in defective sorting of vacuolar proteins (Schu *et al.*, 1993). This suggested that the Vps34p lipid kinase activity and consequently the lipid product PtdIns3*P*, was an important signal transducer in protein trafficking. In addition, genetic and biochemical studies revealed that Vps34p exists as a complex *in vivo* with Vps15p, a 170 kDa serine/threonine kinase (Herman *et al.*, 1991a; Herman *et al.*, 1991b). Vps15p is responsible for the membrane localisation of Vps34p and the activation of its lipid kinase activity. Mutational inactivation of Vps15p protein kinase activity stops its association with Vps34p, blocks activation of Vps34p lipid kinase activity and prevents recruitment of Vps34p to the appropriate membrane site, (Stack and Emr, 1994; Stack *et al.*, 1993). This data illustrated the importance of a functional Vps15p as a regulator of vesicle traffic mediated by Vps34p.

The discovery that in yeast, PtdIns 3-kinase activity is involved in protein and vesicle trafficking raised two important questions: first whether PI kinases might be involved in analogous processes in mammalian cells and second, whether there is an equivalent Vps15p/Vps34p complex in mammalian cells. The use of degenerate primers based on conserved amino acid sequences in the catalytic domain of Vps34p and bovine p110α enabled the cloning of PtdIns 3-kinase, the human homologue of Vps34p (Volinia *et al.*, 1995). To identify any PtdIns 3-kinase associated molecules, an affinity purified rabbit anti-PtdIns 3-kinase antibody was prepared. This antibody was used to immunoprecipitate endogenous PtdIns 3-kinase from human Jurkat 6 and U937 cell lines. In this way, three candidate binding partners of 120 kDa, 150 kDa and 200 kDa

were detected for PtdIns 3-kinase (Volinia *et al.*, 1995). The similarity in molecular mass of the 150 kDa protein with the 160 kDa Vps15p, suggested that the 150 kDa protein which co-immunoprecipitated with PtdIns 3-kinase could be the human homologue of Vps15p. To investigate this further, the 150 kDa protein was co-immunoprecipitated with the PtdIns 3-kinase, digested in the gel using endoproteinase Lys-C and the amino acid sequences of five HPLC-purified peptides were determined (Volinia *et al.*, 1995). With the identity of a human Vps34p homologue established, the discovery of a Vps15p homologue would demonstrate the existence of a human Vps15p/Vps34p complex and further implicate a role for PtdIns 3-kinase activity in mammalian membrane trafficking events.

This chapter describes the cloning of the 150 kDa PtdIns 3-kinase associated protein, termed p150. Using the information derived from the five p150 peptide sequences, a DNA probe was generated and used to screen a cDNA library. This facilitated the isolation of a cDNA clone with an open reading frame (ORF) which had extensive amino acid homology to the Vps34p associated protein Vps15p.

3.2 Results

3.2.1 cDNA cloning of p150

3.2.1.1 Generation of a human p150 cDNA probe by PCR

As described in section 3.1 the partial amino acid sequence of p150 was determined yielding five amino acid sequences: 1) kVF/TAIQDPXLP, 2) kLLALK, 3) kETFLSADEEk, 4)kVVTLLSDPENIVFK and kXLNMENRHLN (Volinia *et al.*, 1995). No significant sequence similarities were identified when the p150 peptide sequences were compared with the protein sequences of the Swissprot database. Using the program TFASTA; however, (Devereux *et al.*, 1984) which compared the p150 peptides with the six possible translations of the database DNA entries, the short anonymous EST HS8804, human clone HFBEP44 (Adams *et al.*, 1993) was shown to encode a sequence identical to that of peptide 1 (figure 3.1). The amino acid sequence from the reading frame of the HFBEP44 cDNA which contained peptide 1, was used to rescreen the Swissprot database using the program FASTA (Devereux *et al.*, 1984). This new search identified the N-terminus (amino acids 11 to 135) of the yeast Vps15p protein kinase as having the greatest sequence homology to the predicted protein fragment encoded in HFBEP44 (25% identity and 45.9% similarity). The forward and reverse primers, 5'-GTGAATTCCAGATCCTTTCT and 5'-GTGAATTGAATGGACGGGTAC, respectively were designed based on the human HFBEP44 clone (figure 3.1). Using these primers and RT-PCR (section 2.1.1.2) the 332bp HFBEP44 partial cDNA was

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  1   CCCTCCCAGATCCTTTCTGTAGAGAGTTATTTTTCAGATATTCATGACTTTGAATATGAT    60
      GGGAGGGTCTAGGAAAGACATCTCTCAATAAAAAGTCTATAAGTACTGAAACTTATACTA

      P   S   Q   I   L   S   V   E   S   Y   F   S   D   I   H   D   F   E   Y   D

  61  AAAAGCCTGGGGAGTACTCGGTTTTTTAAAGTTGCTCGAGCCAAGCACCGAGAAGGCCTG   120
      TTTTCGGACCCCTCATGAGCCAAAAAATTTCAACGAGCTCGGTTCGTGGCTCTTCCGGAC

      K   S   L   G   S   T   R   F   F   K   V   A   R   A   K   H   R   E   G   L

 121  GTCGTTGTGAAGGTTTTTGCAATTCAGGATCCCACATTGCCTTTAACCAGCTATAAACAA   180
      CAGCAACACTTCCAAAAACGTTAAGTCCTAGGGTGTAACGGAAATTGGTCGATATTTGTT

      V   V   V   K   V   F   A   I   Q   D   P   T   L   P   L   T   S   Y   K   Q

 181  GAGCTGGAGGAACTGAAAATCAGGCTTAATTCTGCACAGAATTGTCTACCTTTCCAGAAA   240
      CTCGACCTCCTTGACTTTTAGTCCGAATTAAGACGTGTCTTAACAGATGGAAAGGTCTTT

      E   L   E   E   L   K   I   R   L   N   S   A   Q   N   C   L   P   F   Q   K

 241  GCATCAGAAAAAGCATCTGAGAAAGCAGCTATGCTCTTTAGGCAGTATGTGCGAGACAAT   300
      CGTAGTCTTTTTCGTAGACTCTTTCGTCGATACGAGAAATCCGTCATACACGCTCTGTTA

      A   S   E   K   A   S   E   K   A   A   M   L   F   R   Q   Y   V   R   D   N

 301  CTCTATGATCGCATCAGTACCCGTCCATTCTTGAATAACATTGAGAAGCGCTGGATTGCT   360
      GAGATACTAGCGTAGTCATGGGCAGGTAAGAACTTATTGTAACTCTTCGCGACCTAACGA

      L   Y   D   R   I   S   T   R   P   F   L   N   N   I   E   K   R   W   I   A

 361  TTCCAGATCCTGACAGCTGTGGACCA   386
      AAGGTCTAGGACTGTCGACACCTGGT

      F   Q   I   L   T   A   V   D
```

Figure 3.1 The EST HS8804, human clone HFBEP44.
Using the program TFASTA, the human clone HFBEP44 was identified.
This encoded a sequence identical to peptide 1 (shown in red), identified
from peptide sequencing of the 150kDa protein co-immunoprecipitated
with PtdIns 3-kinase (Volinia *et al.*, 1995). Primers based on the HFBEFP44
nucleotide sequence (underlined) which included Eco RI sites were designed
and used in a RT-PCR procedure to obtain HFBEP44 cDNA.
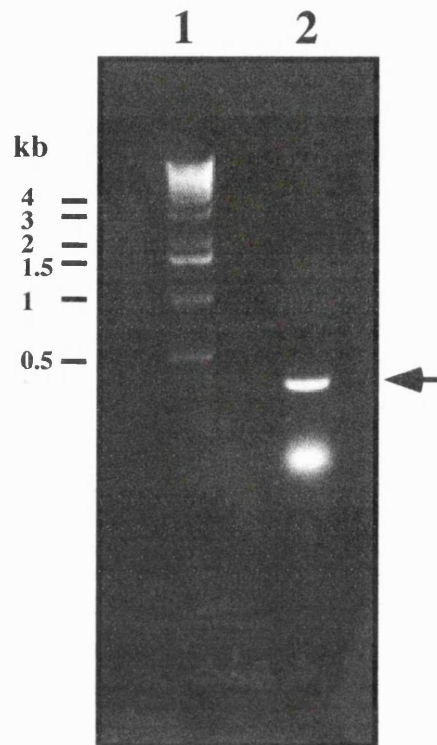
Figure 3.2 **PCR of HFBEP44 cDNA.**
Forward and reverse primers based on the HFBEP44 nucleotide sequence
(see figure 3.1) were designed and used in a PCR procedure from which
a 332bp HFBEP44 partial cDNA was amplified using U937 cDNA library
as template. The PCR product was electrophoresed on a 1% TAE gel (lane 2)
together with 1kb DNA ladder (lane 1). This 332bp PCR product was then
used as a DNA probe to screen a U937 cDNA library to obtain the p150 cDNA
sequence (as described in Experimental procedures)

amplified using U937 cDNA library as template (figure 3.2). To facilitate subcloning of the amplified fragment an *Eco* RI restriction site was incorporated into the 5'-end of each primer (shown underlined). The 332bp PCR product was digested with *Eco* RI and subcloned into *Eco* RI digested pBluescript SK vector (section 2.1.1), this construct was termed pSK-HFBEP44. The nucleotide sequence of the PCR product was determined by sequencing from either side of the pBluescript multiple cloning site with T7 and T3 primers. The DNA sequence obtained from pSK-HFBEP44 was identical to the HFBEP44 cDNA. This 332 bp PCR product was then used as a probe to screen a cDNA library.

3.2.1.2   Screening a U937 cDNA library for p150 clones

The HFBEP44 cDNA was excised from pSK-HFBEP44 by digestion with *Eco* RI, radiolabelled with $[\alpha\text{-}^{32}P]dCTP$ and random primers to generate a probe which was used to screen a primary Lambda Zap U937 cDNA library using the techniques described in section 2.1.5. A U937 cDNA library was chosen since p150 was originally identified in PtdIns 3-kinase immunoprecipitates from U937 cell lysates. After screening $3x10^6$ clones three putative positives were isolated, however using a secondary screen only one of the three clones remained as a positive. From the tertiary screen fifteen positive bacteriophage clones were picked and excised from the Lambda Zap vector by infection with *E. coli* helper phage to generate double-stranded pBluescript SK phagemids (pSK) containing insert cDNA sequences. To identify which of the fifteen clones contained authentic HFBEP44 cDNA, DNA from the fifteen pSK phagemids were digested using *Eco* RI and Xho I (which releases the cDNA insert from the 2.9kb pBluescript SK vector) Southern blotted (section 2.1.5.7) and probed with radiolabelled HFBEP44 cDNA (figure 3.3). Of the fifteen clones, fourteen of the tertiary plaques picked were true positives, whereby the probe bound to cDNA fragments of 3.1 kb and 0.55 kb (figure 3.3A). The *Eco* RI/Xho I digest gave a restriction pattern which included the 2.9kb fragment of the pBluescript vector and fragments of 3.1 kb, 1.2 kb, 0.55 kb and 0.12 kb which indicated that the clones isolated had a cDNA insert of approximately 5 kb. Since all the positive clones gave the same restriction pattern with an *Eco* RI/Xho I digest, one clone, termed pSKp150(cDNA) was chosen for further analysis.

3.2.2   Sequence analysis of pSKp150(cDNA)

The nucleotide sequence of the 5kb cDNA insert in the pSKp150(cDNA) clone was determined and found to contain the HFBEP44 cDNA sequence. In addition, this 5kb cDNA contained a 4077bp ORF with a putative start codon preceded by a 5'-Kozak consensus site (Kozak, 1987) and an in-frame stop codon. These features suggested that this clone represented a full length cDNA for this particular ORF. The ORF encoded a protein of 1358 amino acids (figure 3.4), which included all five of the peptides
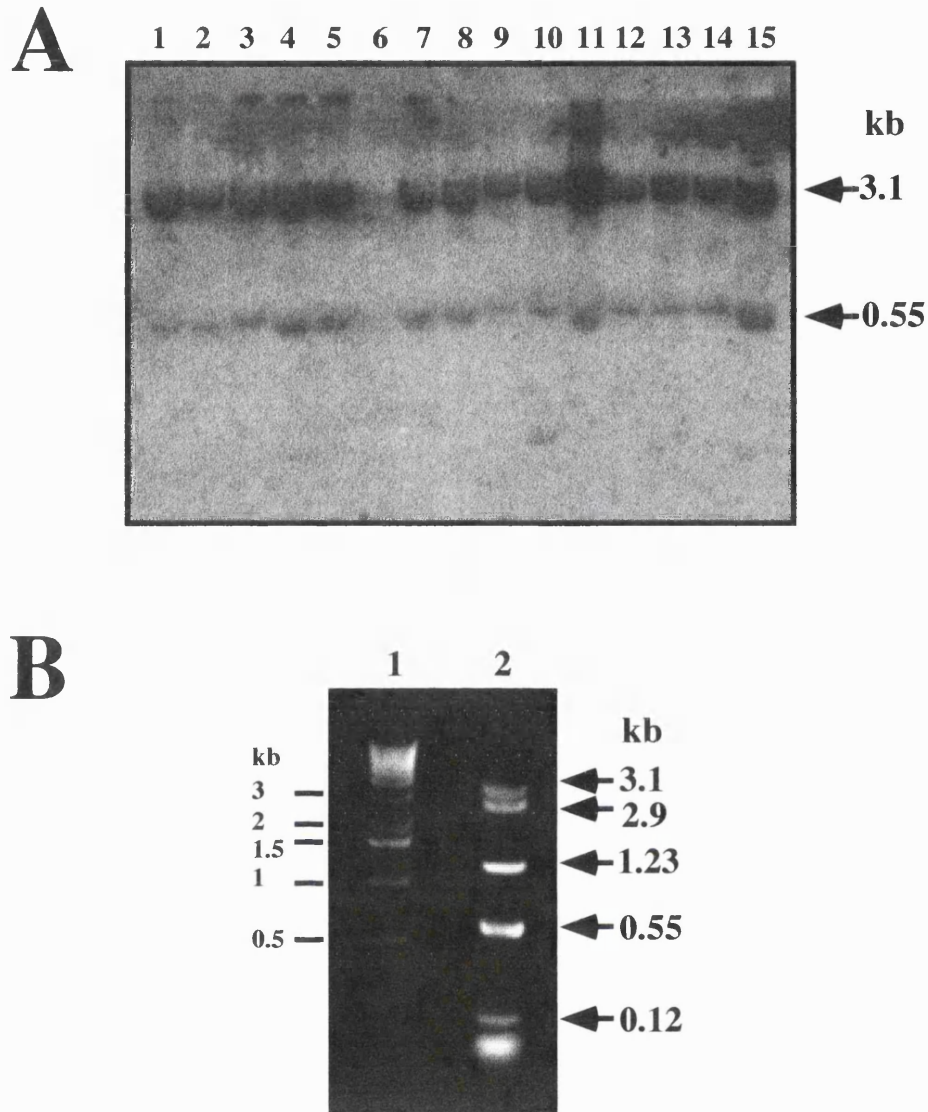
Figure 3.3 Southern blot and restriction digest analysis of putative positive clones.

A. From a tertiary U937 library screen fifteen positive bacteriophage clones were picked and pBluescript SK (pSK) phagemids containing insert cDNA seqences isolated. To identify which of the clones contained authentic HFBEP44 cDNA, DNA from the pSK phagemids were digested with Eco RI and Xho I and Southern blotted (as described in Experimental procedures) with radiolabelled HFBEP44 probe. 3.1kb and 0.55kb cDNA fragments which the probe hybridised to are indicated.

B. The DNA from the pSK phagemids were digested with Eco R1 and Xho I and electrophoresed on a 1% TAE agarose gel. Lane 2 shows a restriction pattern representative of that observed for all the clones (except number 6), with 3.1kb, 1.23kb, 0.55kb and 0.12kb fragments of the putative p150 cDNA insert indicated. A 1kb DNA ladder was run in parallel in lane 1.

Figure 3.4 DNA and amino acid sequence for p150

The full length cDNA sequence isolated for p150 (nucleotides 1-5085) is shown in addition to the longest open reading frame (amino acids 1-1358), including all five of the sequenced peptides (underlined) from the 150kDa protein co-immuno-precipitated with PtdIns 3-kinase (Volinia *et al* 1995).

```
p150:    1 MGNQLA.GIAPSQILSVESYFSDIHDFEYDKSLGSTRFFKVARAKHREGLVVVKVFAIQDPTLPLTSYKQELEELKIRLN    79
           ||.||. .:.:|. :.: ||:. :.:..|  |.|.||:|..:| ..:| :|:|||  ... ..| .: | :  .:::|.
Vps15p:  1 MGAQLSLVVQASPSIAIFSYIDVLEEVHYVSQLNSSRFLKTCKALDPNGEIVIKVFIKPKDQYSLRPFLQRIRAQSFKLG    80

        80 SAQNCLPFQKASEKASEKAAMLFRQYVRDNLYDRISTRPFLNNIEKRWIAFQILTAVDQAHKSGVRHGDIKTENVMVTSW   159
           .:.| : |  |   .::|: ::||.::|||||:| ||:|.:|| ::|||||:.|:.: |..: |||||||||::||||
        81 QLPHVLNYSKLIE..TNRAGYMIRQHLKNNLYDRLSLRPYLQDIELKFIAFQLLNALKDIHNLNIVHGDIKTENILVTSW   158

       160 NWVLLTDFASF.KPTYLPEDNPADFNYFFDTSRRRTCYIAPERFVDGGMFATELEYMRDPSTPLVDLNSNQRTRGELKRA   238
           ||.:|||||.| ||.|||||||::| ::||:|:|||||:|||||          |..  | .||. | |..:.
       159 NWCILTDFAAFIKPVYLPEDNPGEFLFYFDTSKRRTCYLAPERF...............NSKLYQDGKSNN...GRLTKE   220

       239 MDIFSAGCVIAELFTEGVPLFDLSQLLAYRNGHF..FPEQVLNKIEDHSIRELVTQMIHREPDKRLEAEDYLKQQRGNAF   316
           ||||| |||||.|| ::||||| |:.:|| | :| .:|:.::. .:|:|| :||.:|:.||..:|... || |
       221 MDIFSLGCVIAEIFAEGRPIFNLSQLFKYKSNSYDVNREFLMEEMNSTDLRNLVLDMIQLDPSKRLSCDELLNKYRGIFF   300

       317 PEIFYTFLQPYMAQFAKETFLSA..................DERILVIRKDLGNIIHNLCGHDLPEKAEGEP........   370
           |:.|||| |  .|: .:..| .:.| |.|
       301 PDYFYTFIYDYFRNLVTMTTSTPISDNTCTNSTLEDNVKLLDETTEKIYRDFSQICHCLDFPLIKDGGEIGSDPPILESY   380

       371 .................................KENGLVILVSVITSCLQTLKYCDSK.LAALELILHLAPRLSVE       412
                                            ||:. :::::| :. ::..: .:|||: :: |
       381 KIEIEISRFLNTNLYFPQNYHLVLQQFTKVSEKIKSVKEECALLFISYLSHSIRSIVSTATKPVKNLELLAVFAQFVSDE   460

       413 ILLDRITPYLLHFSNDSVPRVRAEALRTLTKVLALVKEVPRNDINIYPEYILPGIAHL...AQDDATIVRLAYAENIALL   489
           :||:.||:: ||::  :||   |.| ||.: .| ||:..:|||:|::.| |.:.| :
       461 NKIDRVVPYFVCCFEDSDQDVQALSLLTLIQVLTSVRKLNQLNENIFVDYLLPRLKRLLISNRQNTNYLRIVFANCLSDL   540

       490 AETALRFLELVQLKNLNMENDPNNEEIDEVTHPNGNYDTELQALHEMVQQKVVTLLSDPENIVKQTLMENGITRLCVFFG   569
           | .  || |:. .| : |.:|   :. |. :|:.:|..|
       541 AIIINRFQEFTFAQHCNDNSMDNNTEIMESSTKYS......AKLIQSVEDLTVSFLTDNDTYVKMALLQN.ILPLCKSFG   613

       570 RQKANDVLLSHMITFLNDKNDWHLRGAFFDSIVGVAAYVGWQS.SSILKPLLQQGLSDAEEFVIVKALYALTCMCQLGLL   648
           |::.||::||||:|:||||:.  ||..:.| |:. ..:|  ::| ||| |..|.|.||:|:.| .|.:: ||:
       614 RERTNDIILSHLITYLNDKDP.ALRVSLIQTISGISILLGTVTLEQYILPLLIQTITDSEELVVISVLQSLKSLFKTGLI   692

       649 QKPHVYEFASDIAPFLCHPNLWIRYGAVGFITVVARQISTADVYCKLMPYLDPYITQPIIQIERKL.VLLSVLKEPVSRS   727
           .|... .:.....|:| ||| ||| .: :|.. :. ..:|.|:||| |.: |::.  :::| .:  ::|. |:|||||
       693 RKKYYIDISKTTSPLLLHPNNWIRQFTLMIIIEIINKLSKAEVYCILYPIIRPFFE...FDVEFNFKSMISCCKQPVSRS   769

       728 IFD..YALRSKDITSLFRH.............................LHMRQKKRNGSLPDCPPP.........EDPAIA   768
           ::: :.:. :. .|||:.                            ::.|:|..:||.:.... | |.|
       770 VYNLLCSWSVRASKSLFWKKIITNHVDSFGNNRIEFITKNYSSKNYGFNKRDTKSSSSLKGIKTSSTVYSHDNKEIPLTA   849

       769 Q...LLKKLLSQGMTEEEEDKLLALKDFMMKSNKAKANIVD....QSHLHDSSQKGVIDLAALGITGRQVDLVKTKQEPD   841
           :   ::.|:   |:||.:  |:::||:::::||:...:. .|    .|:.:.  |:.. :|. .| .|.:.  .  .|..
       850 EDINWIDKFHIIGLTEKDIWKIVALRGYVIRTARVMAANPDFPYNNSNYRPLVQNSPPNLNLTNIMPRNIFFDVEFAEES   929

       842 DKRARKHVKQDSNVNEEWKSMFGSLDPPNMPQALPKGSDQEVIQTGKPPRSESSAGICVPLSTSSQVPEVTTVQNKKPVI   921
           .. :   ||||:::: .::. |       |:|:.  |..:|...  . .. ...||  .|  |||| | |  |  | ||:
       930 TSEG.....QDSNLENQQIYKYDESE.........KDSNKLNINGSKQLSTVMDINGSLIFKNKS...IATTTSNLKNVF   992

       922 PVLSST...ILPSTYQIRITTCKTELQQLIQQKREQCNAERIAK.........QMMENAEWES.....KPPPPGWRPKGL   984
           . |..|   : .....|.  ..::  .. .:.:|.|.|     ..:.:  |::. :.|  | |
       993 VQLEPTSYHMHSPNHGLKDNANVKPERKVVVSNSYEGDVESIEKFLSTFKILPPLRDYKEFGPIQEIVRSPNMGNLRGKL  1072

       985 LVAHLHEHKSAVNRIRVS.DEHSLFATCSNDGTVKIWNSQKMEGKTTTTRSILTYSRIGGRVKTLTFCQGSHYLAIASDN  1063
           :... :.:.....:.  || :| ..: |.|::||| ..: .| |||.  .:: |. :|:.... . :|:.|.:
      1073 IATLMENEPNSITSSAVSPGETPYLITGSDQGVIKIWNLKEIIV.GEVYSSSLTYD.CSSTVTQITMIPNFDAFAVSSKD  1150

      1064 GAVQLLGIE......ASKLPKSPKIHPLQSRILDQKEDGCVVDMHHFNSGAQSVLAYATVNGSLVGWDLRSSSNAWTLKH  1137
           |.: :|  ::          . |: .:. |:.: : ::..|  :.|  | :.:: :|:|.    :.:: :|:|. ..  .::.
      1151 GQIIVLKVNHYQQESEVKFLNCECIRKINLKNFGKNE..YAVRMRAFVNEEKSLLVALTNLSRVIIFDIRTLERLQIIEN  1228

      1138 DLKSGLITSFAVDIHQCWLCIGTSSGTMACWDMRFQLPISSHCHPSRARIRRLSMHPLYQSWVIAAVQGNNE..VSMWDM  1215
           . : |  |.:|..:. .  | |.:||..|.:...||:||||.: |.| : ..:|.| ::.: .:|.. :  .|.|... :.:|::
      1229 SPRHGAVSSICIDEECCVLILGTTRGIIDIWDIRFNVLIRSWSFGDHAPITHVEVCQFYGKNSVIVVGGSSKTFLTIWNF  1308

      1216 ETGDRRFTLWASSAPP...........LSELQ.PSPHSVHGIYCSPADGNPILLTAGSDMKIRFWDLAYPERSYVVAGST  1283
           .|. .:.: .|...|           |.||. .: :|::::  . ....:.||||.:.. .| ::.|.       :.:|.
      1309 VKGHCQYAFINSDEQPSMEHFLPIEKGLEELNFCGIRSLNALSTISVSNDKILLTDEATSSIVMFSLN......ELSSSK  1382

      1284 SSPSVSYYRKIIEGTEVVQEIQNKQKVGPSDDTPRRGPESLPVGHHDIITDVATFQTTQG.FIVTASRDGIVKVWK      1358
           .  |.| :..:: .|:|..:: :    ....|.. :.   .: |||||..:.| :..:. ::|.....|:: ::.
      1383 AVISPSRFSDVFIPTQVTANLTMLLRKMKRTSTHSVDD...SLYHHDIINSISTCEVDETPLLVACDNSGLIGIFQ      1455
```

Figure 3.5 Comparison of human p150 and yeast Vps15p predicted protein sequences.

The optimal alignment of p150 (upper sequence) and Vps15p (lower sequence) was obtained using the GAP program (Devereux *et al.*, 1984). Identical residues are indicated by vertical lines and conserved residues by colons.

sequenced from p150 and had a calculated molecular mass of 150,000 Da. The predicted amino acid sequence was used to search the Swissprot database. This search identified the yeast Vps15p protein as having the greatest homology with 29.6% identity and 53% similarity (figure 3.5).

3.2.3 Regions of conserved sequence in p150

A comparison of the human p150 and yeast Vps15p amino acid sequences using the program COMPARE (Devereux *et al.*, 1984) defined four main areas of colinear homology between the two proteins (figure 3.6 panels A and B): homology region (HR) 1, (amino acids 1-10); this N-terminal region contains a consensus site for the attachment of myristic acid. HR 2, (amino acids 11-300); is conserved between p150 and Vps15p and shows similarity to the catalytic region of protein kinases. HR 3, amino acids (400-700); this central region contains repeating units found in a number of proteins known as HEAT repeats and finally HR 4, (amino acids 1000-1350); which has WD repeat units.

3.2.3.1 HR 1: A conserved myristoylation site

Myristic acid is a 14 carbon saturated fatty acid ($C_{14:0}$) that is co-translationally added to a N-terminal glycine residue of specific eukaryotic proteins via an amide linkage. The addition of myristic acid follows removal of the initiating methionine to which the glycine is adjacent (Towler *et al.*, 1988b). An analysis of the substrate specificity of the yeast myristoyl CoA: protein N-myristoyltransferase (NMT) has suggested a consensus sequence for N-terminal myristoylation $(M)^1G^2X^3Z^4Z^5S^6Z^7$, where uncharged residues are indicated by X and neutral residues by Z (Towler *et al.*, 1987). The Vps15p sequence $(M)^1G^2A^3Q^4L^5S^6L^7$, fits this consensus. Experiments which involved radiolabelling yeast cells with tritated myristic acid and then immunoprecipitating Vps15p have shown that Vps15p does become myristoylated *in vivo* (Herman *et al.*, 1991b). Mammalian NMTs also have substrate specificities similar to that of the yeast NMT (Towler *et al.*, 1988a). The p150 sequence of $(M)^1G^2N^3Q^4L^5G^6I^7$ contains homology to the consensus sequence for N-terminal myristoylation suggesting that like Vps15p, p150 might also be post-translationally modified by the addition of myristic acid..

The main function ascribed to lipid modifications such as myristoylation is to increase the hydrophobicity of a protein, thereby increasing the propensity of the protein to associate with cellular membranes. Examples of proteins which are myristoylated and have been demonstrated to be targeted to lipid membranes include, Myristoylated alanine-rich C kinase substrate (MARCKS) (McLaughlin and Aderem, 1995), pp60^{src} (Buser *et al.*, 1994) and the Gα subunit of heterotrimeric G proteins (Song *et al.*, 1996).
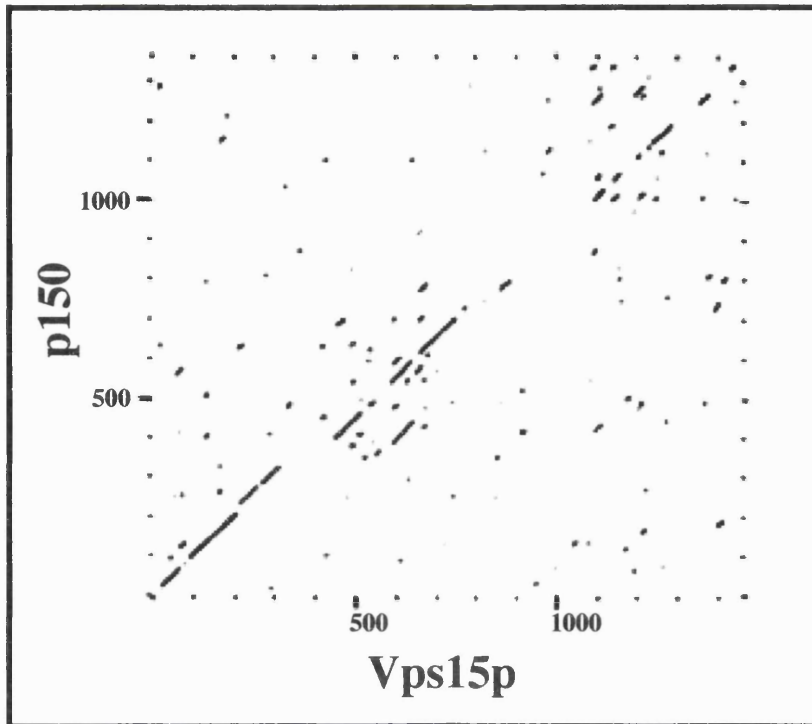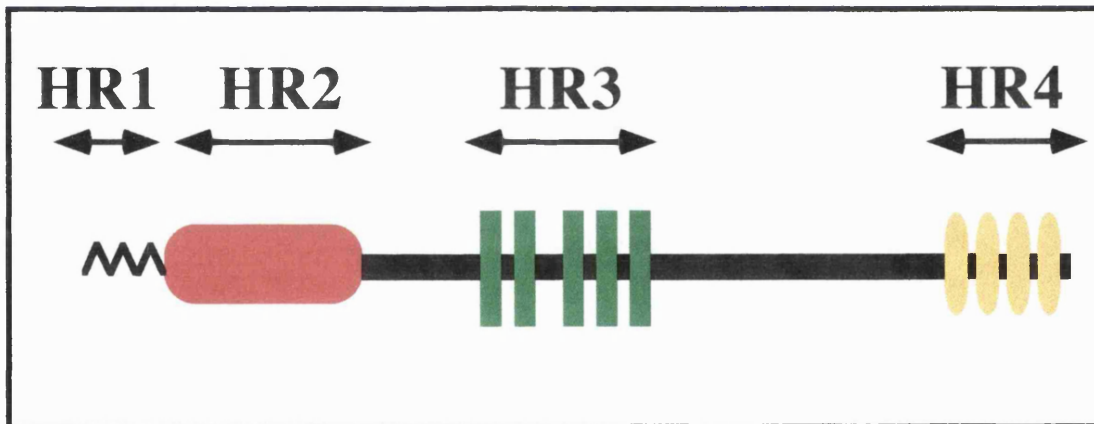
Figure 3.6 Vps15p and p150 have a similar domain structure.

A. A dot plot comparison of Vps15p (1455 amino acids; horizontal axis) and p150 (1358 amino acids; vertical axis). using the COMPARE programe (Devereux *et al* 1984).

B. A schematic representation of Vps15p and p150 domain structure. Homology regions (HR) represented are: HR1: an N-terminal myristoylation consensus site, HR2: a ser/thr protein kinase domain, HR3:HEAT repeats, and HR4: WD-40 repeat motifs, four of which are present in p150 but are less well defined in Vps15p.

3.2.3.2 HR 2: A conserved protein kinase domain

Protein kinases are defined as enzymes that transfer a phosphate group from a phosphate donor such as ATP onto an acceptor amino acid in a substrate protein (Hanks and Quinn, 1991; Hunter, 1991). Protein kinases act as "signal amplifiers" through a process where one kinase can phosphorylate itself or a number of protein molecules in response to a signal. The phosphorylation event itself can then activate or attenuate the activity of the substrate. As a result, protein kinases play pivotal roles in regulating and coordinating aspects of metabolism, gene expression, cell growth, cell motility, cell differentiation and cell division (Johnson *et al.*, 1996). Multiple sequence alignments using members of the extensive family of protein kinases, have been used to show that these enzymes have conserved structural features. This has been confirmed by recent crystal structure determinations of enzymes such as cyclic AMP-dependent kinase (Knighton *et al.*, 1991), Cyclin-dependent kinase CDK2 (De Bondt *et al.*, 1993) and the mitogen activated protein kinase (MAPK), ERK-2 (Zhang *et al.*, 1995a). Conserved features have been identified in twelve subdomain regions. Protein kinase catalytic domains range from 250 to 300 amino acids and residues from particular subdomains have been implicated in essential aspects of enzyme structure and function.

The first N-terminal 300 amino acids of both Vps15p and p150 show extensive amino acid sequence homology (47% identity and 67% similarity). This region in p150 and Vps15p, in turn has homology to the catalytic region of known ser/thr protein kinases (figure 3.7) such as CDK6 and p124^{cdc7} (Fankhauser and Simanis, 1994; Meyerson *et al.*, 1992). Protein kinases are generally classified into two relatively broad groups based on their substrate specificity: those that phosphorylate serine or threonine residues and those that modify tyrosine (Hanks *et al.*, 1988). The catalytic domains of both the ser/thr and tyrosine protein kinases are modular structures in which regions of very high sequence conservation are interspersed with regions of little similarity (Hanks *et al.*, 1988; Hunter, 1987). The highly conserved subdomains are presumably important for catalytic function either directly as constituents of an active site or indirectly as structural elements required for the formation of the active site. The observed N-terminal sequence similarity between Vps15 and p150 is clustered around those regions that are most highly conserved in different protein kinase catalytic domains, most notably, subdomains VI, VII and VIII (figure 3.7). These sequence elements have been implicated in the ATP binding and phosphotransferase activities associated with protein kinases. The invariant or nearly invariant residues D-F-A/G in subdomain VII are associated with ATP binding. These residues also appear to be conserved in a variety of phosphotransferase enzymes, including protein kinases and aminoglycoside phosphotransferases (Brenner, 1987). Furthermore, the Vps15 and p150 sequences contain the consensus triplet A-P-E, a conserved sequence element that is often refered to as an indicator of protein catalytic

protein kinase
subdomain:

consensus
sequence:


```
                                                          V
                                              ←——————
                                                    FQI
     p150: 101 -LFRQYVRDNLYDRISTRPFLNNIEKRWIAFQI 132
   Vps15p: 101 --IRQHLKNNLYDRLSLRPYLQDIELKFIAFQL 131
   p124cdc7: 101 ------------GKIP-ENLVALYT-----FQV 115
     CDK6: 101 ---VDQDLTTYLDKVP-EPGVPTETIKDMMFQL 129


                                    VI
                →       ←——————————————————→

                                   L P
               L         H       H DIKTEN
          133 LTAVDQAHKSGVRHGDIKTENVMVTSWNWVLLT 165
          132 LNALKDIHNLNIVHGDIKTENILVTSWNWCILT 164
          116 LQGLLYLHNQGVIHRDIKGANILTTKDGTIKLA 148
          130 LRGLDFLHSHRVVHRDLKPQNILVTSSGQIKLA 162


               VII
              ←———→

                 G
               DFA
          166 DFASF-KPTYLPEDNPADFNYFFDTSRRRTCYI 197
          165 DFAAFIKPVYLPEDNPGEFLFYFDTSKRRTCYL 197
          144 DFGVATKIN-ALEDHSVVGSPYW---------M 171
          163 DFGLARIYSFQMALTSVVVTLWY---------R 186


               VIII
              ←———→

               APE
          198 APERFVDGG
          198 APERF-NSK
          172 APEVIELVG
          187 APEVLLQSS
```

Figure 3.7 Comparison of p150, Vps15p, p124*cdc7* and CDK6 protein kinase domains

Residues identical to the protein kinase subdomain (V-VIII) consensus sequence are written in red. Residues a) identical or b) similar to those of p150 are in green or blue respectively.

domains (Hanks and Quinn, 1991). An especially high degree of sequence conservation is seen in the central core of the protein kinase domain (subdomain VI, see figure 3.7). Included within this region is a short stretch of amino acids that has been used as an indicator of protein substrate specificity (Hanks *et al.*, 1988). In this case, ser/thr specific kinases possess a sequence resembling the consensus element DLKPEN, in which the lysine residue is absolutely conserved, in contrast tyrosine-specific kinases have either DLRAAN or DLAARN. The sequence DIKTEN within Vps15p and p150 (figure 3.7) closely resembles the ser/thr consensus element, suggesting that p150 and Vps15 belong to the ser/thr family of protein kinases.

Although a high degree of sequence conservation exists between the consensus sequence of protein kinases and Vps15p/p150, relatively weak similarity is detected in the most N-terminal regions of the kinase domain of both Vps15p and p150. These sequence differences suggest that Vps15p and p150 may be novel kinase homologues. This region of the kinase catalytic domain (subdomain I) usually contains a GXGXXG/S sequence element (where X refers to any amino acid). This short motif has been termed a "nucleotide fold" (Walker et al., 1982). A similar sequence motif has been observed in both ATP and GTP binding proteins and it is suggested that this subdomain interacts with the phosphate groups of the bound nucleotide (Dever *et al.*, 1987; McCormick *et al.*, 1985; Walker *et al.*, 1982). In Vps15p and p150 the first and second glycine residues of this motif are replaced with serine/alanine and isoleucine/serine respectively. Since it has been suggested that an essential feature of the conserved glycines in this region is their small size (de Vos *et al.*, 1988) it is possible that the conservative sequence substitutions observed in Vps15p and p150 may not alter the function of this kinase subdomain. The observation that Vps15p is able to autophosphorylate and that phosphopeptide mapping has shown this phosphorylation to occur on a serine residue (Stack and Emr, 1994) demonstrated the presence of a functional ser/thr kinase domain. This discounts a previous suggestion (Herman *et al.*, 1991a) that Vps15p has only a partial protein kinase domain and that it may be part of a hetero-oligomeric complex in which Vps15p contributes the majority of the kinase domain and a second protein in the complex contributes the nucleotide-fold region. The biochemical data demonstrating that Vps15p is a ser/thr kinase and the homology between p150 and various protein kinase domains suggest that p150 may also have a protein kinase activity.

3.2.3.3 HR 3: HEAT repeats

Huntingdon's disease (HD) is an autosomal dominant neurodegenerative disease . The disorder is caused by a CAG/polyglutamine repeat expansion in the first exon of a gene encoding a large 350 kDa protein called huntingtin (Group, 1993). Although the precise function of the protein is unknown, immunohistochemistry, electron microscopy and

subcellular fractionation have shown that huntingtin is primarily a cytosolic protein. A small proportion however is associated with vesicles and/or microtubules, suggesting that it might play a functional role in cytoskeletal anchoring or transport of vesicles (DiFiglia *et al.*, 1995). Analysis of the Huntingtin protein (Andrade and Bork, 1995), has shown that it contains tandem arrays of a repeat, termed HEAT after four functionally characterised proteins in which the repeat was first detected: Huntingtin, Elongation factor 3 (Qin et al 1990 JBC), the 65 kDa regulatory subunit A of protein phosphatase 2A, PP2A (Hemmings *et al.*, 1990) and TOR 1, a target of rapamycin that is essential for progression of the G1 phase of the cell cycle and which has a C-terminal domain homologous to the catalytic region of PI-3 kinase (Sabatini *et al.*, 1994).

HEAT repeats vary in length between 37 and 43 amino acids and follow the consensus pattern LLP-L---L-D----VR-L--L. HEAT motifs occur as no fewer than three consecutive repeats in every protein and each repeat appears to consist of two α helices. In the case of Vps15p and p150, the central region of both proteins (amino acids 400-700) contains five of these HEAT repeat units (figure 3.8). The rather hydrophobic nature of HEAT repeats suggests they are tightly packed against each other and may also contribute to an interaction with other proteins. This is supported by experimental data on HEAT repeat containing A subunits of PP2A, which form rod-like helical structures and bind to T antigens of several viruses, as well as to the PP2A B subunit (Ruediger *et al.*, 1994). In addition to the sequence similarity detected by the alignments of HEAT repeats, proteins containing this motif share a number of features. i) The homologous regions are all predicted to adopt an α-helical topology. ii) Although the HEAT repeats themselves are rather divergent, they always occur as consecutive units multiple times within each protein (eg. huntingtin has 10 repeats, the A subunit of PP2A 15, TOR has 20 and Vps15p/p150 have 5). iii) All proteins of the HEAT family seem to be very large (eg. huntingtin is 350 kDa, TOR is 200 kDa, Vps15p is 160 kDa and p150 is predicted to be 150 kDa). iv) The functionally characterised proteins containing HEAT repeats are eukaryotic regulatory cytoplasmic proteins; most of them seem to be involved in cytoplasmic transport processes, PP2A acts during the transport-intensive cell cycle events (Hemmings *et al.*, 1990), as does TOR (Sabatini *et al.*, 1994), Vps15p is required for the accurate sorting of proteins to the yeast vacuole and huntingtin has been implicated in microtubule mediated vesicle transport. It appears therefore, that the function of HEAT repeats might be to facilitate the creation of a helical rod conformation that provides binding sites for other proteins generating a potential scaffold for the formation of protein complexes. HEAT repeats may also have a general role in cytoplasmic transport processes.

```
HEAT repeat
consensus:
                     LLP L    L D        VR      L  L
      p150   412 EILLDRITPYLLHFSNDSV----PRVRAGALRTLTKVLALVKE 451    1
      Vps15p 460 ENKIDRVVPYFVCCFEDSD----QDVQALSLLTLIQVLTSVRK 498

             457 NIYPEYILPGIAHLAQDDA----TIVRLAYAENIALLAETALR 495    2
             505 NIFVDYLLPRLKRLLISNR----NYLRIVFANCLSDLAIIINR 546

             531 QALHEMVQQKVVTLLSDPE----NIVKQTLMENITRLCVFFGR 570    3
             576 AKLIQSVEDLTVSFLTDND----TYVKMALLQNILPLCKFFGR 614

             571 QKANDVLLSHMITFLNDKD----WHLRGAFFDSIVGVAAYVGW 610    4
             615 ERTNDIILSHLITYLNDKD----PALRVSLIQTISGISILLGT 653

             611 WQSSSILKPLLQQGLSDAE----EFVIVKALYALTCMCQLGLL 648    5
             655 VTLEQYILPLLIQTITDSE----ELVVISVLQSLKSLFKTGLI 692
```

Figure 3.8 Five HEAT repeat motifs occur in p150 and Vps15p.

The amino acids 400-700 in both p150 and Vps15p were examined for
for HEAT repeat units. The HEAT repeat consensus pattern was used
(LLP L L D VR   L L). Residues present in p150 and Vps15p which
are identical to the consensus, are in red and those that are similar,
in blue. The p150 and Vps15p HEAT repeats numbered 1-5 are indicated.

3.2.3.4 HR 4: WD-40 repeats

The guanine nucleotide binding G proteins, transduce extracellular signals to intracellular effectors through coupling with transmembrane receptors (Gilman, 1987). The heterotrimeric G proteins are a complex of α, β, and γ subunits, the α subunit binds to and hydrolyses GTP and the $\beta\gamma$ subunit is required for the replacement of GDP by GTP as well as for membrane anchoring and receptor recogniton (Gilman, 1987). In higher eukaryotes, the heterotrimeric G protein transducin exists as a small multigene family of highly conserved proteins of about 340 aimino acid residues. Structurally, the $\beta\gamma$ subunit of transducin is a seven bladed propeller (Sondek *et al.*, 1996). The approximate sevenfold symmetry of the β-propeller is reflected in the amino acid sequence which contains 7 tandem repeats of about 40 residues, each containing a central Trp-Asp motif also known as a WD-40 repeat. These repeats have a region of variable length (6-94 amino acids) followed by a core of more constant length (23-41 amino acids), which is bracketed by two characteristic pattern elements, a conserved His and WD: $X_{6-94}[HX_{23-41}WD]$.

The amino acid sequence for p150 contains four such WD-40 repeats at the C-terminus (amino acids 1000-1300): 1) H_{1102}-(WD)$_{1124-1125}$, 2) H_{1137}-(WD)$_{1168-1169}$, 3) H_{1193}-(WD)$_{1213-1214}$, and 4) H_{1243}-(WD)$_{1268-1269}$. Analysis of Vps15p (Neer *et al.*, 1994) indicates that repeating elements similar but not matching the consensus pattern for WD repeats are also present at the C-terminus of this protein. It has been postulated that using the β subunit of transducin as a model (Sondek *et al.*, 1996), other proteins containing WD repeats will also form β-propellers. This suggests that p150 could have a four-bladed propeller like structure at its C-terminus. To date WD-repeats have been found in about 60 other eukaryotic proteins and all display a functional diversity which has precluded the identification of a precise role for this domain. Proteins containing WD-repeats have however, been implicated in the following functions: i) signal transduction, ii) RNA processing, iii) gene regulation/development, iv) vesicular traffic and v) regulation of cytoskeletal assembly/cell cycle (Neer *et al.*, 1994).

A common feature of proteins containing WD-40 domains is that they form multimeric complexes, suggesting that the formation of protein-protein interactions could be a possible function for the WD-repeat region. Crystal structure determination of the α and $\beta\gamma$ subunits of transducin has shown that α subunit interacts with the top and side of the β propeller domain (Lambright *et al.*, 1996; Sondek *et al.*, 1996) demonstrating that WD-40 repeats can act as binding sites. The $\beta\gamma$ subunit also brings the β-adrenergic receptor kinase, (β-ARK), to the β-adrenergic receptor, via an interaction with the PH domain of β-ARK (Touhara *et al.*, 1994). The PH domain in other proteins bind $\beta\gamma$ less well than β-ARK, perhaps because they are meant to interact with different WD-repeat proteins

(Wang *et al.*, 1995; Wang *et al.*, 1994). As a result, the wide variety of functions carried out by WD-repeat proteins indicate that the repeating units may have become differentiated and functionally specialised. The ability of WD proteins to facilitate the assembly of multiprotein complexes may give an insight into the function of WD repeats within proteins such as Vps15p and p150. Interestingly, the deletion of 30 amino acids from the C-terminus of Vps15p, a region which contains WD-40 like repeats, results in a protein that causes missorting of soluble yeast hydrolases (section 1.5.3.3) (Herman *et al.*, 1991b). This suggests that WD-40 repeats may have a functional significance in vacuolar protein sorting.

3.2.3.5 The Chediak Higashi protein, Vps15p and p150 share a similar modular architecture

Chediak Higashi Syndrome (CHS) is a rare autosomal recessive disorder characterised by hypopigmentation and severe immunodeficiency with neutropenia and lack of natural killer cells (Higashi, 1954). The hallmark of CHS is the occurance of giant inclusion bodies and organelles in a variety of cell types and the observation of protein sorting defects involving these organelles (Jones *et al.*, 1992). Similar abnormalities occur in the *beige* mouse, which has been proposed as an experimental model for human CHS (Lutzner *et al.*, 1966). The screening of human cDNA libraries with mouse *beige* probes identified the gene for CHS (Nagle *et al.*, 1996). The ORF of the CHS gene predicts a large polypeptide of 430 kDa. This CHS protein is found to consist of several hydrophobic helices, which closely resemble HEAT and ARM (Peifer *et al.*, 1994) repeat motifs that tend to form long rods. ARM repeats were originally found in the *Drosophila melanogaster* segment polarity gene product armadillo and share considerable similarity with HEAT motifs (Bork and Koonin, 1996; Peifer *et al.*, 1994). The CHS protein is typical of a HEAT repeat containing protein. It is large and has a number of repeats that occur in tandem (section 3.2.3.3). Furthermore, the C-terminal region of the CHS polypeptide contains seven WD-40 motifs. The only known proteins that contain HEAT repeats (or helical regions that resemble HEAT and ARM repeats) and C-terminal consecutive WD-40 motifs is Vps15p and p150. Given the similarity in modular architecture of Vps15p, p150 and the CHS protein, it is tempting to speculate that these proteins may share a similar function. This is consistent with the observation that mutation of the CHS protein leads to a similar phenotype as mutation of Vps15p, namely defective vesicular transport to and from the lysosome (analagous to the yeast vacuole) and late endosome (Burkhardt *et al.*, 1993). A mutated CHS protein also causes aberrant compartmentalisation of lysosomal and granular enzymes (Holcombe *et al.*, 1994). The modular architecture illustrated by the CHS protein, Vps15p and p150, implicates HEAT repeats and WD-40 domains as having a function in vesicle trafficking, possibly in the

formation of membrane associated signal transduction complexes which regulate intracellular protein trafficking.

3.3 Discussion

The p150 protein was initially observed as a 150 kDa protein found in complex with human PtdIns 3-kinase, when immunoprecipitated from Jurkat cells (Volinia *et al.*, 1995). The results described in this chapter demonstrate that the 5kb cDNA clone isolated encodes the human p150 protein. The 4077bp ORF of the clone encodes a protein with a predicted molecular mass of 150 kDa which contains all five peptides isolated from microsequencing analysis of the original p150 obtained from Jurkat cells. The predicted amino acid sequence encoded by the p150 cDNA clone has extensive homology (29.6% identity and 53% similarity) to the yeast protein Vps15p which associates with the yeast homologue of PtdIns 3-kinase, Vps34p. In addition, structural features common to both p150 and Vps15p such as a conserved protein kinase domain, HEAT repeats and WD-40 motifs have been identified. Further biochemical analysis is required to confirm that the ORF for the p150 cDNA clone can indeed be used to express the predicted 150 kDa protein and whether p150 functions biochemically in a manner similar to its yeast homologue Vps15p.

CHAPTER 4

Expression of p150

CHAPTER 4

Expression of p150

4.1 Introduction

The previous chapter described the cloning of a 5kb cDNA whose open reading frame can encode p150 which was shown to display similarity at the amino acid level to the yeast ser/threonine kinase Vps15p and which has a predicted molecular mass of 150 kDa. The tissue distribution of p150 was investigated by Northern blot analysis using poly(A)$^+$ RNA isolated from human tissues. In addition, to initiate biochemical characterisation, the ORF of p150 was expressed as a recombinant protein and its ability to associate with PtdIns 3-kinase analysed.

4.2 Results

4.2.1 RNA analysis

To investigate the tissue distribution of p150, Northern blot analysis was performed using a multiple human RNA blot (Clontech). A probe was generated by using the 330bp PCR fragment HFBEP44. This cDNA fragment was originally used to screen the cDNA library from which the p150 clone was obtained (section 3.2.1.1) To screen the RNA blot, HFBEP44 was labeled with [α-^{32}P]dCTP by random priming (as described in section 2.1.5.4). Hybridisation of the probe to the Northern blot RNA revealed that the cDNA encoding p150 hybridised to a single 5.5 kb mRNA species (figure 4.1). p150 mRNA was detectable in the majority of the RNA extracts from the human tissues analysed. The highest levels of p150 mRNA were detected in skeletal muscle, liver, ovary, testis, thymus, spleen and pancreas with lower levels present in kidney and lung. Although the expression of human PtdIns 3-kinase is high in skeletal muscle (Volinia *et al.*, 1995), the level of PtdIns 3-kinase found in other tissues does not directly mirror that observed for p150. In addition, the Northern blot used to examine the distribution of p150 should ideally be probed with radiolabelled β-actin cDNA as a loading control.

4.2.2 Expression of p150 as a recombinant protein in insect (Sf9) cells

Over the past few years a number of prokaryotic and eukaryotic expression systems have been developed for the production and isolation of recombinant proteins. Prokaryotic systems are able to produce large amounts of protein and have proved particularly useful when expressing protein domains for structural analysis. However, larger proteins (such as those over 150 kDa) can be difficult for prokaryotes to express and could be prone to be incorporated into inclusion bodies. In addition, prokaryotes lack the ability to post-

113
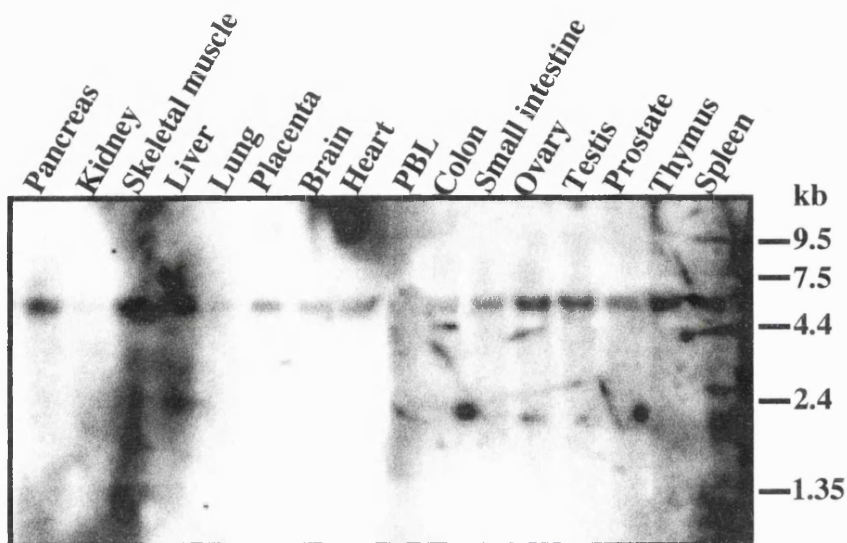
Figure 4.1 Northern blot analysis of p150.

A Northern blot (CLONTECH) of human poly(A)$^+$ RNA was hybridised with full-length p150 cDNA radiolabelled with [α-^{32}P]dCTP. Lanes1-16 contain 2μg of RNA from pancreas, kidney, skeletal muscle, liver, lung, placenta, brain, heart, peripheral blood leukocytes (PBL), colon, small intestine, ovary, testis, prostate, thymus and spleen respectively. Hybridisation was performed at 65^{0}C using the ExpressHyb protocol (CLONTECH). The position of RNA molecular markers (kb), are indicated.

translationally modify proteins. The absence of an important phosphorylation or lipid modification may be detrimental to the stability or functional activity of the protein being expressed. The baculovirus system is a well characterised method of producing heterologous protein in eukaryotic cells (Summers and Smith, 1987). The insect cells which baculoviruses infect, accomplish most eukaryotic post-translational modifications, allowing the production of correctly processed proteins. As a result the baculovirus system was chosen to over-express p150 and to examine its interaction with PtdIns 3-kinase *in vivo* in insect (Sf9) cells. The baculovirus encoding human PtdIns 3-kinase was already available (constructs made by Dr Stefano Volinia and Dr Meredith Layton LICR), the construction of the baculoviruses encoding p150, a GST fusion of p150 (GST-p150) and a C-terminal epitope (EFMPME) tagged p150, p150(EE) is described in this chapter.

4.2.2.1 Construction of the p150 baculovirus transfer vectors

To generate an N-terminal GST-fusion protein of p150 using the baculovirus transfer vector pACGEX-2T, a three step ligation procedure was devised. This was necessary as the pSKp150 cDNA construct contained 568bp and 924bp of 5' and 3' untranslated region respectively which required removal to obtain the p150 ORF. In addition the p150 cDNA contained internal restriction sites that were also present in the multiple cloning site of the vector, resulting in a cloning strategy that required more than one step (summarised in figure 4.2).

The first step of the subcloning procedure involved the introduction by PCR of *Bam* HI and *Kpn* I/*Eco* RI sites at the 5' and 3' ends respectively, of nucleotides 1-350 of the ORF. The following primers were used:

A. Sense: 5'-CGGATCCATGGGAAATCAGCTTGCTGCC   (*Bam* HI site is underlined and the initiating methionine codon is written in bold).

B. Antisense: 5'-GGGAATTCGCGGTACCGAATGGACAAATACTGAT   (*Eco* RI and *Kpn* I sites are underlined respectively).

The resulting 350bp PCR fragment was digested with Bam HI and *Eco* RI and subcloned into pACGEX-2T digested with the same enzymes to create pACGEX-2T/p150-(1-350). In the second step, nucleotides 141-3487 were introduced by digesting pSKp150(cDNA) with *Stu* I and *Kpn* I and inserting this fragment into pACGEX-2T/p150-(1-350) which had been digested with the same enzymes to create pACGEX-2T/p150-(1-3487). Finally, this construct was digested with *Kpn* I to allow insertion of nucleotides 3488-4077, the final PCR-generated *Kpn* I-digested 590bp fragment. This PCR product was created using the following primers:

C. Sense: 5'-CGGTACCATGGCTTGTTGGGA (*Kpn* I site is underlined).

D. Antisense: 5'-CGGTACCTTATTTCCACACCTT (*Kpn* I site is underlined and a translational stop codon is written in bold).
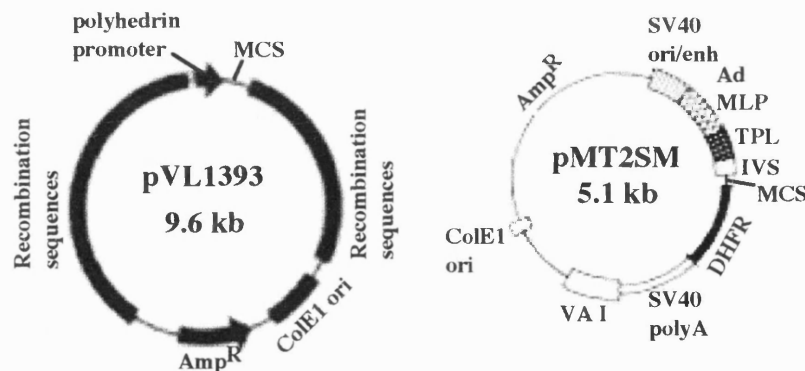
Figure 4.2 Generation of pAcGEX-2T/p150, pSKp150 and pSKp150(EE) constructs.

(abbreviations: ORF, open reading frame; UTR, untranslated region; Ad MLP, Adenovirus major late promoter; Amp^R, ampicillin resistance gene; ColE1 ori, bacterial colicin origin of replication; DHFR, dihydrofolate reductase coding sequence; IVS, intervening sequence; MCS, multiple cloning site; SV40 ori/enh, SV40 origin and enhancer element; SV40 polyA, SV40 early polyadenylation signal; TPL, tripartite leader sequence; VA I, adenovirus VA I gene).

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A similar three-step procedure was used to generate two pBluescript SK constructs, pSKp150 and pSKp150(EE). Both constructs contain 5' and 3' *Not* I sites (an enzyme that does not digest the p150 ORF) which enable the full p150 ORF to be excised as a "*Not* I cassette" and subcloned into other *Not* I containing vectors. Both constructs have also had a Kozak consensus site engineered by PCR, immediately prior to the initiating methionine codon. The Kozak GCCACC motif is thought to enhance the efficiency of translation. This modification increases protein expression in eukaryotic cells by allowing the initiating AUG to be more effectively recognised by the 40S ribosome (Kozak, 1991).

To produce the pSKp150 and pSKp150(EE) constructs, *Not* I and *Not* I/*Kpn* I sites were introduced by PCR at the 5' and 3' ends respectively of nucleotides 1-350 of the ORF. The following primers were used:

E. Sense: 5'-C<u>GCGGCCGC</u>GCCACC<u>**ATG**</u>GGAAATCAGCTTGCTGGC   (*Not* I site is underlined, Kozak site is written in bold and the initiating methione codon is underlined and written in bold).

F. Antisense: 5'-GC<u>GGTACC</u>C<u>GCGGCCG</u>CGAATGGACGGGTACTGAT   (*Kpn* I and *Not* I sites are underlined respectively).

The 350bp PCR fragment was digested with *Not* I and *Kpn* I and subcloned into pBluescript SK digested with the same enzymes to create pSKp150(1-350). In the second step, nucleotides 141-3487 were introduced in the same way as for pACGEX-2T/p150 in that pSKp150(1-3487) is created. Finally this construct was digested with *Kpn*I to allow insertion of nucleotides 3488-4077, the final PCR generated *Kpn* I digested 590bp fragment. To generate the final 590bp for the pSKp150 construct the following primers were used:

G. Sense: 5'-C<u>GGTACC</u>ATGGCTTGTTGGGA   (*Kpn* I site is underlined)

H. Antisense: 5'-C<u>GGTACC</u>A<u>GCGGCCGC</u>TTATTCCACACTT   (*Kpn* I and *Not* I sites are underlined respectively and a translational stop codon is written in bold).

In the case of the pSKp150(EE) construct the following primers were used to generate the final 620bp fragment:

I. Sense: 5'-C<u>GGTACC</u>ATGGGCTTGTTGGGGA   (*Kpn* I site is underlined)

J. Antisense:
5'.CGG<u>GGTACC</u>G<u>GCGGCCG</u>CTTA.**CTG.CAT.CGG.CAT.GAA.CTC.**ACC.ACC.
GGG.TTTCCACACAATCCC   (*Kpn* I and *Not* I sites are underlined , the translation termination codon is in bold, the codons for the six amino acid "EFMPME" tag are underlined and written in bold and a flexible "PGG" linker between the tag and p150 coding region is italicised).

The antisense primer used here, contains a six amino acid epitope "EFMPME" tag, (refered to as an "EE" tag) to enable immunodetection of expressed protein using a mouse monoclonal anti-EE tag antibody (EEmAb). The introduction of *Not* I sites at the 5' and 3' ends of both the pSKp150 and pSKp150(EE) constructs allowed the full length 4077bp ORF from both to be subcloned into the *Not* I digested baculovirus transfer vector pVL1393, thereby enabling expression of p150 with or without a C-terminal epitope tag.

4.2.2.2 Testing expression of p150 in Sf9 cells

Wild type baculovirus *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) was co-transfected into Sf9 cells with the recombinant transfer vectors pACGEX-2T/p150, pVL1393-p150(EE) and pVL1393-p150 using the Baculogold system (described in section 2.2.2.2). Under these conditions homologous recombination between the wild type virus and transfer vector can generate a virus in which the polyhedrin coding region has been replaced by the sequences encoding GST-p150, C-terminal EE tagged p150 or untagged p150. Six days later, cells were harvested and extracellular virions amplified several times to make virus stocks which were tested for protein production in insect Sf9 cells.

Virus collected from the third passage was used to infect Sf9 cells in order to test protein expression. Sf9 cells were infected with varying dilutions of virus stock and a negative control flask of cells was also set up with no virus added. Cells were harvested 2.5 days post-infection, washed in PBS, lysed in 1% Triton X-100 lysis buffer (section 2.3.3) and clarified supernatants incubated with glutathione-Sepharose beads for cells infected with pACGEX-2T/p150 virus. For cells infected with pVL1393-p150(EE) virus, proteins were immunoprecipitated with EEmAb and collected on protein G Sepharose beads. Precipitated proteins were released from glutathione or protein G Sepharose beads by boiling in sample buffer and were separated by SDS-PAGE. The resulting gels were stained with Coomassie Blue. Infection of Sf9 cells with pVL1393-p150(EE) virus, led to the expression of a 150 kDa protein that did not appear in cells which were not infected with virus (figure 4.3A). The molecular mass of 150 kDa corresponds to the predicted molecular mass of 150 kDa for the 4077bp coding region of p150 (section 3.2.2). Sf9 cells infected with pACGEX-2T/p150 virus, expressed a protein of approximately 180 kDa which corresponds to the 150 kDa, p150, tagged with a 27 kDa GST moiety. There appeared to be no large differences in the levels of GST-p150 or EE-tagged p150 expressed when different amounts of virus were used, although a 1:50 dilution of pVL1393-p150(EE) virus did appear to produce protein with fewer contaminating bands (figure 4.3A).
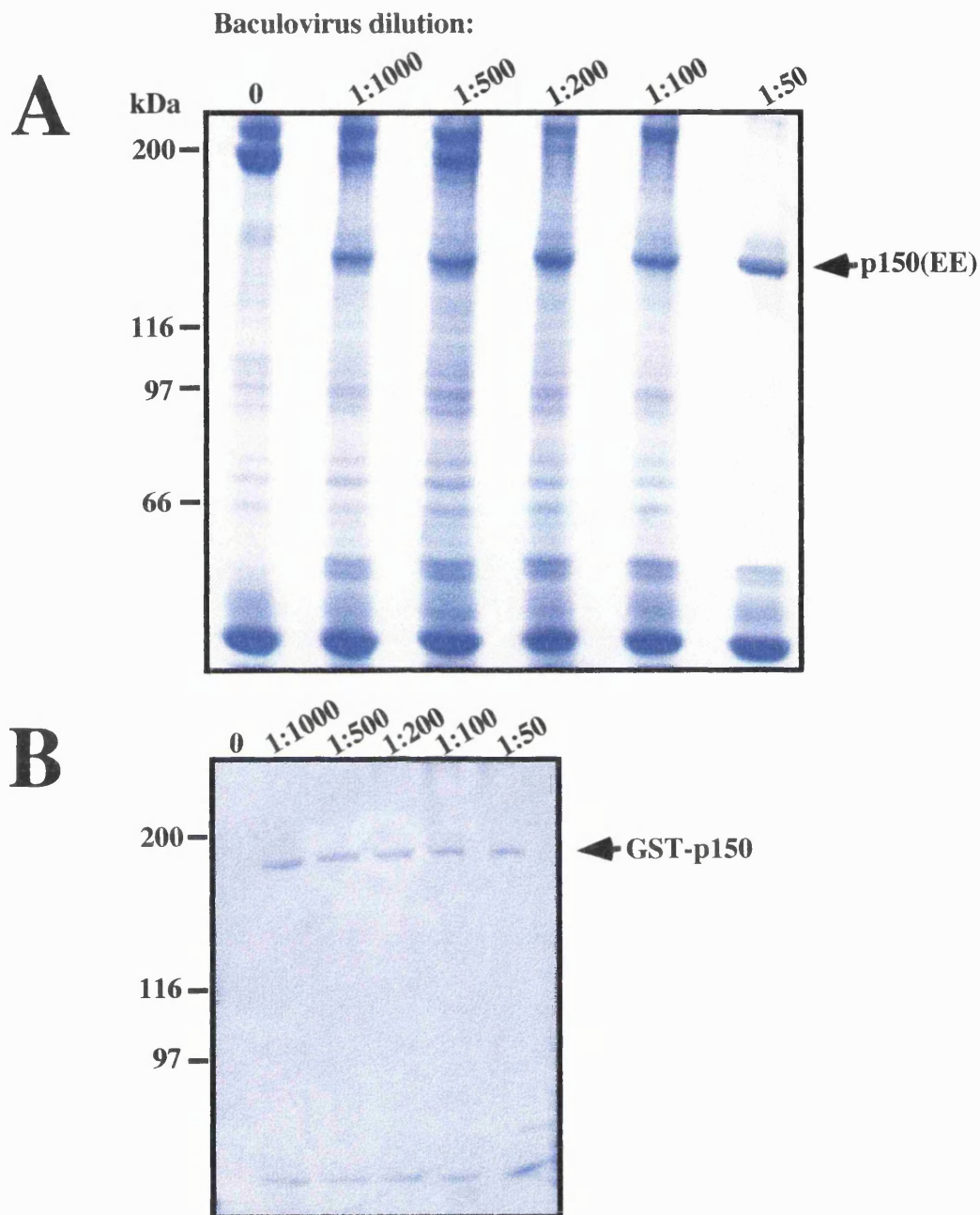
Figure 4.3 Expression of p150(EE) and GST-p150 in insect Sf9 cells.

Sf9 cells were infected with varying dilutons of recombinant baculovirus to express p150(EE) (Panel A), or GST-p150 (Panel B). After 60h, EE-tagged proteins were immunoprecipitated from cell lysates with EEmAb and collected on protein G-Sepharose. GST fusion proteins were affinity purified using glutathione-Sepharose. Immobilised proteins were washed and analysed by 7.5% SDS-PAGE and visualised using Coomassie Blue staining.

4.2.3 Interaction of p150 with PtdIns 3-kinase *in vivo*

To investigate whether p150 can associate with PtdIns 3-kinase *in vivo*, Sf9 cells were co-infected with baculovirus encoding GST-p150 and PtdIns 3-kinase (100 kDa). Affinity purification on glutathione-Sepharose beads was used to demonstrate that GST-p150 was able to associate with PtdIns 3-kinase (figure 4.4A). A 100 kDa protein was observed in complex with GST-p150, which was absent from cells where no PtdIns 3-kinase baculovirus was added (figure 4.4A lane 1). Similarly, when Sf9 cells were co-infected with viruses expressing GST-PtdIns 3-kinase and p150 (figure 4.4B) or PtdIns 3-kinase with p150(EE) (figure 4.4C) a p150/PtdIns 3-kinase complex could be detected. Since PtdIns 3-kinase phosphorylates PtdIns in a Mn^{2+} dependent manner (Volinia *et al.*, 1995), PI 3-kinase assays were performed on the various p150/PtdIns 3-kinase complexes in the presence of Mn^{2+}, using PtdIns as substrate (figure 4.4D). These experiments demonstrated that the isolated complexes had a functional lipid kinase activity, indicating that the GST tag at the N-terminus of p150 or the C-terminal EE-tag epitope did not significantly alter the ability of p150 to bind PtdIns 3-kinase or affect lipid kinase activity.

4.2.3.1 p150 associates specifically with human PtdIns 3-kinase

Co-expression experiments with the human PtdIns 3-kinase and the p110α adaptor protein p85α, had demonstrated that p85α was able to associate in stochiometric amounts with p110α but not with PtdIns 3-kinase (Volinia *et al.*, 1995). To investigate whether p150 could act in a similar fashion, as a specific adaptor protein for the human Vps34p homologue PtdIns 3-kinase, Sf9 cells were co-infected with baculoviruses expressing GST-p110α and p150 or GST-PtdIns 3-kinase and p150 (figure 4.5A). Affinity purification of the resulting cell lysates with glutathione-Sepharose beads showed that whilst GST-PtdIns 3-kinase was able to associate stochiometrically with p150, no association of p150 was observed with GST-p110α (figure 4.5A, lanes 2 and 4 respectively). Of the class III PI 3-kinase members (section 1.3.3; figure 1.2B) the *Drosophila melanogaster* Vps34p homologue (PI 3K_59F), has the greatest amino acid sequence homology with the human Vps34p, (PtdIns 3-kinase), compared with Vps34p homologues from other species (figure 1.2B) (Linassier *et al.*, 1997). This finding raised the possibility that p150 might associate with a PtdIns 3-kinase from a different species. To investigate this further, Sf9 cells were co-infected with baculoviruses expressing GST-PtdIns 3-kinase and p150 or GST-PI 3K_59F and p150. Affinity purification of cell lysates with glutathione-Sepharose beads showed that the *Drosophila* Vps34p homologue was unable to associate with the human p150 protein (figure 4.5B, lane 2), suggesting that the p150 interaction site has not been conserved from flies to humans.
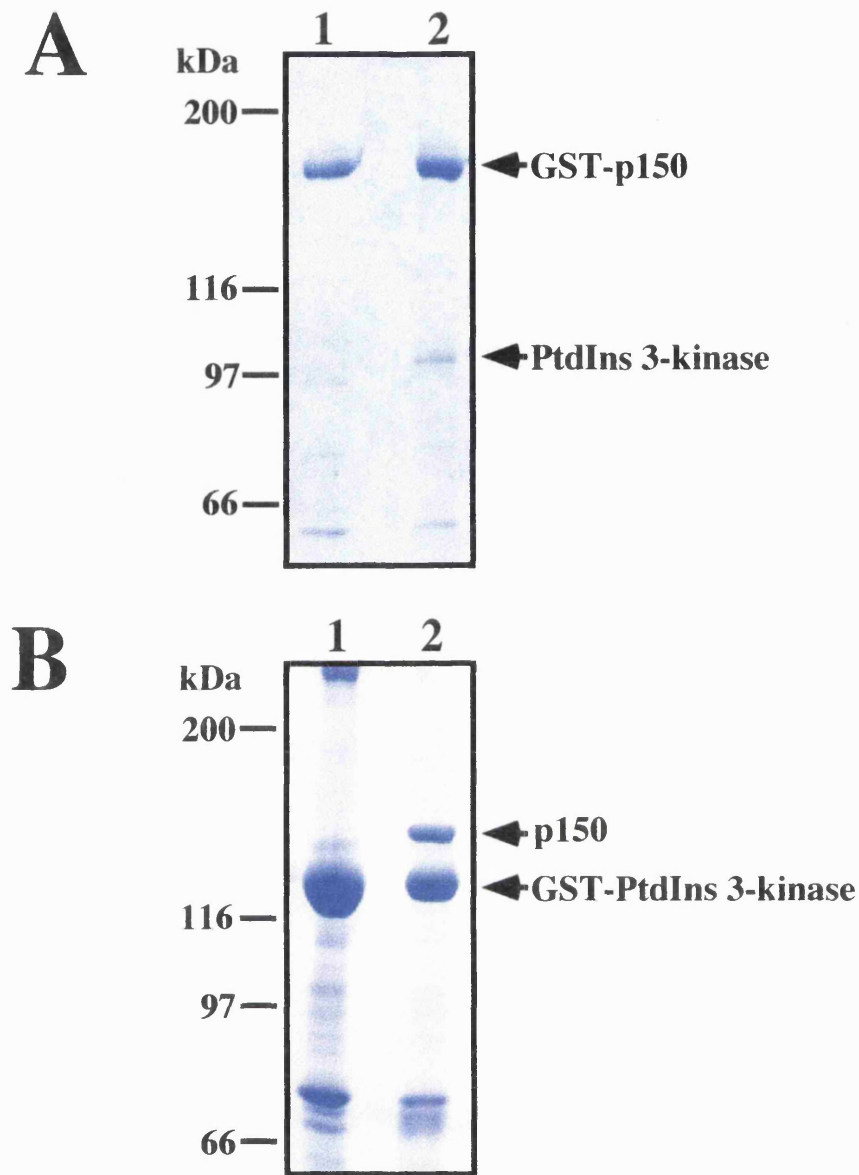
Figure 4.4 Expression and association of p150 with PtdIns 3-kinase.

Sf9 cells were infected with recombinant baculovirus and after 60h, GST fusion proteins were affinity-purified from cells lysates using glutathione-Sepharose beads. EE-tagged proteins were immunoprecipitated from cell lysates using EEmAb and collected on protein G-Sepharose. Immobilised proteins were washed and analysed by 7.5% SDS-PAGE visualised using Coomassie Blue staining.

A. Sf9 cells were infected with recombinant baculovirus to express either GST-150 alone (lane1) or GST-p150 and PtdIns 3-kinase (lane 2).

B. Sf9 cells were infected with recombinant baculovirus to express either GST-PtdIns 3-kinase alone (lane 1) or GST-PtdIns 3-kinase and p150 (lane 2).

C

kDa	1	2	
200 —			
			← p150(EE)
116 —			
97 —			← PtdIns 3-kinase
66 —			
45 —			

D

	1	2	3	4	5	6
PtdIns3P →						
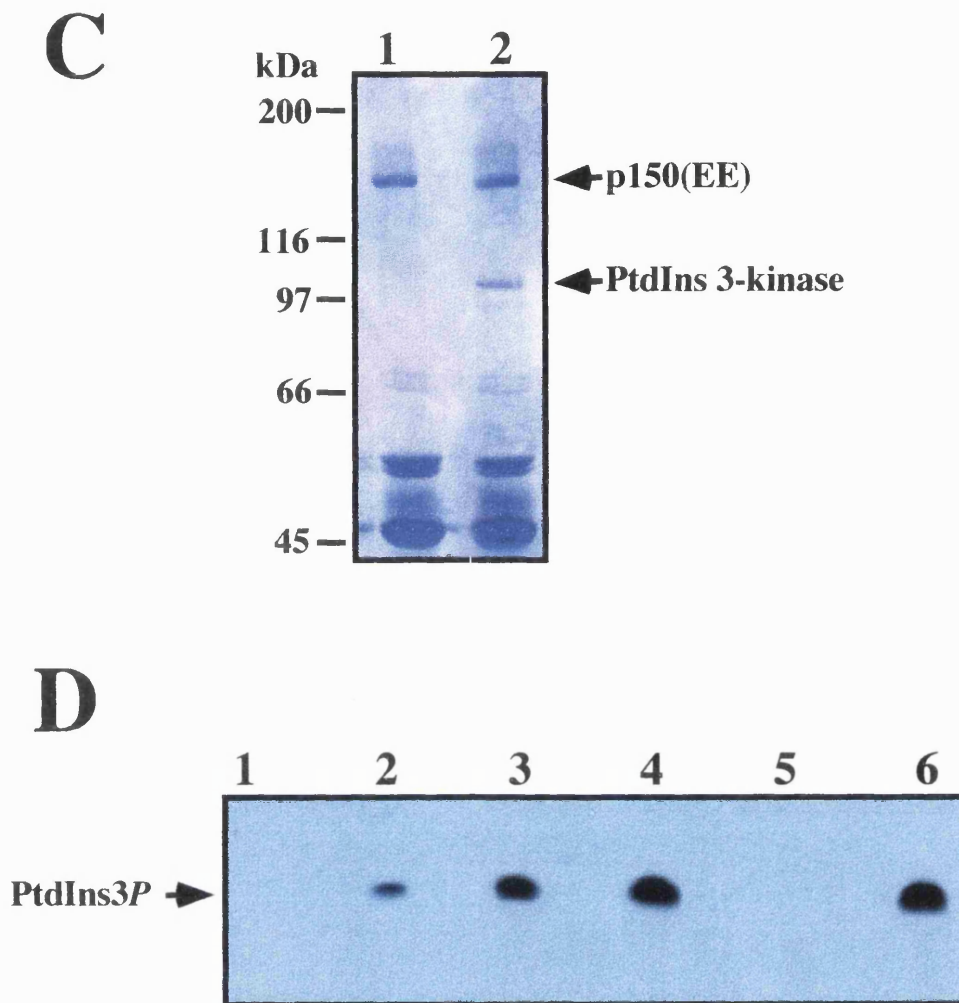
Figure 4.4 Expression and association of p150 with PtdIns 3-kinase.

C. Sf9 cells were infected with recombinant baculovirus to express either p150(EE) alone (lane 1) or p150(EE) and PtdIns 3-kinase (lane 2).

D. PI 3-kinase assays were performed in the presence of Mn^{2+} using PtdIns as substrate on GST-p150 (lane 1), GST-p150/PtdIns 3-kinase complex (lane 2), GST-PtdIns 3-kinase (lane 3), GST-PtdIns 3-kinase/p150 complex (lane 4), p150(EE) (lane 5) and p150(EE)/PtdIns 3-kinase complex (lane 6). Phosphorylated lipids were extracted and analysed using TLC and autoradiography. The position of radiolabelled PtdIns3P is indicated.
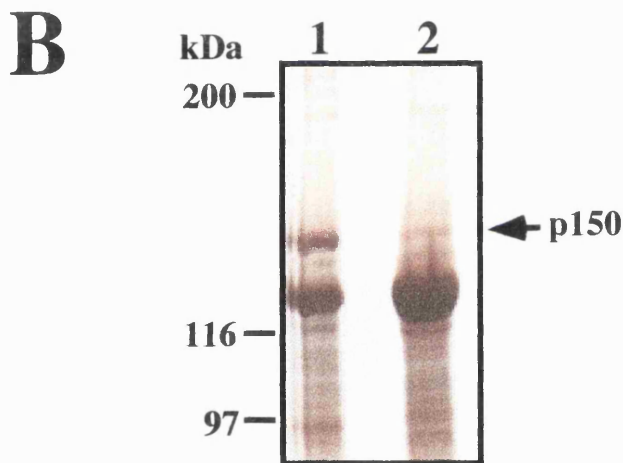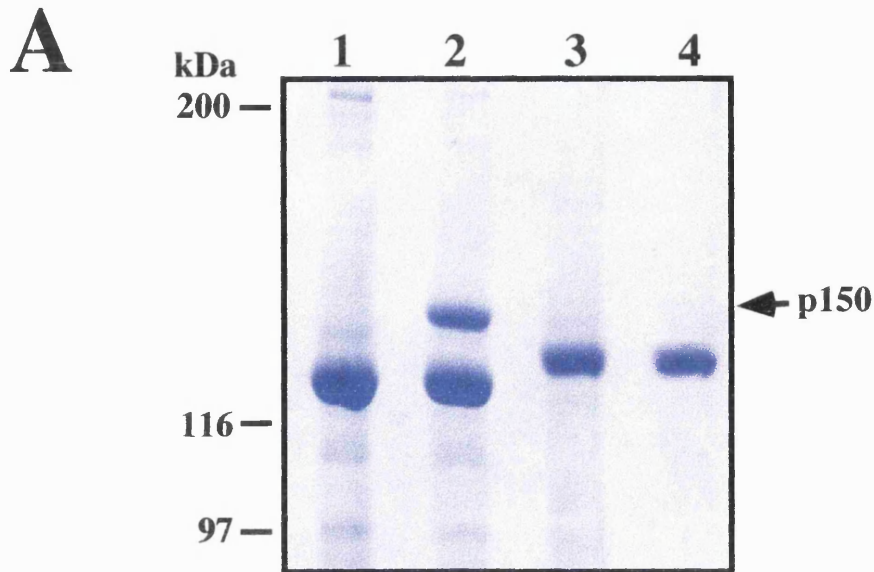
Figure 4.5 p150 associates specifically with human PtdIns 3-kinase.

Sf9 cells were infected with recombinant baculovirus and after 60h, GST
fusion proteins were affinity purified from cell lysates using glutathione-
Sepharose beads. Immobilised proteins were washed and analysed by 6.0%
SDS-PAGE and visualised using Coomassie Blue staining (Panel A)
or silver staining (Panel B).

A. Sf9 cells were infected to express GST-PtdIns 3-kinase alone (lane1),
GST-PtdIns 3-kinase and p150 (lane 2), GST-p110α (lane 3), GST-p110α and
p150.

B. Sf9 cells were infected to express GST-PtdIns 3-kinase and p150 (lane 1)
or GST-PI3 K_59F and p150 (lane 2).

4.2.3.2 Association of p150 increases the lipid kinase activity of PtdIns 3-kinase

Genetic and biochemical techniques have been used to demonstrate that the Vps15p protein kinase and the Vps34p PtdIns 3-kinase interact both functionally and physically to facilitate the delivery of soluble proteins to the yeast vacuole (Stack *et al.*, 1995c). Studies investigating the role of Vps15p in in the regulation of Vps34p lipid kinase activity showed that yeast strains deleted for *VPS15* are extremely defective in PtdIns 3-kinase activity. In such experiments a very low but detectable PtdIns 3-kinase activity was exhibited by $\Delta vps15$ strains (Stack *et al.*, 1993) suggesting that Vps34p lipid kinase activity undergoes a Vps15p mediated activation. To examine whether p150 could regulate the enzymatic activity of PtdIns 3-kinase in a manner analogous to that of Vps15p in yeast, Sf9 cells were infected with baculoviruses to express PtdIns 3-kinase or both p150 and PtdIns 3-kinase (figure 4.6A). Untagged versions of both p150 and PtdIns 3-kinase were used to preclude the possible interference of the epitope tag or GST moiety. Proteins were immunoprecipitated with affinity purified anti-PtdIns 3-kinase antibody (Volinia *et al.*, 1995) and the resulting immune complexes assayed for lipid kinase activity. Lipid kinase assays were performed in the presence of Mn^{2+} using PtdIns as substrate (section 2.4.1). Quantitation of the phosphorylated lipid products using a PhosphorImager (figure 4.6B) demonstrated that the lipid kinase activity of PtdIns 3-kinase increased two fold when associated with p150. Western blotting of anti-PtdIns 3-kinase immunoprecipitates produced in parallel to those used in the lipid kinase assays (figure 4.6C) showed that equal amounts of the enzyme were used in these studies.

4.3 Discussion

The predicted molecular mass of 150 kDa for the protein encoded by the human p150 cDNA (section 3.2.2) was verified when the 4077bp p150 ORF was expressed in Sf9 cells yielding a protein of 150 kDa (figure 4.3A). Co-infection studies with baculoviruses expressing both p150 and PtdIns 3-kinase demonstrated that p150 can stably associate with PtdIns 3-kinase. This indicated that recombinant p150 was identical to the 150 kDa protein initially observed in complex with endogenous human PtdIns 3-kinase when immunoprecipitated with anti-PtdIns 3-kinase antisera from Jurkat cells (Volinia *et al.*, 1995). The human PtdIns 3-kinase is unable to bind the p110α adaptor subunit p85α. Although PtdIns 3-kinase and p110α share amino acid sequence homology at their respective C-terminal lipid kinase domains, the N-terminus of p110α, which is responsible for binding p85α (Dhand *et al.*, 1994a) shares very little amino acid homology with the N-terminus of PtdIns 3-kinase. Interestingly, this N-terminal region of PtdIns 3-kinase is also postulated to contain a binding domain for its adaptor subunit p150 (Volinia *et al.*, 1995). The observation that p150 is unable to bind p110α or the
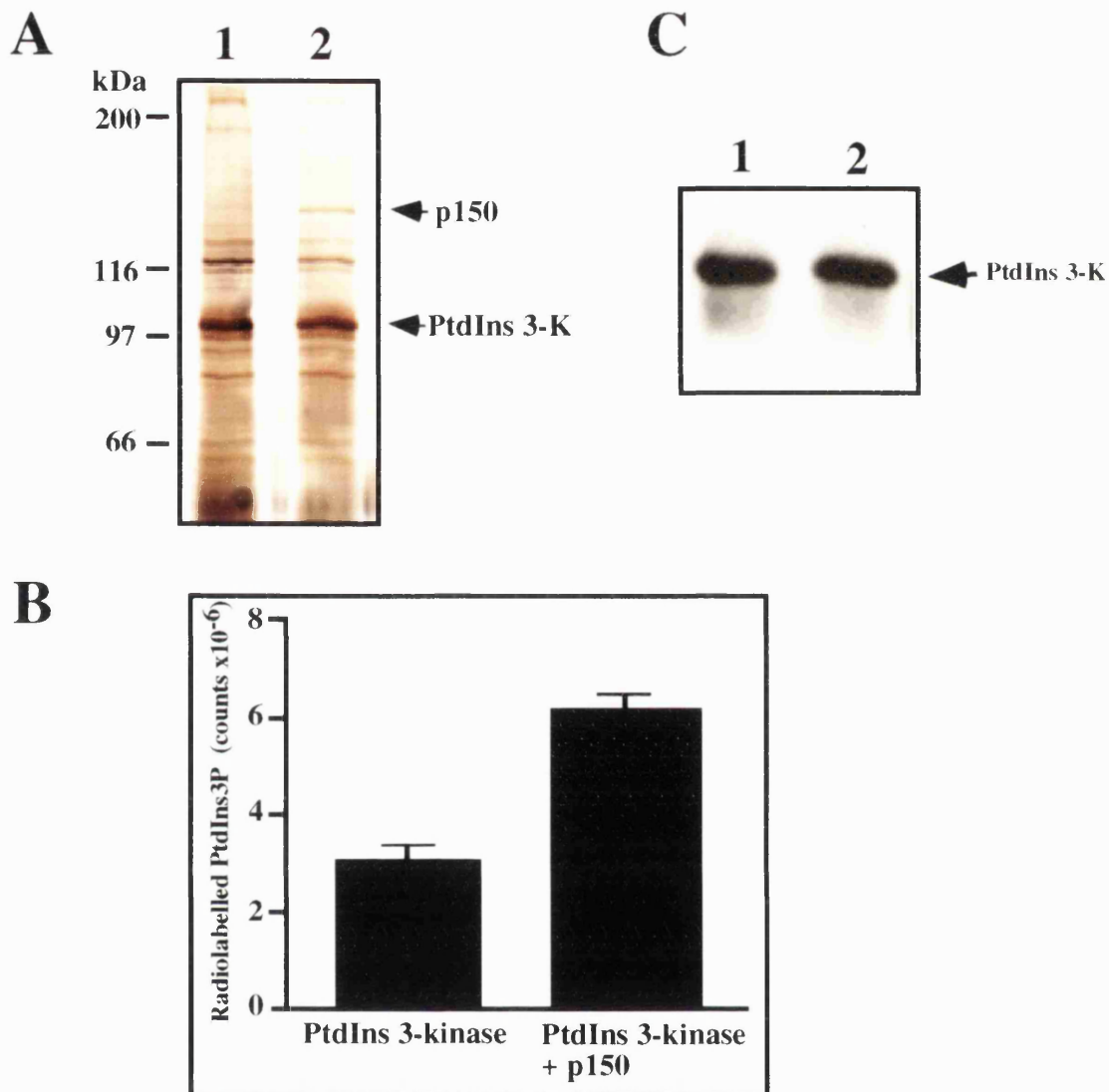
Figure 4.6 Effect of p150 on the lipid kinase activity of
PtdIns 3-kinase.

A. Sf9 cells were infected with baculoviruses to express untagged
PtdIns 3-kinase (lane 1) or p150/PtdIns 3-kinase complex as untagged
proteins (lane 2). After 60h cell lysates were immunoprecipitated with
PtdIns 3-kinase specific antisera and protein complexes collected on
protein A-Sepharose. Immobilised proteins were analysed by 7.5%
SDS-PAGE and silver staining.

B. PtdIns 3-kinase and p150/PtdIns 3-kinase complex (prepared as in
A) were subjected to *in vitro* PI 3-kinase assays in the presence of
Mn^{2+} using PtdIns as substrate. Extracted lipids were resolved by TLC
and quantitated using the PhosphorImager system.

C. Immunoprecipitates of recombinant PtdIns 3-kinase (lane 1) and
p150/PtdIns 3-kinase complex (lane 2) produced in parallel to those
used in B were resolved by 7.5% SDS-PAGE. PtdIns 3-kinase was detected
by Western blotting using PtdIns 3-kinase specific antisera.

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Drosophila Vps34p homologue PI 3K_59F (figure 4.5) suggests that the association of p150 with PtdIns 3-kinase occurs in a species specific manner. This is despite PI 3K_59F sharing extensive amino acid homology (70% similarity, 50% identity) with PtdIns 3-kinase. Since PI 3K_59F and PtdIns 3-kinase are homologous, it is possible that subtle amino acid changes maybe sufficient to alter the interaction surface of PI 3K_59F to preclude p150 binding. Alternatively regions in the p150/Vps15p family of proteins might act as putative, species specific binding sites for its PtdIns 3-kinase partner.

In yeast, the association of Vps15p with Vps34p leads to the activation of lipid kinase activity (Stack *et al.*, 1993). Experiments which examined if p150 functioned as a regulator of PtdIns 3-kinase enzymatic activity, revealed that p150 can only activate the lipid kinase activity of PtdIns 3-kinase by a modest twofold. Possible explanations for this may be that the *in vitro* conditions of the assay may not reflect true physiological conditions. Additional protein-protein interactions (PtdIns 3-kinase immunoprecipitates from Jurkat cells contained 120 kDa and 200 kDa proteins in addition to p150) or possibly protein- lipid membrane interactions may be required for full PtdIns 3-kinase activation. The twofold activation of PtdIns 3-kinase activity however, still contrasts with the effect on lipid kinase activity observed upon formation of the p85α/p110α heterodimer. When p85α associated with p110α *in vitro*, a decrease in lipid kinase activity was detected, which correlated with serine phosphorylation of the p85α adaptor subunit (Dhand *et al.*, 1994b).

CHAPTER 5

Post-translational modification of p150

CHAPTER 5

Post-translational modification of p150

5.1 Introduction

The p150 protein and Vps15p have been shown to be homologous at the amino acid level and share a number of conserved domains (section 3.2.3). Consistent with the presence of an N-terminal myristoylation site, Vps15p is myristoylated *in vivo* (Herman *et al.*, 1991a) In addition, the Vps15p protein kinase domain has been demonstrated to be responsible for its autophosphorylation (Herman *et al.*, 1991b). In this chapter the biochemical characteristics of p150 are examined. [^{3}H]myristic acid is used to investigate whether p150, like Vps15p is myristoylated. The phosphorylation state of p150 and its ability to function as a protein kinase is also examined.

5.2 Results

5.2.1 p150 is myristoylated *in vivo*

The 14-carbon fatty acid myristate is added co-translationally to the N-terminal glycine residue of many cellular proteins following the removal of the initiating methionine (Towler *et al.*, 1988b). The N-terminal sequence of p150, (M)^{1}G^{2}N^{3}Q^{4}L^{5}G^{6}I^7, was observed to be very similar to the proposed consensus sequences for myristic acid addition (section 3.2.3.1) and therefore suggested that p150 could be myristoylated at its N-terminus. To analyse directly whether p150 was myristoylated *in vivo*, Sf9 cells were infected with baculovirus expressing p150(EE) for 24 hours. A control flask was included where no baculovirus was added. The cells were then labelled with [^{3}H]myristic acid for an additional 16 hours and then lysed. Clarified cell lysates were incubated with EEmAb, immunocomplexes were collected on protein G-Sepharose beads and resolved by SDS-PAGE. The presence of a labelled 150 kDa protein and absence of any labelled proteins in the control sample showed that myristic acid was incorporated into p150 (figure 5.1A lane 2). This suggested that p150 is modified by the addition of myristic acid *in vivo*. Further support for this observation was obtained from the analysis of a p150 mutant that possessed alterations in the myristic acid attachment site. In this mutant, the critical glycine residue at position 2 was changed to an alanine, (G^{2}A) and the residue at position 3 changed to an aspartate, (N^{3}D). To generate this mutant, PCR was used to introduce these amino acid substitutions into the N-terminus of p150. The following primers were used to generate a 350bp fragment:

```
Amino acids in wild type p150:            (G)   (A)
                                          GGA.  AAT.
Sense: 5'-TCCCCCGGGAGCGGCCGCGCCACCATG.GCC.GAC.CAGCTTGCTGGC
Amino acids in mutant p150:               (A)   (D)
```

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(A *Sma* I and *Not* I site are underlined respectively, the Kozak consensus sequence is italacised, the initiating methionine codon is underlined and written in bold and the amino acid substitutions G^2A and A^3D are written in bold)

Antisense: 5'- CGAATGGACGGGTACTGAT

The 350bp PCR product containing the codon changes was digested with *Sma* I and *Stu* I and subcloned into pVL1393-p150(EE) digested with the same enzymes to create pVL-Myr⁻/p150(EE). The GGA to GCC (G^2A) and AAT to GAC (N^3D) codon changes were confirmed by nucleotide sequencing. The pVL-Myr⁻/p150(EE) construct was transfected with BaculoGold DNA (section 2.2.2.2) to generate recombinant baculovirus. The expression of Myr⁻/p150(EE) protein was checked by infecting Sf9 cells with varying dilutions of virus stock and harvesting the cells 2.5 days later (figure 5.2). To analyse whether the Myr⁻/p150(EE) mutant is myristoylated *in vivo*, Sf9 cells were infected with baculovirus to express Myr⁻/p150(EE) and radiolabelled with [^{3}H]myristic acid as described for the wild type p150 protein. The absence of radiolabelled Myr⁻/p150(EE) protein in this experiment, (figure 5.1A lane 3), demonstrated that disruption of the N-terminal myristoylation consensus sequence abolished the attachment of myristic acid to p150.

5.2.2 Analysis of p150 and PtdIns 3-kinase protein kinase activities

5.2.2.1 Analysis of p150 and PtdIns 3-kinase phosphorylation state *in vivo*

Experiments using [^{32}P]orthophosphate to label the yeast *Saccharomyces cerevisiae* demonstrated that both Vps15p and Vps34p exist as phosphoproteins in vivo (Stack et al 1994 JBC). To examine whether p150 and PtdIns 3-kinase are similarly phosphorylated *in vivo*, U937 and Jurkat cells were radiolabelled with [^{32}P]orthophosphate. To investigate whether the phosphorylation state of p150 or PtdIns 3-kinase changes upon cellular stimulation, the potent agonist phorbol 12-myristate 13-acetate (PMA), was used. During [^{32}P]-orthophosphate labelling, cells were incubated in the presence and absence of PMA, the resulting lysates were then immunoprecipitated with antisera specific to PtdIns 3-kinase (figure 5.3). In U937 cells, both PtdIns 3-kinase and p150 appeared to be phosphorylated. Stimulation with PMA did not significantly alter the phosphorylation of either PtdIns 3-kinase or p150 over that of other proteins. In Jurkat cells, PtdIns 3-kinase and p150 were also found to be phosphorylated. Furthermore, a 120 kDa phosphoprotein was also co-immunoprecipitated from these cell lysates that was absent in PMA stimulated or unstimulated U937 cells.
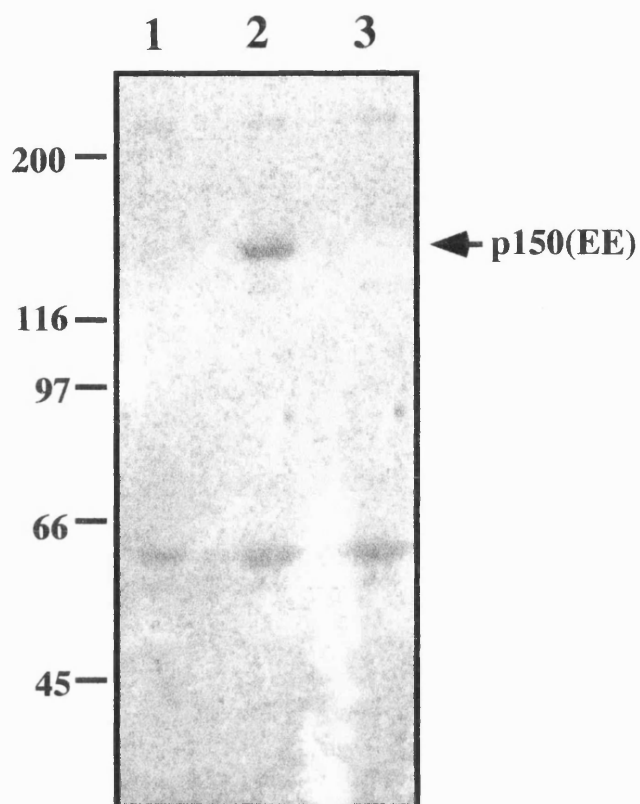
Figure 5.1 p150 is myristoylated *in vivo*.

Sf9 cells were infected with recombinant baculovirus to express
p150(EE) (lane 2), or the myristoylation mutant Myr⁻/p150(EE)
(lane 3). Uninfected Sf9 cells were also included as a control (lane1).
After 24h the control and baculovirus infected cells were labelled
with [^{3}H]myristic acid for 16h at 27^{0}C. EE-tagged proteins were
immunoprecipitated from labelled lysates using EEmAb and collected
on protein G-Sepharose. Immobilised proteins were washed and
resolved by 7.5% SDS-PAGE.Following fluorographic processing,
radioactive bands were visualised by autoradiography.
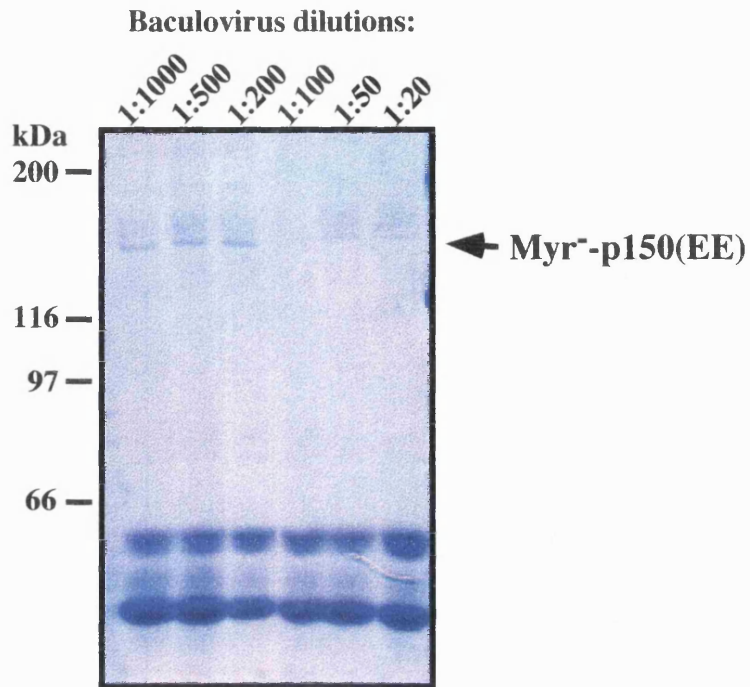
Figure 5.2 Expression of Myr⁻-p150(EE) in insect Sf9 cells.

Sf9 cells were infected with varying dilutions (1:20-1:1000) of recombinant baculovirus to express the p150 myristoylation mutant Myr⁻-p150(EE). After 60h, EE-tagged proteins were immunoprecipitated from cell lysates using EEmAb and collected on protein G-Sepharose beads. Immobilised proteins were analysed using 7.5% SDS-PAGE and visualised by Coomassie Blue staining.

PMA: — — + — —

1 2 3 4 5

kDa

200 —

←p150

116 —

97 —

← PtdIns 3-kinase

66 —
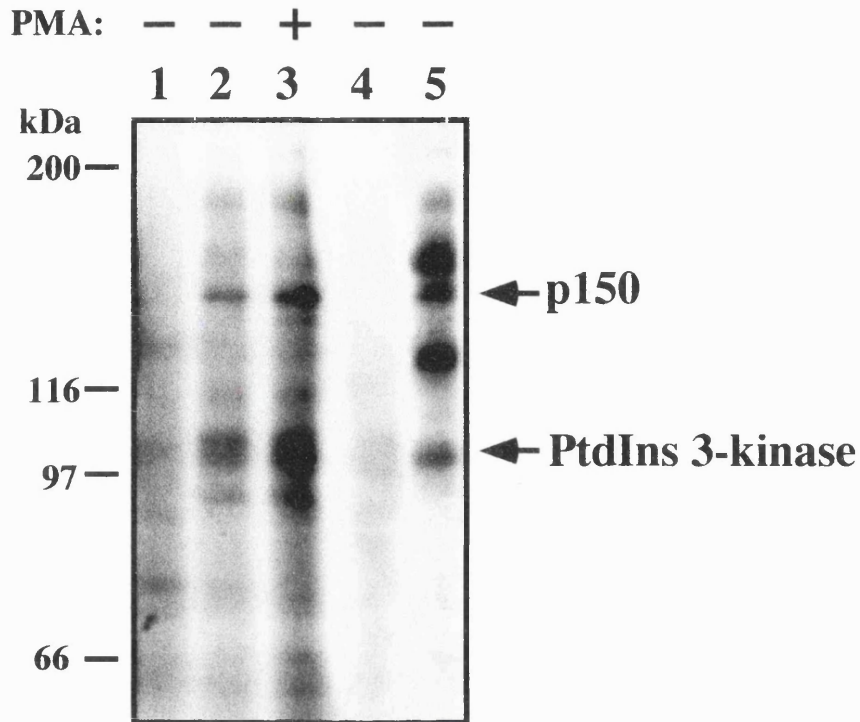
Figure 5.3 *In vivo* phosphorylation of p150 and PtdIns 3-kinase.

U937 and Jurkat cells (2×10^7 cells/sample) were radiolabelled with [^{32}P]orthophosphate for 4h at 37^{0}C and then treated in the presence (+), or absence (-) of phorbol 12-myristate 13-acetate (PMA; 10ng/ml). Using antisera specific to PtdIns 3-kinase, protein complexes were immunoprecipitated from U937 cells (lanes 2 and 3) and Jurkat cells (lane 5). Controls were performed using preimmune serum on radiolabeled U937 (lane 1) and Jurkat (lane 4) cells. Immunoprecipitates were washed extensively, resolved by 7.5% SDS-PAGE and transfered to PVDF. Radioactive proteins were visualised by autoradiography.

5.2.2.2 Analysis of *in vitro* p150 and PtdIns 3-kinase autophosphorylation activity

The use of *in vitro* kinase assays have been used to show that the yeast Vps15p and Vps34p proteins are able to act as protein kinases, since both proteins can undergo autophosphorylation *in vitro* (Stack and Emr, 1994). Vps15p and Vps34p autophosphorylation required the presence of divalent cations, with Mn^{2+} resulting in the highest degree of phosphorylation (Stack and Emr, 1994). Mutation of the Vps15p and Vps34p kinase domains indicated that phosphorylation of these proteins was due their intrinsic protein kinase activities and not a contaminating protein kinase (Stack and Emr, 1994). In addition, phosphoamino acid analysis of *in vitro* and *in vivo* labelled Vps15p or Vps34p showed that both proteins have a functional ser/thr protein kinase activity (Herman *et al.*, 1991a; Stack and Emr, 1994).

To investigate whether p150 and PtdIns 3-kinase are able to carry out autophosphorylation, Sf9 cells were infected with baculoviruses to express PtdIns 3-kinase(EE), p150(EE), the p150(EE)/PtdIns 3-kinase complex and the p85α/GSTp110α heterodimer. EE-tagged proteins were immunoprecipitated from Sf9 cell lysates using EEmAb and collected on protein G-Sepharose beads. Sf9 cells expressing GST fusion proteins were affinity purified using glutathione-Sepharose beads. Immunoprecipitated or affinity purified proteins were washed and incubated with $[\gamma\text{-}^{32}P]ATP$ (as described in section 2.3.4) in the presence of different divalent cations. The labelled proteins were then separated using SDS-PAGE and visualised by autoradiography (figure 5.4). The results show that neither p150 nor PtdIns 3-kinase were able to autophosphorylate irrespective of the divalent cation used. In addition, the formation of the p150/PtdIns3-kinase complex was found to be unable to promote any observable *in vitro* protein kinase activity since both proteins remained unphosphorylated. To verify that the conditions used were suitable for *in vitro* protein kinase activity, the p85α/GSTp110α heterodimer was used as a positive control. It has previously been demonstrated that p110α can act as a ser/thr protein kinase, by specifically phosphorylating the serine 608 residue of p85α (Dhand *et al.*, 1994b). This experiment confirmed the phosphorylation of p85α by GSTp110α. The absence of p150 and PtdIns 3-kinase phosphorylation could be explained if p150 and PtdIns 3-kinase had already been phosphorylated *in vivo*, such that potential sites for autophosphorylation might already be occupied. To preclude this possibility, immunoprecipitated or affinity purified proteins were dephosphorylated with Calf Intestine Alkaline Phosphatase (CIAP) prior to assaying for *in vitro* kinase activity. This treatment however, did not alter the initial observation that the p150/PtdIns 3-kinase complex remained unphosphorylated and that p150 or PtdIns 3-kinase were unable to initiate autophosphorylation (results not shown). *In vitro* protein kinase assays of
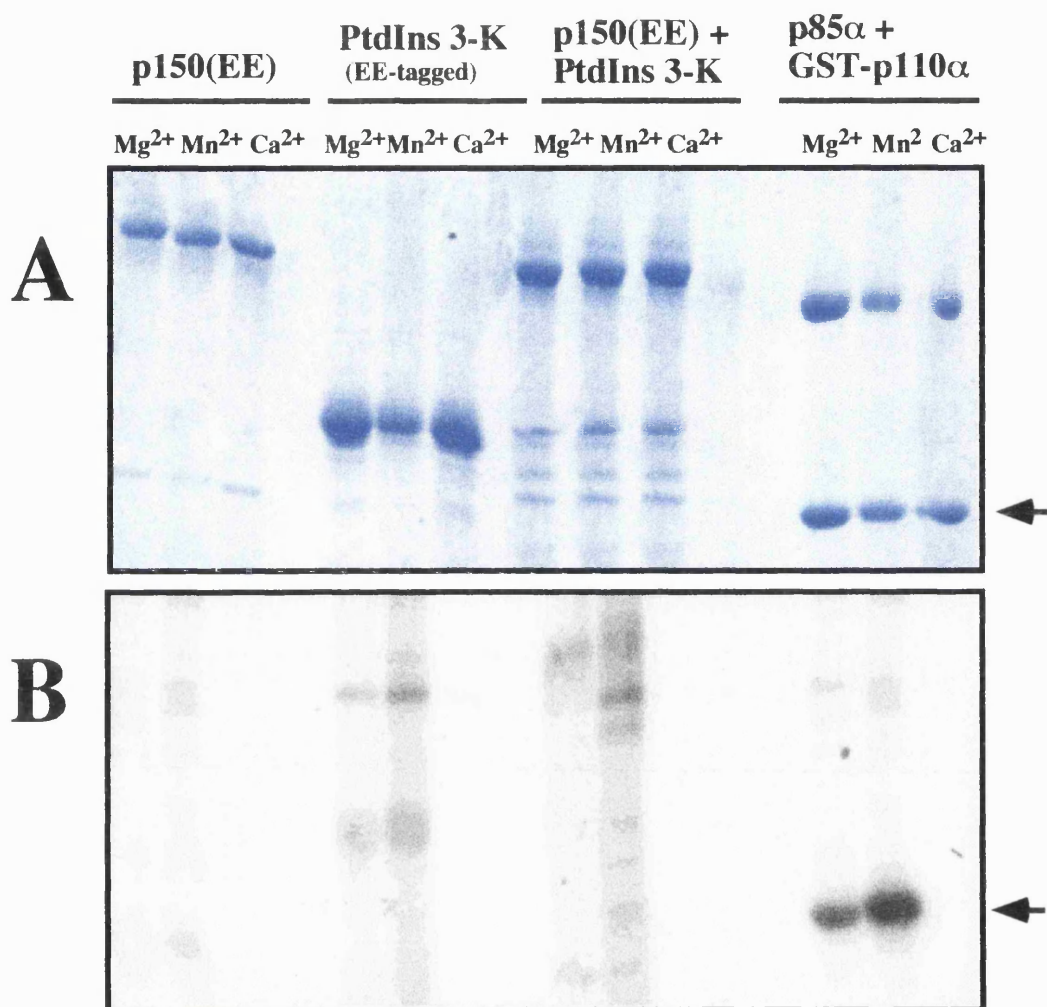
Figure 5.4 Recombinant p150 and PtdIns 3-kinase are not phosphorylated *in vitro*.

Sf9 cells were infected for 60h with baculoviruses to express p150(EE), PtdIns 3-kinase(EE), the p150(EE)/PtdIns 3-kinase complex and the p85α/GST-p110α heterodimer. Cell lysates were immunoprecipitated with EEmAb and collected on protein G-Sepharose, or were affinity purified using glutathione-Sepharose. Immunoprecipitated oraffinity purified proteins were washed and incubated with [γ-^{32}ATP] (as described in 'materials and methods') in the presence of differentdivalent cations (Mg^{2+}, Mn^{2+}, or Ca^{2+}). The proteins were resolved by 7.5% SDS-PAGE and visualised using Coomassie Blue staining (Panel A). Radiolabelled proteins were visualised by autoradiography of the same Coomassie stained gel (Panel B). Arrows indicate the position of p85α protein (panel A) and phosphorylated p85α (panel B)

endogenous p150 and PtdIns 3-kinase immunoprecipitated from Jurkat cells also failed to demonstrate any phosphorylation of these proteins (Volinia *et al.*, 1995).

5.2.2.3 Does p150 or PtdIns 3-kinase have protein kinase activity towards other substrates?

The *in vitro* autophosphorylation of p150 or PtdIns 3-kinase, which has previously been reported in yeast for Vps15p and Vps34p, could not be observed for these human proteins. As a complementary approach therefore, the protein kinase activity of the recombinant p150/PtdIns 3-kinase complex toward peptide and protein substrates was investigated.

These results (Table 5.1), demonstrate that the GST-PtdIns 3-kinase/p150 complex is able to efficiently phosphorylate protein kinase C substrate, Myelin Basic Protein (MBP), and to a lesser extent, other substrates. Interestingly, this protein kinase activity displays a cation preference for Mn^{2+}, which has also been observed for the Vps15p and Vps34p protein kinase activities. To examine whether the protein kinase activity of the GST-PtdIns 3-kinase/p150 heterodimer can be attributed to either a p150 or a PtdIns 3-kinase protein kinase activity, EE-tagged p150 or PtdIns 3-kinase was expressed separately in Sf9 cells, immunoprecipitated and incubated with MBP and $[\gamma\text{-}^{32}P]ATP$. The results from this experiment indicated that there was no significant phosphorylation of MBP over background levels by PtdIns 3-kinase or p150 (data not shown), suggesting that protein kinase activity is only functional when p150 and PtdIns 3-kinase are in complex with each other. Protein kinase activity appears to arise on the formation of the p150/PtdIns 3-kinase complex, however, which of the two proteins is providing the protein kinase activity remains unclear.

5.3 Discussion

Using radiolabelled $[^3H]$myristic acid, p150 expressed in Sf9 cells was shown to be myristoylated. This result was confirmed by the mutagenesis of the N-terminal myristoylation consensus site, where the critical glycine residue at position 2 was changed to an alanine thereby abolishing myristic acid attachment. The post-translational lipid modification observed for p150 is the same as that previously demonstrated for Vps15p (Herman *et al.*, 1991a), suggesting that the myristoylation of both proteins serves a similar function. In yeast, differential centrifugation studies determined the intracellular location of Vps15p to be in a detergent soluble pellet fraction, suggesting that Vps15p is associated with a membrane fraction of yeast cell extracts (Herman *et al.*, 1991a). In addition subcellular fractionation studies revealed that Vps15p exhibited a fractionation profile similar to the late Golgi protein Kex2p (Herman *et al.*, 1991a).

Table 5.1

Phosphorylation of different substrates by GST-PtdIns 3-kinase/p150 in the presence of Mg^{2+} or Mn^{2+}.

Substrate	GST-PtdIns 3-kinase/p150 activity (cpm/assay)	
	Mg^{2+}	Mn^{2+}
Protein kinase C substrate	58	10,363
Myelin basic protein	3328	17,631
Kemptide	44	2458
Tyrosine kinase substrate	170	2183
p34^{cdc2} kinase substrate	93	1325
Histone	2127	3305
Syntide 2	7480	6032

Sf9 cells were infected with GST-PtdIns 3-kinase and p150 baculovirus and after 60 h GST-fusion protein was affinity purified from cell lysates using glutathione-Sepharose beads. The immobilised GST-PtdIns 3-kinase/p150 complex was incubated for 30 min with different protein kinase substrates and 0.1 mCi [γ-^{32}P]ATP as described in section 2.3.4.2. All substrates used were at a final concentration of 3.0 mg/ml. Background phosphorylation has been subtracted to show the phosphorylation attributable to the GST-PtdIns 3-kinase/p150 complex.

The N-terminal myristoylation of several proteins including the product of the c-src proto-oncogene and the α subunits of heterotrimeric G proteins, have been demonstrated to be essential for their membrane association (Buss *et al.*, 1986; Schultz *et al.*, 1988). The addition of myristic acid to Vps15p may function in targeting Vps15p to the late Golgi compartment and other vesicular intermediates. By analogy, the myristoylation of p150 may provide a similar membrane targeting function in mammalian cells.

Experiments radiolabelling Jurkat and U937 cells with [^{32}P]orthophosphate, demonstrated that p150 and PtdIns 3-kinase become phosphorylated *in vivo*. In addition, a 120 kDa protein was found to be phosphorylated with the p150/PtdIns 3-kinase complex from Jurkat cells. This 120 kDa phosphoprotein was not observed in immunoprecipitates from U937 cells and at present, the identity of this protein is unknown. The presence of an additional phosphorylated protein associated with the p150/PtdIns 3-kinase heterodimer from Jurkat T-cells, may indicate that there are cell specific regulatory or functional differences compared to the same complex immunoprecipitated from the U937 B-cells.

The existence of p150 and PtdIns 3-kinase as phosphoproteins *in vivo* raises the possibility that these proteins could undergo autophosphorylation, as has been demonstrated for their respective yeast homologues, Vps15p and Vps34p. When recombinant p150, PtdIns 3-kinase or the p150/PtdIns 3-kinase complex were used for *in vitro* protein kinase assays no autophosphorylation was observed. The Vps15p autophosphorylation activity however, is poor and has a low specific activity (Jeff Stack, personal communication). It is possible, therefore that other proteins may be required to fully activate the p150 protein kinase activity. However, *in vitro* kinase assays on endogenous p150 and PtdIns 3-kinase immunoprecipitated from Jurkat cells did not show that these proteins were phosphorylated (Volinia *et al.*, 1995). Nevertheless, a protein kinase activity could only be demonstrated for the recombinant GST-PtdIns 3-kinase/p150 complex when Protein Kinase C substrate or MBP were used as substrates. A protein kinase activity could not be observed for p150 or PtdIns 3-kinase in isolation suggesting that a functional protein kinase activity can only be achieved when both proteins are complexed. Site directed mutagenesis can be used to change essential residues in the kinase domains of p150 and PtdIns 3-kinase to abrogate any protein kinase activity. These mutants could then be used to investigate whether p150 or PtdIns 3-kinase contributes to the protein kinase activity of the p150/PtdIns 3-kinase complex.

Although there is a high degree of sequence conservation between protein kinases and Vps15p and p150 (section 3.2.3.2), this homology is weaker in the N-terminal region of the kinase domain. This area of the catalytic domain contains a GXGXXG sequence

element also termed a nucleotide fold (section 3.2.3.2) and is required for correct binding of ATP. This sequence is poorly conserved in both Vps15p and p150 which may explain why Vps15p is reported to be a poor kinase and the kinase activity of p150 alone, cannot be detected. Indeed, p150 may constitute only a partial protein kinase domain, a suggestion that was initially proposed for Vps15p (Herman *et al.*, 1991a). It is possible that p150 may be part of a hetero-oligomeric complex in which p150 contributes the majority of the kinase catalytic domain and a second protein in the complex contributes the nucleotide-fold region. This is supported by the finding that protein kinase activity using MBP as substrate can only be observed for the GST-PtdIns 3-kinase/p150 complex and not for the individual proteins.

It is clear that p150 and PtdIns 3-kinase exist as phosphoproteins *in vivo*. The absence of autophosphorylation by the recombinant proteins *in vitro* and the lack of phosphorylation observed from *in vitro* kinase assays on Jurkat cell immunoprecipitates suggests that another kinase could phosphorylate p150 and PtdIns 3-kinase *in vivo*. Alternatively, membrane binding of p150 by its myristic acid modification maybe required for protein kinase activation. Membrane binding is often required for the biological activity of myristoylated proteins. Non-myristoylated $pp60^{v-src}$ does not transform cells (Resh, 1993) and nonacylated $G\alpha_t$ (the α subunit of the heterotrimeric G protein transducin) does not associate with $\beta\gamma$ complexes (Wedegaertner *et al.*, 1995). Furthermore, it has been demonstrated that a non-myristoylated Vps15p molecule has a defective ser/thr protein kinase activity, as the phosphorylation signal is only 35-50% of that observed with the wild type Vps15p protein (Herman *et al.*, 1991b). It is possible therefore that the absence of a membrane environment may explain a lack of p150 or PtdIns 3-kinase phosphorylation *in vitro* .

CHAPTER 6

Analysis of 3'-phosphorylated lipid production by PtdIns 3-kinase and substrate presentation to the p150/PtdIns 3-kinase complex

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CHAPTER 6

Analysis of 3'-phosphorylated lipid production by PtdIns 3-kinase and substrate presentation to the p150/PtdIns 3-kinase complex

6.1 Analysis of 3-phosphorylated lipid production

6.1.1 Introduction

Despite the wealth of information regarding participation of PI 3-kinases in agonist signalling cascades (Domin and Waterfield, 1997; Vanhaesebroeck *et al.*, 1997a), comparatively little is known about the functions of these enzymes in resting cells. In contrast with the agonist activated class I enzymes, the class III PI 3-kinases (also referred to as 'PtdIns 3-kinases') are characterised operationally by their constitutive activity in cells and by a more restricted substrate specificity (section 1.3.3). The class III PI 3-kinases which include the yeast Vps34p and human PtdIns 3-kinase, specifically phosphorylate PtdIns to form PtdIns3P and apparently function to maintain constant levels of this phosphoinositide in both resting and actively growing cells. Although PtdIns3P is constitutively present at substantial levels in many different cell types, including yeast, the functional significance of this metabolic pathway has remained elusive. PtdIns3P, as well as the other D-3 phosphorylated lipids such as PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 are resistant to cleavage by phospholipase C (Serunian *et al.*, 1989), and to date, no cellular effects of PtdIns3P have been conclusively identified.

The yeast lipid kinase Vps34p has been demonstrated to possess PtdIns 3-kinase activity *in vitro* and *in vivo*, producing PtdIns3P as its major phospholipid product (Schu *et al.*, 1993). The human PtdIns 3-kinase also exhibits an activity *in vitro* that is selective for PtdIns as substrate (Volinia *et al.*, 1995). However, the effects on *in vivo* cellular phosphoinositide metabolism of PtdIns 3-kinase expressed in mammalian cells has not been examined. In addition, the novel D-3 phosphoinositide PtdIns(3,5)P_2 has been identified as a product of an agonist independent pathway in resting mouse cells (Dove *et al.*, 1997; Whiteford *et al.*, 1997). This suggested that PtdIns(3,5)P_2 was formed by phosphorylation of PtdIns3P at the D-5 position, implicating the lipid product of PtdIns 3-kinase as a substrate for another lipid kinase and PtdIns(3,5)P_2 as a potential lipid signalling molecule.

To investigate the effects of PtdIns 3-kinase on cellular phosphoinositide metabolism, this section describes the radioactive labelling and isolation of phospholipids from

mammalian COS-7 (monkey kidney) cells transiently transfected to express PtdIns 3-kinase.

6.2 Results

6.2.1 Expression of PtdIns 3-kinase in mammalian cells

To examine the effect of elevated PtdIns 3-kinase expression on the levels of cellular phospholipids in mammalian cells, transient transfection assays were chosen as an analytical method. These experiments require the use of a cell line in which relatively high transfection efficiencies and high levels of exogenous protein expression can be achieved. COS-7 cells have DNA encoding the SV40 T antigen stably integrated into their genome. This was achieved by the infection of the simian CV-1 cell line, permissive for lytic growth of SV40, with a replication defective mutant of SV40 (Gluzman, 1981). Consequently, transfection of COS-7 cells with plasmids containing an SV40 origin of replication, together with foreign DNA under the control of the adenovirus major late promoter results in high copy numbers of the transfected plasmid, and high expression levels of the exogenous gene. The pMT2SM mammalian expression vector was constructed for this purpose (Kaufman *et al.*, 1989) and can be transiently transfected into COS-7 cells using electroporation (Knutson and Yee, 1987). To achieve transient expression of PtdIns 3-kinase in mammalian cells the ORF for this lipid kinase was subcloned into pMT2SM via *Eco* RI sites to create the construct pMT2-PtdIns 3-K (made by Dr Stefano Volinia LICR). To verify the expression of recombinant protein from this construct, 5μg of pMT2-PtdIns 3-K DNA was used to transfect COS-7 cells using electroporation (section 2.2.1.4). As a control, COS-7 cells were also transfected with pMT2SM vector alone. After 36 hours, whole cell lysates were prepared and separated using SDS-PAGE, transferred onto PVDF membrane and immunoblotted using PtdIns 3-kinase specific antisera (section 2.3.6). The 100 kDa recombinant PtdIns 3-kinase was expressed in pMT2-PtdIns 3-K transfected cells (figure 6.1, lane 2) and the presence of a small amount of endogenous PtdIns 3-kinase was observed in control cells transfected with pMT2SM vector (figure 6.1, lane 1). To examine the transfection efficiency of pMT2-PtdIns 3-kinase using electroporation, transfected cells were plated onto glass coverslips. Cells expressing PtdIns 3-kinase were visualised using indirect immunofluorescence (section 2.6.3) and counted. Routinely, this demonstrated that 20-30% of cells transfected with the pMT2-PtdIns 3-K construct would express recombinant PtdIns 3-kinase protein.

6.2.2 *In vivo* phosphoinositide analysis

To investigate the phosphoinositides generated *in vivo* by PtdIns 3-kinase, COS-7 cells were transiently transfected with pMT2-PtdIns 3-K construct or pMT2SM vector as a
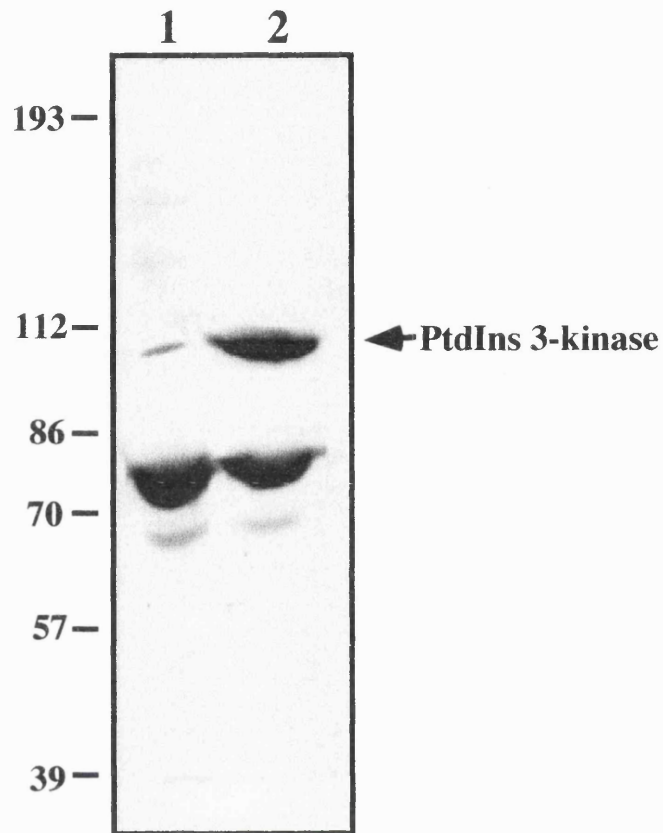
Figure 6.1 Expression of PtdIns 3-kinase in COS-7 cells.

COS-7 cells were transfectedwith either empty pMT2SM vector (lane 1) or pMT2-PtdIns 3-kinase construct (lane 2). After 36h cells were lysed and crude lysate samples resolved using 7.5% SDS-PAGE. PtdIns 3-kinase was detected by Western blotting using PtdIns 3-kinase specific rabbit polyclonal antisera.

control. After 36 hours the cells were labelled with [^{32}P]orthophosphate (200 μCi/ml) for 1 h at 37°C. The labelled lipids were extracted as described in section 2.4.2.1 and deacylated using 33% methylamine in ethanol/water/n-butanol (50:15:5). The resulting glycerophosphoinositols (GroPIns) were separated by anion exchange HPLC. [^{32}P]-labelled GroPIns3P, GroPtIns(3,4)P$_2$, and GroPIns(3,4,5)P$_3$ were also run on HPLC as standards. These were generated by GST-p110α in lipid kinase assays using PtdIns, PtdIns4P and PtdIns(4,5)P$_2$ as lipid substrates. After incubation, the phosphorylated lipids were extracted and separated on a TLC system using propan-1-ol/2M acetic acid (65:35). Phosphorylated lipids were identified by autoradiography, scraped from the TLC plate and deacylated to generate GroPIns standards (section 2.4.2.2). Figure 6.2A illustrates the elution profile of these standards when run on anion exchange HPLC. The profile shows that GroPIns3P eluted first after 24 minutes, then GroPIns(3,4)P$_2$ after 52.5 minutes, and GroPIns(3,4,5)P$_3$ after 80 minutes. In addition, two ^{3}H-labelled standards were also run, these were GroPIns(3,5)P$_2$ [Frank Cooke ICRF, (Dove et al., 1997)] which is eluted just before GroPIns(3,4)P$_2$ at 51 minutes and GroPIns(4,5)P$_2$ which is eluted after 55.5 minutes. The second profile (figure 6.2B) is shown as an example of HPLC analysis of deacylated [^{32}P]-labelled phosphoinositides from pMT2SM transfected (control) COS-7 cells. The cellular deacylated phosphoinositides are identified by comparison of their elution times against the standards which were run in parallel. Experiments involving quantification of GroPIns levels in deacylated extracts of [^{32}P]-labelled control or pMT2-PtdIns 3-K transfected cells were performed in duplicate and repeated at least twice. Levels of the cellular lipids were normalised to the amount of GroPIns present in each sample. Figure 6.3 shows the changes in the levels of PtdIns 3P (panel A), PtdIns(3,5)P$_2$ (panel B), PtdIns(3,4)P$_2$ (panel C) and PtdIns(3,4,5)P$_3$ (panel D) which were observed when PtdIns 3-kinase was expressed in COS-7 cells. The expression of PtdIns 3-kinase in COS-7 cells leads to a 3-fold increase in PtdIns 3P, a 2-fold increase in PtdIns(3,5)P$_2$ and 1.5-fold increase in PtdIns(3,4)P$_2$ and PtdIns(3,4,5)P$_3$ levels respectively when compared to control cells.

6.3   Discussion

As described in section 6.2.2, expression of PtdIns 3-kinase in COS-7 cells was found to produce 1.5-fold increases in PtdIns(3,4)P$_2$ and PtdIns(3,4,5)P$_3$ levels. Since the COS-7 cells used in these experiments were not stimulated by any agonist it is perhaps surprising that PtdIns(3,4)P$_2$ and PtdIns(3,4,5)P$_3$ were observed at all, since these 3'-phosphorylated lipids are usually undetectable in quiescent cells (Stephens et al., 1993b). It is possible however, that in some cell lines such as COS-7, which is transformed with SV40, certain signal transduction pathways might be constitutively active and the generation of PtdIns(3,4)P$_2$ and PtdIns(3,4,5)P$_3$ is observed. Furthermore, the production of PtdIns3P in cells overexpressing PtdIns 3-kinase may serve as a substrate
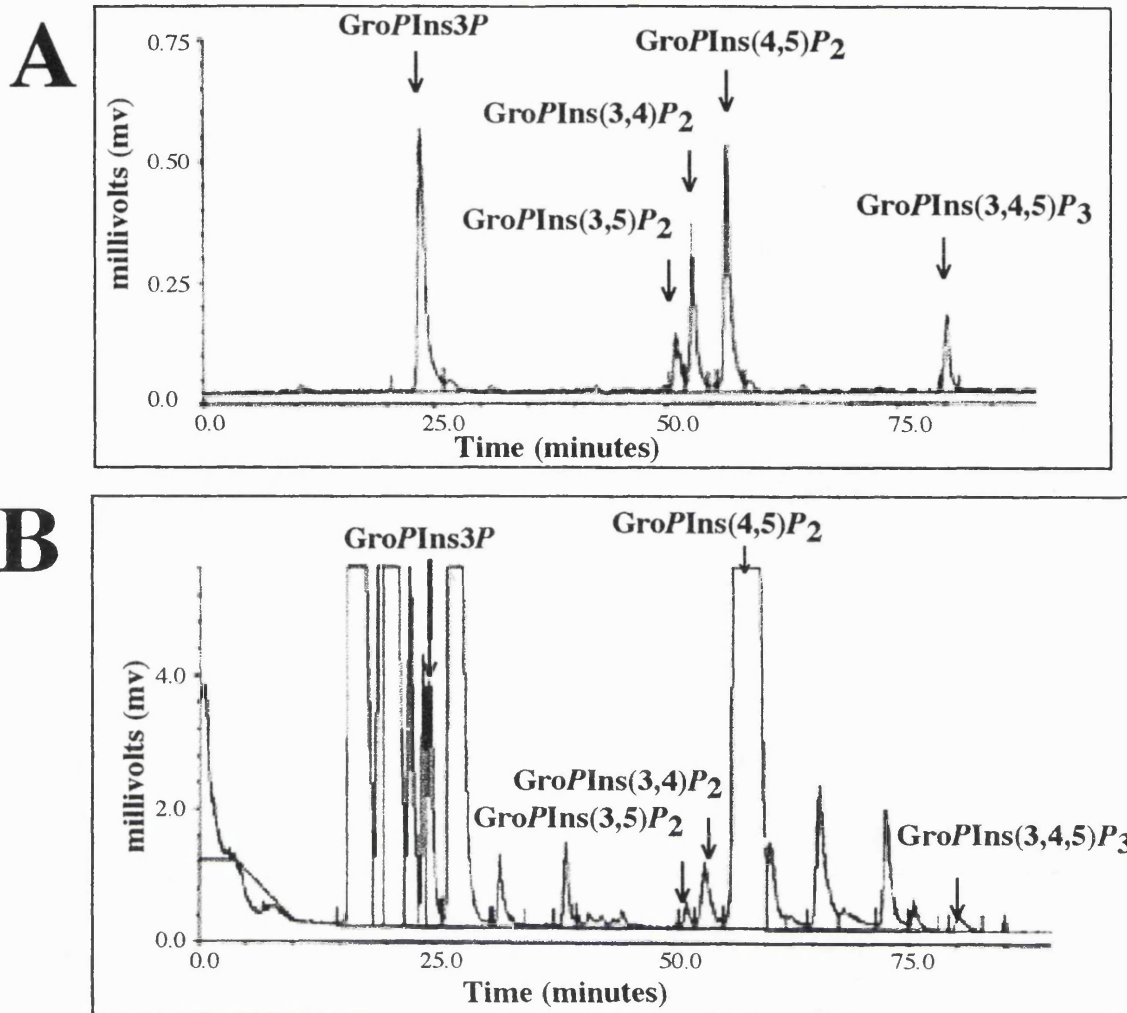
Figure 6.2 Elution profiles of [^{32}P]-labelled glycerophosphoinositols (Gro*P*Ins).

3'-phophoinositides from PI 3-kinase assays resolved by TLC analysis or extracted lipids from [^{32}P]-labelled COS-7 cells were de-acylated (as described in Experimental procedures) and analysed on anion exchange HPLC using a Partisphere Sax column. Samples were eluted using a linear gradient of $(NH_4)_2HPO_4$ at 1ml/min. The elution gradient from 0-1M $(NH_4)_2HPO_4$ pH 3.8 was developed using pumps A (HPLC grade H_2O) and B [1M $(NH_4)_2HPO_4$ pH 3.8] Eluate from the HPLC column flowed into an on-line continuous flow scintillation detector for isotope detection, chromatograms were electronically recorded (panels A and B) showing elution time (minutes; x-axis) and 'radioactive counts' (millivolts; y-axis) of eluted products.

A. Elution profile of Gro*P*Ins standards. PtdIns3*P*, PtdIns(3,4)*P*$_2$ and PtdIns(3,4,5)*P*$_3$ were generated from PI 3-kinase assays, deacylated and run on anion HPLC as described, together with tritiated Gro*P*Ins(4,5)*P*$_2$ and Gro*P*Ins(3,5)*P*$_2$ standards. The separated Gro*P*Ins standards are indicated and have the following elution times: Gro*P*Ins3*P* (24 min), Gro*P*Ins(3,5)*P*$_2$ (51 min), Gro*P*Ins(3,4)*P*$_2$ (52.5 min), Gro*P*Ins(4,5)*P*$_2$ (55.5 min) and Gro*P*Ins(3,4,5)*P*$_3$ (80 min).

B. Elution profile of [^{32}P]-labelled Gro*P*Ins from control COS-7 cells. COS-7 cells were mock transfected with empty pMT2SM vector and labelled with [^{32}P]orthophosphate, lipids were extracted and deacylated (as described in Experimental procedures) and run on anion exchange HPLC. The positions of eluted Gro*P*Ins3*P*, Gro*P*Ins(3,5)*P*$_2$, Gro*P*Ins(3,4)*P*$_2$, Gro*P*Ins(4,5)*P*$_2$ and Gro*P*Ins(3,4,5)*P*$_3$ are indicated, according to the elution times observed for known standards.

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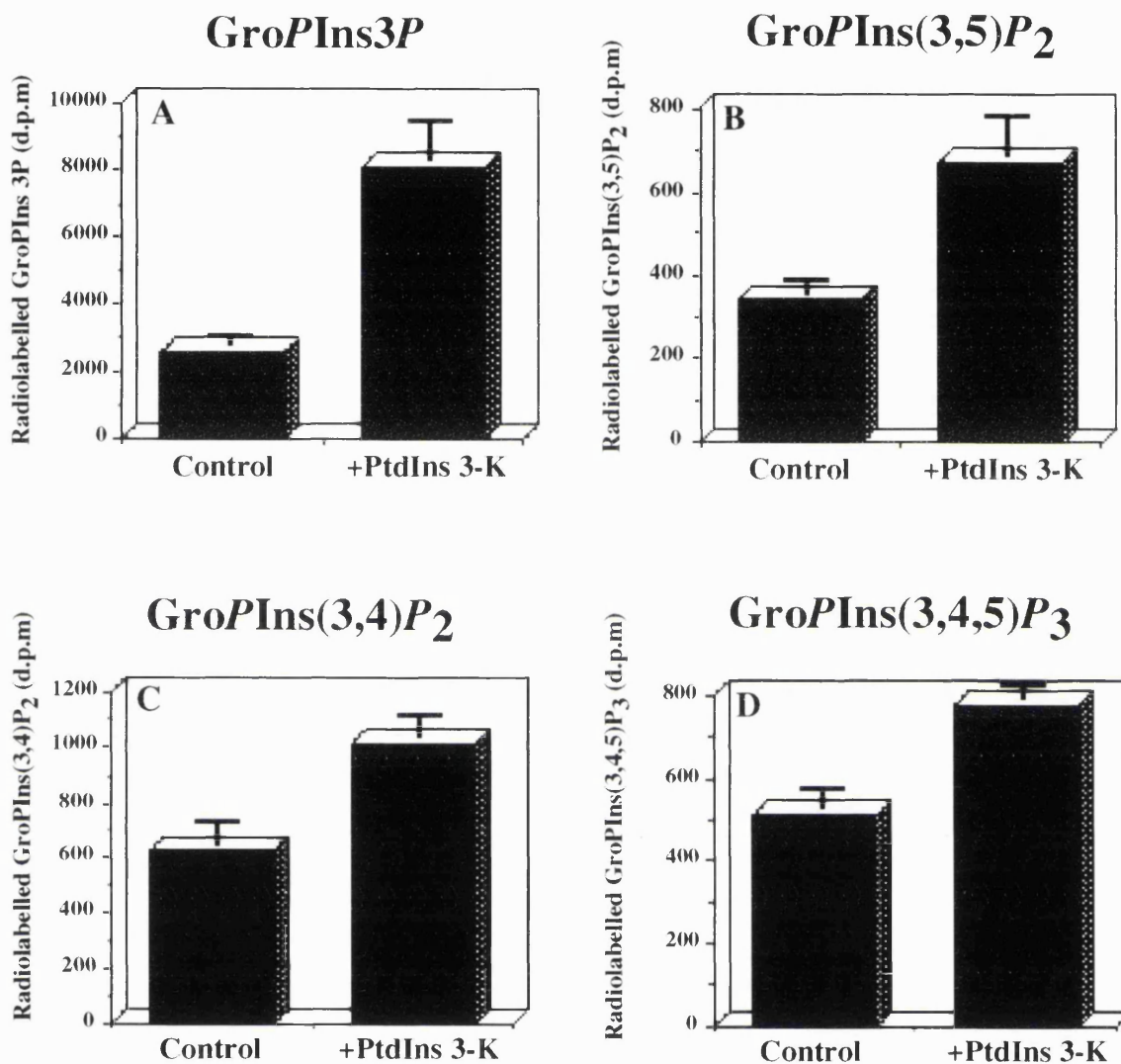
Figure 6.3 Comparison of [^{32}P]- labelled phosphoinositides generated from control and pMT2-PtdIns 3-kinase transfected (+PtdIns 3-kinase), COS-7 cells.

COS-7 cells were transiently transfected with pMT2-PtdIns 3-kinase or transfected with pMT2SM vector as a control. After 36h, cells were radiolabelled with [^{32}P]orthophosphate. Labelled lipids were extracted and deacylated (as described in Experimental procedures). Glycerophosphoinositides (Gro*P*Ins) were separated by anion exchange HPLC. Levels of the cellular lipids were normalised to the amount of Gro*P*Ins present in each sample. The levels of Gro*P*Ins3*P* (Panel A), Gro*P*Ins(3,5)P_2 (Panel B), Gro*P*Ins(3,4)P_2 (Panel C) and Gro*P*Ins(3,4,5)P_3 (Panel D), when PtdIns 3-kinase is expressed (+PtdIns 3-K), are shown in comparison to Gro*P*Ins levels in control cells.

for PI 4-kinases and PI 5-kinases, resulting in the elevated levels of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 observed in PtdIns 3-kinase expressing cells. It has, for example, been demonstrated that a PtdIns5P 4-kinase (Rameh *et al.*, 1997b) can phosphorylate PtdIns3P to form PtdIns(3,4)P_2 (Yamamoto *et al.*, 1990; Zhang *et al.*, 1997).

The 3- and 2-fold increases in PtdIns3P and PtdIns(3,5)P_2 respectively in PtdIns 3-kinase transfected cells observed in this study, may not initially appear to represent a considerable change in phospholipid levels. However, it should be noted that 20-30% of the total cell population express the PtdIns 3-kinase protein. Consequently, after normalising for a 20-30% transfection efficiency there is in fact an 8-fold increase in PtdIns3P and a 6-fold increase in PtdIns(3,5)P_2 in cells transiently expressing the PtdIns 3-kinase enzyme. Using *in vitro* assays, the p150/PtdIns 3-kinase complex has a 2-fold higher lipid kinase activity compared to PtdIns 3-kinase alone (section 4.2.3.2). It is possible therefore, that co-expression of PtdIns 3-kinase and p150 in COS-7 cells may lead to an even greater increase in the *in vivo* levels of PtdIns3P and PtdIns(3,5)P_2 phosphoinositides. In addition, the overexpression of PtdIns 3-kinase in COS-7 cells could result in the inappropriate localisation of this protein. Consequently, PtdIns 3-kinase may act on membranes which this lipid kinase would not usually encounter in a normal physiological setting. The co-expression of p150 with PtdIns 3-kinase might allow the correct subcellular targeting of this lipid kinase, thereby minimising the possibility of non-specific lipid phosphorylation.

Experiments were performed to examine the phosphoinositides produced when p150 and PtdIns 3-kinase were co-expressed in COS-7 cells. However, due to the very low number of cells co-expressing both proteins (2-5%), any changes in PtdIns3P or PtdIns(3,5)P_2 levels due to the expression of the p150/PtdIns 3-kinase complex could not be observed above the background of cells expressing PtdIns 3-kinase alone (20-30%). Ideally for such an experiment, a stable cell line should be used in which p150 and PtdIns 3-kinase could be stably expressed, or in which one protein could be stably expressed and the other transiently transfected. Attempts at making such cell lines however, proved that they were difficult to derive.

The 8-fold increase in PtdIns3P observed in PtdIns 3-kinase expressing cells strongly suggests that PtdIns 3-kinase acts as a PtdIns specific lipid kinase *in vivo*, as does its yeast homologue, Vps34p. The data obtained from these experiments however, cannot be used to determine whether the 6-fold increase in PtdIns(3,5)P_2 levels that is also observed is due to: a) the action of a PI 5-kinase using the PtdIns 3P produced by PtdIns 3-kinase as substrate, b) PtdIns 3-kinase using PtdIns5P as a substrate to produce PtdIns(3,5)P_2 or c) that expression of PtdIns 3-kinase somehow leads to an up-

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regulation of a 4-phosphatase activity which metabolises PtdIns(3,4,5)P_3 leading to an increase in PtdIns(3,5)P_2.

Experiments which initially demonstrated PtdIns(3,5)P_2 to be a product of an agonist independent pathway in resting mouse fibroblasts (Whiteford et al., 1997), suggested that PtdIns(3,5)P_2 could not be derived from the 4'-dephosphorylation of PtdIns(3,4,5)P_3 due to the very low levels of this lipid in the cells used. Data was provided, however, to show that PtdIns(3,5)P_2 was formed by the phosphorylation of PtdIns3P at the D-5 position by a PtdIns3P 5-kinase. Short [^{32}P]orthophosphate labelling times were used to ensure that the the last phosphate group added to polyphosphoinositides would be labelled to the highest specific activity (Stephens and Downes, 1990; Stephens et al., 1991). The majority of the radioactivity associated with Gro$P$Ins(3,5)P_2 was present at the D-5 position of the inositol ring, indicating that addition of the phosphate to the D-5 position occured subsequent to phosphorylation at the D-3 position. Furthermore addition of wortmannin, an inhibitor of PI 3-kinase activity (Arcaro and Wymann, 1993) resulted in decreased cellular levels of both PtdIns3P and PtdIns(3,5)P_2, whereas PtdIns, PtdIns4P and PtdIns(4,5)P_2 levels remained unaffected. Although these cellular studies could not rule out the possibility of a wortmannin-sensitive PtdIns3P 5-kinase, the PtdIns3P used as substrate for this kinase was shown to be generated by the action of a wortmannin-sensitive PI 3-kinase, which human PtdIns 3-kinase (wortmannin IC$_{50}$: 2nM) could be a candidate. Further labelling studies also revealed that the 3' and 5' phosphates in PtdIns(3,5)P_2 reached isotopic equilibrium even more quickly than in PtdIns(4,5)P_2, indicating that PtdIns(3,5)P_2, once formed, was rapidly reconverted to PtdIns 3P by the action of a specific 5'-phosphatase activity. These results pointed to the interesting possibility that PtdIns(3,5)P_2 and not PtdIns3P may mediate the signalling functions which are anticipated of an agonist-independent PI 3-kinase such as PtdIns 3-kinase (Whiteford et al, 1997).

Dove et al, have recently demonstrated that when the yeasts Saccharomyces cerevisiae and Schizosaccharomyces pombe are hyperosmotically stressed, they rapidly synthesise PtdIns(3,5)P_2 by a process that involves activation of a PtdIns3P 5-kinase (Dove et al., 1997). This PtdIns(3,5)P_2 accumulation only occurs in yeasts that have an active VPS34-encoded PtdIns 3-kinase, showing that this lipid kinase produces the PtdIns3P for PtdIns(3,5)P_2 synthesis (Dove et al., 1997). S.cerevisiae responses to hyperosmotic stress are controlled through a pathway involving the MAP kinase homologue Hog 1 (Schuller et al., 1994; Wurgler Murphy and Saito, 1997). Mutants were examined in which components of this signalling cascade were knocked out (Dove et al., 1997). These included the Sho 1 osmosensor, the Ssk 1 response regulator element of the Sln 1 two-component osmosensor and the Hog 1 protein kinase (Wurgler Murphy and Saito,

1997). These knockouts showed normal PtdIns(3,5)P_2 accumulation responses, indicating that the Hog 1 pathway does not control hyperosmotically induced PtdIns(3,5)P_2 synthesis. To define the synthetic route to PtdIns(3,5)P_2, S.cerevisiae were briefly labelled with [^{32}P]orthophosphate during hyperosmotic stress. Under these conditions the D-5 phosphate of GroPIns(3,5)P_2 was added last indicating that PtdIns(3,5)P_2 is synthesised by 5'-phosphorylation of PtdIns3P. A rapid metabolic turnover of PtdIns(3,5)P_2 was also observed, which corresponds to the data obtained from resting mouse fibroblasts (Whiteford et al., 1997). These results give further credence to the pathway:

PtdIns → PtdIns 3P → PtdIns(3,5)P_2 (see figure 6.4)

The defective protein trafficking observed in yeast strains mutated in Vps34p activity suggest that PtdIns(3,5)P_2 may have a role in vesicular protein sorting.

The PtdIns(3,5)P_2 accumulation observed in hyperosmotically stressed yeast cells, is in contrast to that observed in COS-7 cells (Dove et al., 1997). In this case, hyperosmotic shock was demonstrated to decrease PtdIns(3,5)P_2 levels in COS-7 cells whereas hypo-osmotic shock enhanced PtdIns(3,5)P_2 in these cells. The synthesis of PtdIns(3,5)P_2 in this way, suggests the existence of an osmotically regulated PtdIns3P 5-kinase activity. Candidates for such a phosphoinositide kinase include the S.cerevisiae Tor 1 and Tor 2 proteins Rad 3 (S.Pombe) and FRAP (human) which contain putative lipid kinase domains, but as yet have not had a lipid kinase activity ascribed to them. In addition the products of the two yeast PtdIns4P 5-kinase like genes mss 4 and Fab 1 and in mammalian cells the type I PI 5-kinases could also be enzymes which use PtdIns3P to make PtdIns(3,5)P_2 (Rameh et al., 1997a). It is interesting to note that some of these candidates for an osmotically regulated PtdIns3P 5-kinase have previously been postulated to function in some kind of osmoregulatory capacity. For example, Fab1p is an important regulator of vacuole homeostasis in budding yeast (Yamamoto et al., 1995), whereas the inactivation of Mss4p and the yeast PtdIns 4-kinase, Stt4p, results in an osmosensitivity similar to that observed in yeast PKC mutants (Flanagan and Thorner, 1992; Yoshida et al., 1994). In addition to this, vps34 and vps15 mutants not only exhibit severe defects in the sorting of soluble vacuolar hydrolases but also demonstrate an increased sensitivity to conditions of osmotic stress (Herman and Emr, 1990; Herman et al., 1992). It is therefore tempting to speculate that the human Vps15p/Vps34p heterodimer, p150/PtdIns 3-kinase, may behave as an osmosensitive complex producing PtdIns3P substrate for a similarly regulated PtdIns 3P 5-kinase, generating PtdIns(3,5)P_2 as a potential lipid signalling molecule.

The results from section 6.2.2 show that an overexpression of PtdIns 3-kinase in COS-7 cells produced increased levels of PtdIns3P and PtdIns(3,5)P_2 phosphoinositides,
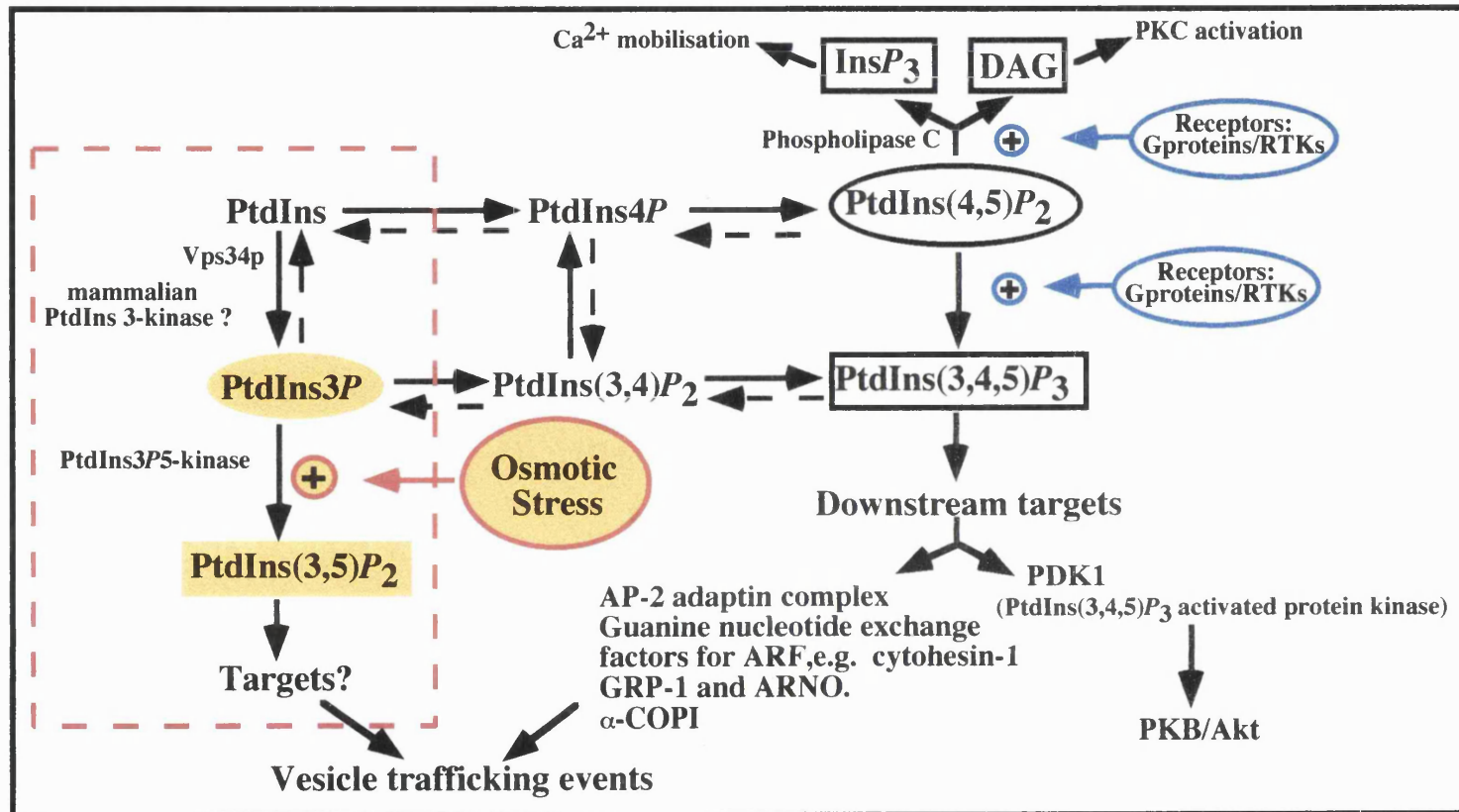
Figure 6.4 Scheme to illustrate the stress activated synthesis of PtdIns(3,5)P_2.

Production of PtdIns(3,5)P_2 is described (boxed, left) and previously known inositol lipid-based signalling mechanisms. Substrates of signalling reactions and their activators are enclosed in ovals and proven or potential second messengers in rectangles. All these reactions occur in animal cells, but the PtdIns(4,5)P_2 3-kinase pathway appears to be absent from yeasts and plants, and the status of the phospholipase C pathway is uncertain in yeast (Dove *et al* 1997). (RTKs: receptor tyrosine kinases; InsP_3: Ins(1,4,5)P_3; DAG: diacylglycerol, ARF: ADP-ribosylation factor, GRP-1: general receptor for phosphoinositides 1; ARNO: ARF nucleotide-binding site opener).

suggesting that like Vps34p, human PtdIns 3-kinase uses PtdIns as its main substrate *in vivo*. Data from Whiteford *et al* , and Dove *et al*, have demonstrated that PtdIns(3,5)P_2 is likely to be a product of PtdIns3P phosphorylation through the action of a PI 5-kinase (Dove *et al.*, 1997; Whiteford *et al.*, 1997). The observation that hypo-osmotic shock elevates PtdIns(3,5)P_2 labelling in COS-7 cells (Dove *et al.*, 1997) raises the question of whether there would be an additional increase in PtdIns(3,5)P_2 levels in COS-7 cells overexpressing PtdIns 3-kinase that are also osmotically stressed. The fact that hyperosmotic stress in yeast cells and hypo-osmotic stress in COS-7 cells provokes a rapid burst of PtdIns(3,5)P_2 synthesis, might indicate that enhanced PtdIns(3,5)P_2-dependent vesicle trafficking is somehow involved in an acute osmotic adaptation response. This could involve an acceleration or intensification of the normal Golgi-to-vacuole/lysosome flux or the recruitment of some other vesicle trafficking event.

6.4   Substrate presentation to the p150/PtdIns 3-kinase complex

6.4.1   Introduction

The phospholipid transfer proteins (PL-TPs) are known for their ability to act *in vitro* as specific carriers of single phospholipid molecules between membranes (Liscovitch and Cantley, 1995; Wirtz, 1997). Mammalian phosphatidylinositol transfer proteins (PI-TPs) appear to play an important role in vesicle flow, both in budding from the *trans*-Golgi network and in fusion with the plasma membrane (Wirtz, 1997). The yeast phosphatidylinositol transfer protein, Sec14p, catalyses exchange of PtdIns and phosphatidylcholine (PtdCho) between membrane bilayers *in vitro* (Bankaitis *et al.*, 1990). *In vivo*, Sec14p activity is essential for the process of vesicle budding from the Golgi complex (Kearns *et al.*, 1997). Recent data has suggested that the PtdIns and PtdCho-bound forms of Sec14p execute independent, yet complementary functions which maintain a Golgi diacylglycerol (DAG) pool, essential for Golgi-derived secretory vesicle biogenesis (McGee *et al.*, 1994; Skinner *et al.*, 1995). It is thought that PtdCho-bound Sec14p negatively regulates Golgi CDP-choline pathway activity and prevents DAG consumption by the PtdCho biosynthetic pathway. PtdIns-bound Sec14p then stimulates PtdIns metabolism through which Golgi DAG may be replenished (Kearns *et al.*, 1997). This latter action of PtdIns-bound Sec14p may explain how the PtdIns-transfer activity of mammlian PI-TP enables mammalian PI-TP to rescue *sec14-1*[ts] yeast mutants (Alb *et al.*, 1995; Tanaka and Hosaka, 1994).

Mammalian PI-TPα was identified by its ability to rescue mutant *sec14-1*[ts] yeast strains. The mammalian isoform PI-TPβ (which has 77% identity and 94% similarity with PI-TPα) was isolated by a similar method (Tanaka and Hosaka, 1994). Although PI-TPα

and PI-TPβ were only able to partially rescue *sec14-1^{ts}* mutants, both Sec14p and mammalian PI-TPs share the ability to act as phospholipid transfer proteins *in vitro* (Liscovitch and Cantley, 1995; Wirtz, 1997). However, since the mammalian PI-TPs have not been implicated in preserving DAG Golgi pools and have very little amino acid sequence homology with Sec14p, the mechanism by which the mammalian PI-TPs function *in vivo* remains unclear. The identification of a human Sec14p homologue, Sec14L (Chinen *et al.*, 1996) suggests an evolutionary conservation between yeast and mammals. Nevertheless, the lack of PI-TPα and PI-TPβ homologues in yeast illustrates an evolutionary divergence which raises further questions as to the precise role these PI-TPs have in mammalian cells.

The presence in mammalian cells of the highly homologous PI-TPα and PI-TPβ isoforms, which have similar properties in biochemical assays, suggest that a functional redundancy may exist. The mouse *vibrator* mutation (*vb*) manifests itself as a progressive whole body action tremor which is caused by a decrease in PI-TPα expression (Hamilton *et al.*, 1997). Levels of PI-TPβ appear to be independent of *vb* genotype, therefore loss of PI-TPα in *vb* mice is not compensated by increased expression level of the other known PI-TP isoform (Hamilton *et al.*, 1997). This data suggests that despite their sequence similarity, PI-TPα and PI-TPβ may serve different functions *in vivo*. The Drosophila retinal degeneration B (*rdgB*) gene, encodes an integral membrane protein which contains an N-terminal PI-TP domain that has more than 40% identity to PI-TPα (Vihtelic *et al.*, 1993). Expression of RdgB's PI-TP domain as a soluble protein in *rdgB2* mutant flies is sufficient to completely restore normal phenotype. Substitution by PI-TPα, a classical PtdIns-transfer protein, for RdgB's PI-TP domain could not restore a normal phenotype to mutant *rdgB2* flies (Milligan *et al.*, 1997). This data therefore suggests that although PI-TPs may have similar PtdIns/PtdCho transfer activity *in vitro*, certain functions cannot be substituted *in vivo*.

The mammalian PI-TPs have been implicated as components of intracellular membrane traffic. Blocked intracellular transport is proposed to account for some of the neurodegenerative pathology observed in *vibrator* mice which lack PI-TPα (Hamilton etal'97). In mammalian cells vesicle flow also appears to be dependent on PI-TP. PI-TP was identified as a cytosolic factor required for the ATP-dependent priming of secretory granules in PC12 cells (Hay and Martin, 1993). PI-TP also participates in the synthesis of phosphoinositides by presenting PtdIns to PtdIns 4-kinase (Kauffmann Zeh *et al.*, 1995; Liscovitch and Cantley, 1994). Studies which examined the biogenesis of immature secretory vesicles from the TGN in cell-free systems have led to the isolation of mammalian PI-TP as a factor required for vesicle budding (Ohashi *et al.*, 1995). Such studies have implicated mammalian PI-TP in the process of vesicular transport from the

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TGN. Furthermore, the requirement for PI-TP in Golgi complex function may reflect a general role for this protein in PtdIns substrate presentation (De Camilli *et al.*, 1996) not only to PtdIns 4-kinase, but also to other PtdIns-specific lipid kinases such as the human PtdIns 3-kinase.

In this section therefore, the effect of PI-TP on human PtdIns 3-kinase activity was investigated and the possible role of PI-TP in PtdIns substrate presentation to the p150/PtdIns 3-kinase complex examined.

6.5 Results

6.5.1 The effect of PI-TP on the lipid kinase activity of the p150/PtdIns 3-kinase complex

To investigate the possible effect of PI-TP on PtdIns 3-kinase activity, the GST-p150/PtdIns 3-kinase complex was expressed in Sf9 cells and affinity purified using glutathione-Sepharose beads (section 4.2.3). Before examining the dose-dependent effects of PI-TP on lipid kinase activity, a time course assay was carried out to ensure that a reaction time was chosen which was within the linear range of the PtdIns 3-kinase enzymatic activity. Since endogenous PI-TP in human neutrophils has been estimated at 0.5μM, this concentration of recombinant PI-TP was used for the time course experiments (Kular *et al.*, 1997). PI-TPα, PI-TPβ or Sec14p at a concentration of 0.5μM was preincubated with 1mM PtdIns for 5mins at 37°C and then added to the GST-p150/PtdIns 3-kinase complex. The radiolabelled PtdIns3*P* produced was extracted and quantified using PhosphorImager analysis (section 2.4.1). Figure 6.5 illustrates the increase in PtdIns 3-kinase activity over time which is greater in the presence of PI-TP. From the time course experiment, a 15 min reaction time was chosen for subsequent assays (figure 6.5). This time point was within the linear range and gave a maximal fold increase in lipid kinase activity for those samples to which 0.5 μM PI-TP had been added. A 3-fold increase in PtdIns 3-kinase activity was obtained in the presence of PI-TPα and PI-TPβ and 4-fold in the case of Sec14p. To examine the dose-dependent effect of PI-TP on PtdIns 3-kinase activity, 0-2.6μM PI-TPα, PI-TPβ or Sec14p was preincubated with 1mM of PtdIns and added to the GST-p150/PtdIns 3-kinase complex in a dose-dependent manner. Following the lipid kinase reaction radiolabelled PtdIns3*P* was extracted and quantified as described previously (section 2.4.1). Enzymatic activity was expressed as a percentage change over that detected in reactions performed in the absence of PI-TP. Results presented in figure 6.6 show that a dose-dependent increase in lipid kinase activity took place upon the addition of PI-TP. At concentrations above 1μM, both PI-TPα and PI-TPβ exhibited slight inhibitory effects. Sec14p produced the greatest
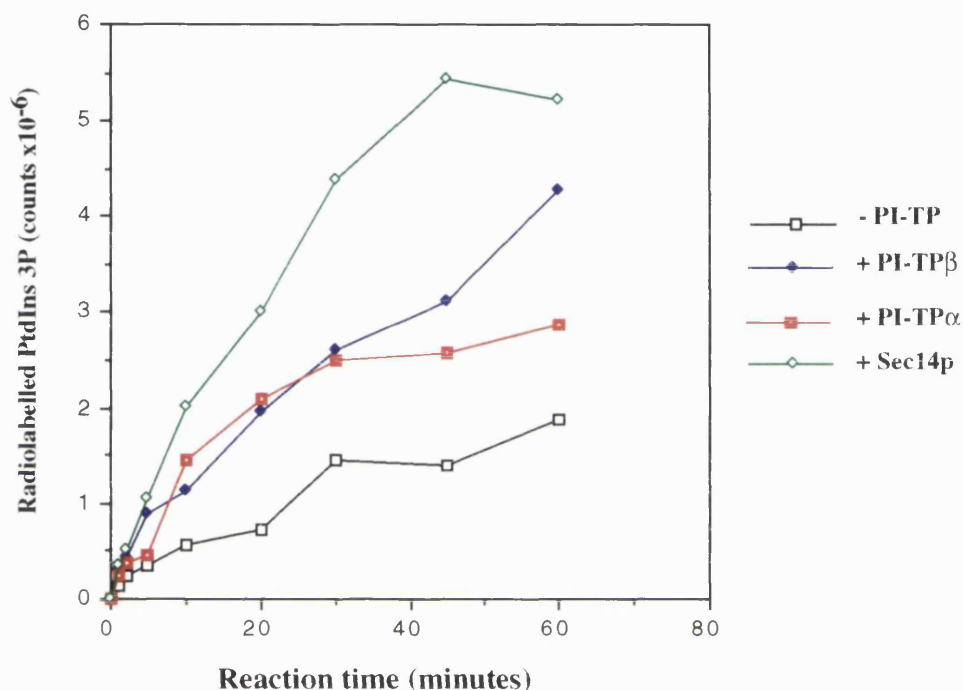
Figure 6.5 Time course assay of PtdIns 3-kinase
activity in the presence or absence of PI-TP.

Sf9 cells were infected with recombinant baculoviruses to express
GSTp150/PtdIns 3-kinase complex. After 60h, GST fusion protein
was affinity purified using glutathione-Sepharose beads. PI 3-kinase
assays were performed on the immobilised protein in the presence of
Mn^{2+} using PtdIns as substrate. For the time course experiments,
1mM PtdIns was incubated for 5 mins at 37^0C with 0.5µM PI-TPα,
PI-TPβ, Sec14p or in the absence of PI-TP. This was added to
GSTp150/PtdIns 3-kinase and PI 3-kinase assays performed over a
period of 0-60 mins. The radiolabelled PtdIns3P produced was extracted,
resolved using TLC and quantified using PhosphorImager analysis.
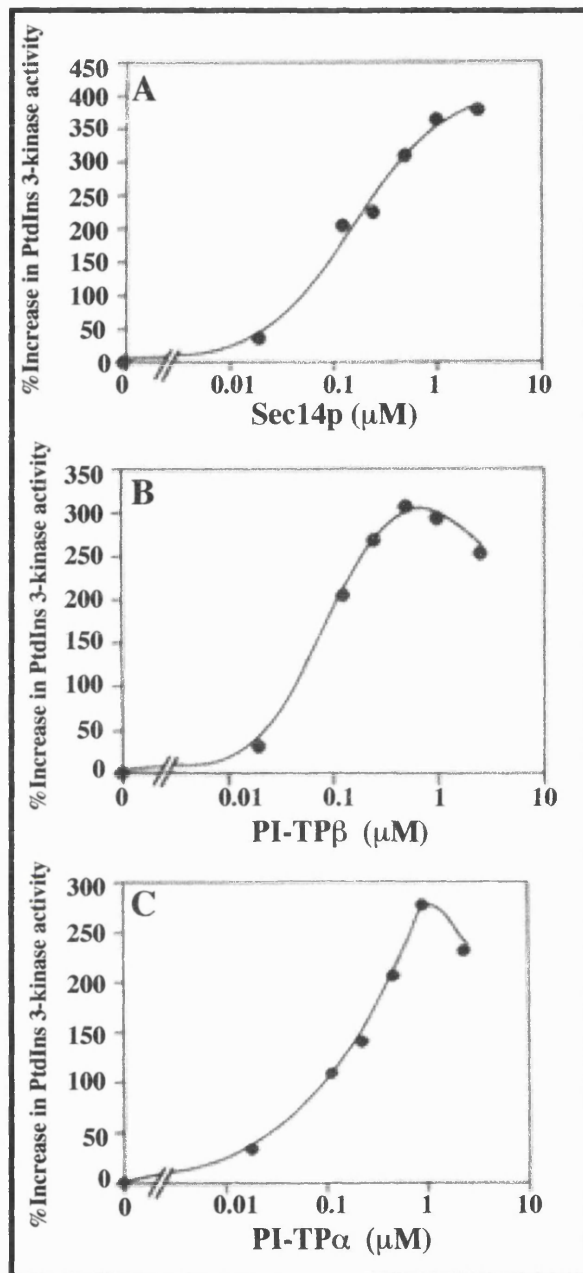
Figure 6.6 Effect of PI-TP on PtdIns 3-kinase activity.

Sf9 cells were co-infected with GST-p150 and PtdIns 3-kinase expressing baculoviruses. After 60h, co-expressed protein complexes were affinity purified from cell lysates using glutathione-Sepharose beads. PI 3-kinase assays were performed on the immobilised protein in the presence of Mn^{2+} using PtdIns as substrate. To investigate the effect of PI-TP on the lipid kinase activity of the complex, increasing amounts of Sec14p (Panel A), PI-TPβ (Panel B), and PI-TPα (Panel C) were preincubated with 1mM PtdIns for 5 min at 37^0C. This was then added to the GST-p150/PtdIns 3-kinase complex and the PI 3-kinase reaction initiated. Extracted lipids were resolved by TLC and quantitated using the PhosphorImager. The increase in lipid kinase activity is expressed as a percentage of that given by the GST-p150/PtdIns 3-kinase complex in the absence of PI-TP.

increase in PtdIns 3-kinase activity (4-fold); PI-TPβ gave a β-fold increase and PI-TPα increased lipid kinase activity by 3-fold. These maximal increases in PtdIns 3-kinase activity were observed at physiological PI-TP concentrations (0.5-1μM range). The absence of any contaminating proteins in the PI-TPα, PI-TPβ and Sec14p samples (figure 6.7), precluded the possibility that other proteins mediated the observed increases in PtdIns 3-kinase activity.

6.5.2   Interaction of PI-TP with the GST-p150/PtdIns 3-kinase complex

The ability of PI-TP to increase PtdIns 3-kinase activity *in vitro* suggested that a physical interaction might occur between the two proteins, thereby enabling PtdIns substrate bound by PI-TP, to be transfered to PtdIns 3-kinase. To investigate if PI-TP can physically associate with the GST-p150/PtdIns 3-kinase complex, recombinant mammalian PI-TP (PI-TPα and PI-TPβ) was incubated with either GST protein or GST-p150/PtdIns 3-kinase. Protein complexes were collected on glutathione-Sepharose beads and separated using SDS-PAGE. Western blot analysis (figure 6.8) indicated that both PI-TPα and PI-TPβ were able to associate with the GST-p150/PtdIns 3-kinase complex *in vitro,* but not with GST alone.

6.5.3   A PtdIns-specific PI 3-kinase associates with PI-TP *in vivo*

To examine whether PI-TP associated with PtdIns 3-kinase *in vivo*, PI-TPα was immunoprecipitated from Jurkat cells and assayed for lipid kinase activity using PtdIns as substrate in the presence of Mn^{2+}. Extracted lipids were separated using a borate solvent system (Walsh *et al.,* 1991), which can separate PtdIns4P from PtdIns3P. A lipid kinase activity co-immunoprecipitating with PI-TPα was detected and found to be a PI 3-kinase and not a PtdIns 4-kinase (figure 6.9A). The human PtdIns 3-kinase, however, could not be detected by Western blot analysis of parallel PI-TPα immunoprecipitates. This could be due to low antibody sensitivity, which as a consequence may fail to detect the presence of low levels of PtdIns 3-kinase in these immunoprecipitates. An alternative possibility is that a PtdIns 3-kinase isoform may be co-immunoprecipitated with PI-TPα that cannot be detected with the PtdIns 3-kinase antisera used.

To investigate the substrate specificity of this PI-TP-associated PI 3-kinase further, PI-TPα was immunoprecipitated from Jurkat cells and the immune complex was subjected to PI 3-kinase assays in the presence of Mg^{2+} or Mn^{2+} using PtdIns, PtdIns4P or PtdIns(4,5)P_2 as substrate (Figure 6.9B). The PI 3-kinase activity associated with PI-TPα primarily used PtdIns as substrate and exhibited a cation preference for Mn^{2+}. This biochemical profile parallels that of the human Vps34p homologue PtdIns 3-kinase,
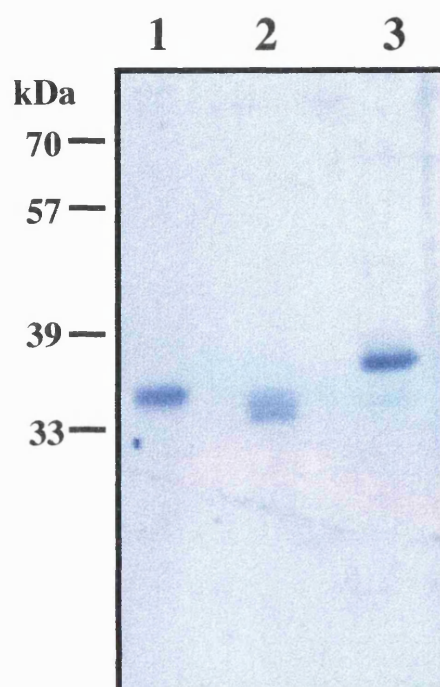
Figure 6.7 Recombinant PI-TPα, PI-TPβ and Sec14p proteins.

PI-TPα (lane 1), PI-TPβ (lane 2) and Sec14p (lane 3) were expressed in *Escherichia coli* as Histidine-tagged proteins and purified as described in Cunnigham *et al.*, 1996.
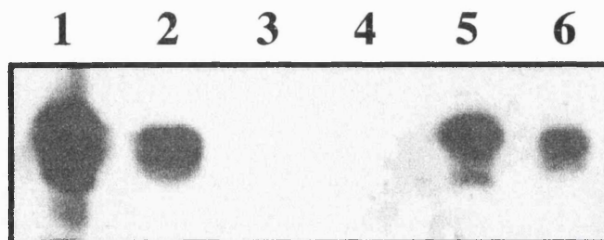
Figure 6.8 Association of PI-TPα and PI-TPβ
with the GST-p150/PtdIns 3-kinase complex *in vitro*.

Sf9 cells were co-infected with recombinant baculoviruses to
express GST-p150 and PtdIns 3-kinase. Co-expressed protein
complexes were affinity purified from cell lysates using glutathione-
Sepharose beads. To investigate the association of PI-TPα or PI-TPβ
with the GST-p150/PtdIns 3-kinase complex, 5μg of PI-TPα or PI-TPβ
was preincubated with 1 mM PtdIns for 5min at 37⁰C. This PI-TPα
or PI-TPβ was added to immobilised GST protein (lanes 3 and 4
respectively) or GST-p150/PtdIns 3-kinase (lanes 5 and6 respectively)
in 300 μl of EB for 1 h at 4⁰C. Protein complexes were washed and resolved
by 10.5% SDS-PAGE. PI-TP was detected by Western blotting using
PI-TP-specific antibody. Recombinant PI-TPα (lane 1) and PI-TPβ (lane 2)
were run as controls.

A
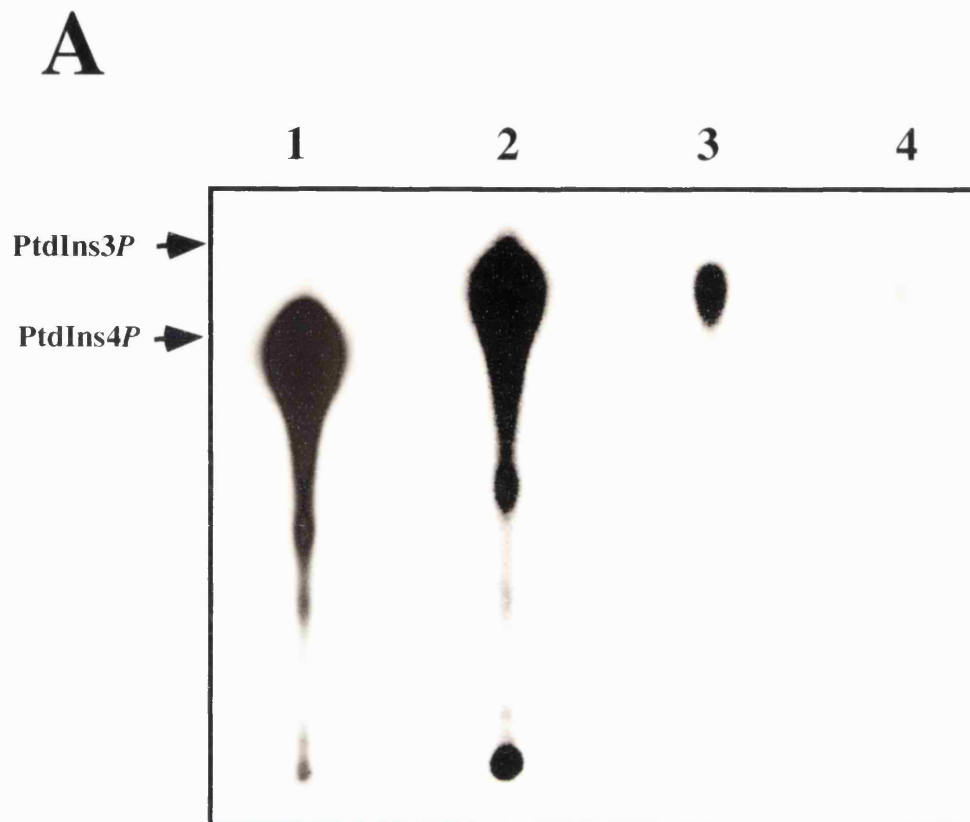
Figure 6.9 A PtdIns-specific activity associates with PI-TP *in vivo*.

A. PI 3-kinase activity was immunoprecipitated from Jurkat cells (2×10^7cells) using anti-PI-TP and anti-PtdIns 3-kinase antisera. Lipid kinase assays were performed on the immune complexes in the presence of Mn^{2+}, using PtdIns as substrate. Phosphorylated lipids were extracted, resolved using TLC and visualised by autoradiography. The position of PtdIns4*P* standard (lane 1), generated using A431 lysate and PtdIns3*P* standard (lane 2), generated using recombinant PtdIns 3-kinase is indicated. Lane 3 shows the lipid kinase activity associated with anti-PI-TPα immunoprecipitates from Jurkat cells. Lane 4 shows the lipid kinase activity associated with protein G-Sepharose alone.
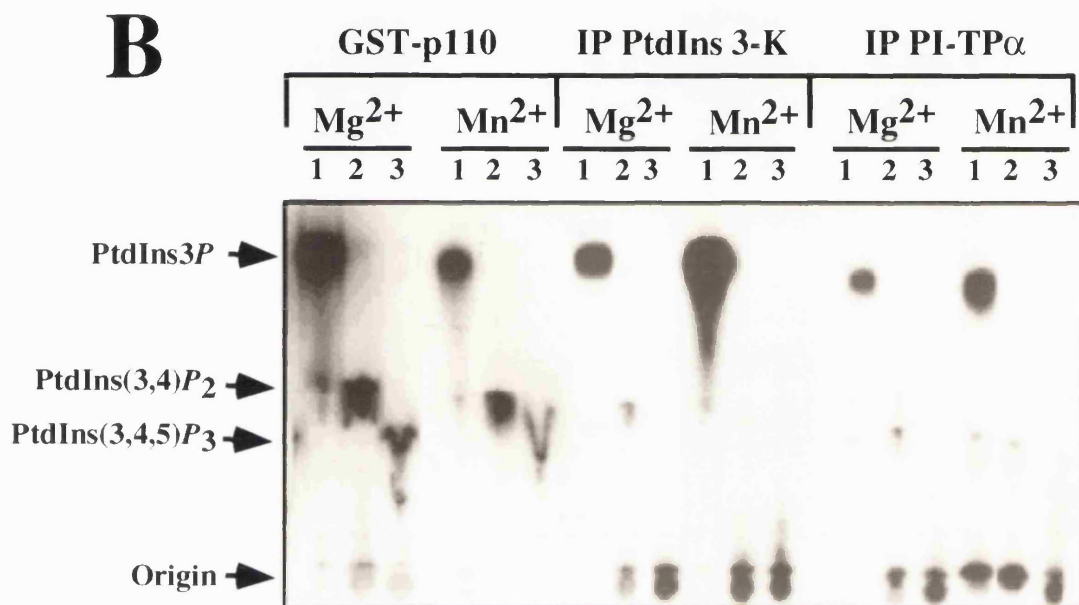
Figure 6.9 A PtdIns 3-kinase specific PI 3-kinase activity associates with PI-TP *in vivo*.

B. Lipid kinase assays were performed on recombinant GST-p110 and immunoprecipitates (IP) obtained from Jurkat cells using either anti-PtdIns 3-kinase (PtdIns 3-K) or anti-PI-TP antisera. PI 3-kinase assays were performed in the presence of 10 mM Mg^{2+} or Mn^{2+} using PtdIns (lanes 1), PtdIns4P (lanes 2) or PtdIns(4,5)P_2 (lanes 3) as the lipid substrate. Phosphorylated lipids were extracted, resolved by TLC and visualised by autoradiography.

which uses PtdIns as its prefered substrate and has a cation preference for Mn^{2+}. These results demonstrate biochemically, that PI-TP most likely associates with a PtdIns-specific 3-kinase *in vivo* such as the human PtdIns 3-kinase

6.6 Discussion

Mammalian PI-TP isoforms have been identified as factors required in triggering vesicle budding and fusion from the TGN (Ohashi *et al.*, 1995). Although yeast Sec14p has little sequence homology to mammalian PI-TP at the amino acid level, it is able to substitute for mammalian PI-TP in secretory vesicle formation. Since the role of Sec14p in yeast is to functon in maintaining a Golgi DAG pool essential for Golgi-derived vesicle formation (Kearns *et al.*, 1997), the precise way in which PI-TP operates in mammalian cells is still in question. A function for PI-TP has been proposed in which it replenishes agonist-sensitive pools of inositol lipids by transferring PtdIns from its site of synthesis to sites of consumption (Currie *et al.*, 1997). Consequently events requiring PI-TP, such as receptor-stimulated phospholipase activity are explained by the ability of PI-TP to transfer PtdIns/PtdCho from one membrane to another, replenishing PtdIns substrate at the plasma membrane (Downes and Batty, 1993). Other studies however, suggest that PI-TP functions as a co-factor which stimulates the activity of PI kinases and PLC by presenting protein-bound lipid substrates to these enzymes. For example, it has been observed that the addition of PI-TP to permeabilised A431 cells restores PLCγ activity and stimulates the synthesis of PtdIns(4,5)P_2 in the presence of EGF (Kauffmann Zeh *et al.*, 1995). These studies showed that PtdIns 4-kinase was regulated by EGF but required PI-TP for full activity (Kauffmann Zeh *et al.*, 1995) This result suggested that PI-TP may function in presenting PtdIns substrate to PtdIns 4-kinase. Similarly, permeabilised HL60 cells have been demonstrated to require PI-TP for GTPγS-mediated PLCβ activity which also results in a stimulated PtdIns(4,5)P_2 synthesis (Cunningham *et al.*, 1996; Thomas *et al.*, 1993). These investigations have been used to propose that receptor-activation triggers the assembly of a signal-producing complex consisting of inositol lipid kinases and PLC's with which PI-TP interacts to deliver PtdIns for phosphorylation.

In an analogous fashion, PtdIns 3-kinase could also be part of a complex in which PI-TP functions by presenting PtdIns as substrate to this lipid kinase. The resulting PtdIns3P substrate may in turn serve as substrate for a PI 5-kinase generating PtdIns(3,5)P_2, a potential lipid signalling molecule (as discussed in section 6.3).

The results described in section 6.5.1 demonstrate that there is a dose dependent increase in the lipid kinase activity of the p150/PtdIns 3-kinase complex which takes place upon the addition of PI-TP. PI-TPα, PI-TPβ and Sec14p were all found to be effective in activating PtdIns 3-kinase activity, suggesting that the ability shared by all three proteins

to bind and transfer PtdIns is sufficient for presenting PtdIns as substrate *in vitro*. The potential role of PI-TP in presenting PtdIns substrate to PtdIns 3-kinase could be investigated further by using PI-TP mutants in which the phospholipid binding properties of the protein has been abrogated. For example, experiments could be performed where the PtdIns-transfer activity of PI-TPα is specifically eliminated by mutation of the consensus site of phosphorylation by protein kinase C (PKC), Thr^{59}, whose surrounding motif represents the most highly conserved region in metazoan PI-TPs (Alb *et al.*, 1995). In addition, deletions to the C-terminus of PI-TPα have been shown to reduce PtdIns/PtdCho transfer activities (Hara *et al.*, 1997; Prosser *et al.*, 1997). The use of such mutants would provide greater insight into the way in which PI-TP is able to activate the lipid kinase activity of the p150/PtdIns 3-kinase complex.

In this study, the association of PI-TPα and PI-TPβ with the p150/PtdIns 3-kinase complex *in vitro* was demonstrated (section 6.5.2) and the interaction of PI-TPα, *in vivo*, with a PtdIns 3-kinase was also observed (section 6.5.3). It was found that PI-TPα immunoprecipitates contained a lipid kinase activity which had a biochemical profile that closely matched that of human PtdIns 3-kinase. This data however, cannot exclude the possibility of PI-TPα associating with other PtdIns specific lipid kinases such as that identified by Stephens *et al*, or other as yet unidentified PtdIns 3-kinase isoforms (Stephens *et al.*, 1994a). There has been at present, no genetic or biochemical data from budding yeast to suggest that the Vps15p/Vps34p complex functions in conjunction with the yeast phosphatidylinositol transfer protein Sec14p. In this context however, evolutionary divergence, such as the absence of PI-TPα and PI-TPβ like proteins in yeast should also be considered. As a result therefore, a role for mammalian PI-TP in presenting PtdIns substrate to PtdIns 3-kinase, in a manner similar to that already suggested for PtdIns 4-kinase could still remain a possibility.

Western blot analysis and immunofluorescence microscopy have been used to investigate the subcellular localisation of PI-TPα and PI-TPβ. Immunofluorescence studies on mouse Swiss 3T3 fibroblasts, have demonstrated that PI-TPα is localised in the cytoplasm and in the nucleus and that PI-TPβ is preferentially associated with the Golgi system and punctate vesicles, that suggested an association with secretory vesicles (de Vries *et al.*, 1995; Snoek *et al.*, 1993). This supports biochemical data implicating PI-TP as a factor required for vesicle formation at the TGN, for GTPγS stimulated protein secretion in HL60 cells (Fensome *et al.*, 1996) and Ca^{2+}-activated secretion of noradrenalin in PC12 cells (Hay and Martin, 1993). Further investigations however, have demonstrated that the subcellular localisation of PI-TPα and PI-TPβ was not absolute, since some PI-TPα was associated with the Golgi and some PI-TPβ situated in the nucleus and cytoplasm (De Vries *et al.*, 1996). This could explain the relatively low

levels of PtdIns 3-kinase activity obtained from the PI-TPα immunoprecipitates. It is possible that like its yeast Vps34p homologue, human PtdIns 3-kinase is most likely to be localised at the TGN or on vesicles involved in protein trafficking from the TGN such as vesicular compartments implicated in endocytic/endosomal or secretory pathways. As a result it is most likely that PtdIns 3-kinase could function to a greater extent with the PI-TPβ isoform, which has a more TGN/vesicular localisation. Whether more PtdIns 3-kinase activity can be selectively immunoprecipitated with PI-TPβ however, remains to be established.

Subsequent to the studies presented in this chapter (Panaretou *et al.*, 1997), a PtdIns 3-kinase and PI-TP protein have been shown to act synergistically in the formation of constitutive transport vesicles from the TGN of rat hepatocytes (Jones *et al.*, 1998b). The PtdIns 3-kinase investigated in this study has a biochemical profile similar to that of the human Vps34p homologue, since in PI 3-kinase assays it used PtdIns as its only substrate to generate PtdIns3P in a Mn^{2+} dependent manner. However, this PtdIns 3-kinase activity was sensitive to wortmannin at micromolar concentrations (IC$_{50}$: 3.5 μM) whilst PtdIns 3-kinase (the human Vps34p homologue) is sensitive to wortmannin at nanomolar concentrations (IC$_{50}$: 2.5 nM). In addition, the PtdIns 3-kinase identified by Jones *et al*, is suggested to function with a 62 kDa phosphoprotein and the 25 kDa GTPase rab6 (collectively called p62^{cplx}) (Jones *et al.*, 1993). The 100 kDa PtdIns 3-kinase and p62^{cplx} were proposed to assemble at the cytoplasmic domain of TGN 38 (an integral membrane protein of the TGN) where they facilitate exocytic vesicle formation (Banting and Ponnambalam, 1997). Whether the PtdIns 3-kinase implicated in this event is the human Vps34p homologue or another PI 3-kinase family member, is currently unknown. However, the observation that mammalian PI-TP functionally co-operates with this potentially novel PtdIns 3-kinase to drive exocytic vesicle formation, further suggests a role for PI-TP in facilitating PtdIns3P production.

CHAPTER 7

Subcellular localisation of the p150/PtdIns 3-kinase complex and mutational analysis of p150

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7.1 Subcellular localisation of the p150/PtdIns 3-kinase complex

7.1.1 Introduction

The efficient sorting and delivery of proteins from a common cytoplasmic site of synthesis to their final intracellular location is a fundamental process that occurs in all eukaryotic cells. This process has been examined in detail using the yeast *S.cerevisiae*, and it has been shown that the Vps15p protein kinase and the Vps34p PtdIns 3-kinase are required for the sorting of soluble hydrolases to the yeast vacuole (Stack *et al.*, 1995c). To support this functional role, differential centrifugation and protease protection experiments (Herman *et al.*, 1991a), were used to show that Vps15p is associated with the cytoplasmic face of an intracellular membrane. Further subcellular fractionation studies using sucrose density gradients, showed that like Vps15p, Vps34p associated with a membrane fraction. In addition, Vps15p and Vps34p demonstrated nearly identical gradient fractionation characteristics with that of Kex2p, a membrane protein of the late Golgi (Stack *et al.*, 1993). These data suggested that the Vps15p/Vps34p complex was localised to the cytoplasmic face of an intracellular membrane such as that of a late TGN compartment or vesicular transport intermediates which exist between the Golgi complex and the vacuole.

The extensive amino acid homology between the yeast Vps15p/Vps34p and human p150/PtdIns 3-kinase complexes respectively (Panaretou *et al.*, 1997; Volinia *et al.*, 1995), suggested that p150 and PtdIns 3-kinase might also play a role in vesicle mediated protein trafficking. As has been postulated for the yeast Vps15p/Vps34p complex, p150 and PtdIns 3-kinase could also be involved in sorting of mammalian lysosomal enzymes from the TGN to endosomal/lysosomal compartments. Consistent with this proposed physiological role, the p150/PtdIns 3-kinase complex could be localised to these organelles and/or their transport vesicles. Treatment of mammalian NRK cells with the PI 3-kinase inhibitor wortmannin, resulted in a redistribution of the lysosomal type I integral membrane proteins lgp 110 and lgp 120, suggesting a role for phosphoinositide 3-kinase activity in regulating membrane traffic late in the endocytic pathway (Reaves *et al.*, 1996). In addition, treatment of NRK cells with wortmannin also caused mistargeting of

the mammalian soluble hydrolase, procathepsin D implicating a PI 3-kinase in vesicular transport to lysosomes (Brown *et al.*, 1995b; Davidson, 1995).

In this chapter the techniques of indirect immunofluorescence and confocal microscopy are used in experiments to examine the subcellular localisation of the p150/PtdIns 3-kinase complex. A detailed characterisation of where p150 and PtdIns 3-kinase reside in intact cells would provide more information on their function and support any biological role proposed for these proteins in mammalian cells.

7.1.1.1 Immunofluorescence

Immunofluorescence microscopy is a powerful technique that has been used to study aspects of cellular organisation. It allows rapid visualisation of organelles and membrane compartments and in particular, has been a valuable tool for examining the intracellular localisation of numerous proteins. This method been used extensively to study membrane traffic within the cell and enables the subcellular distribution of up to three proteins to be visualised simultaneously in the same cell. In particular, immunofluorescence microscopy enables the pattern of labelling for a particular protein to be assessed at the whole cell level (Griffiths *et al.*, 1993).

Immunofluorescence can be used to examine the localisation of endogenous proteins recognised by specific antibodies. The key to the success of this approach is the availability of specific antisera which retain their ability to recognise their antigenic epitope under the conditions used. In the absence of a specific antibody, an epitope tag can be genetically engineered onto the N-or C-terminus of a protein. The modified protein can be expressed in a cell using transfection and detected using an antibody recognising the epitope tag. Direct immunofluorescence involves the use of a primary antibody that is chemically bonded to a fluorophore. This technique can be time consuming and wasteful, as each antibody recognising a protein of interest would require fluorophore-conjugation, using a method which is not 100% efficient and requires milligrams of antibody (when only microgram amounts may be available). Consequently, a more common approach used is indirect immunofluorescence, in which a fluorophore-conjugated anti-antibody is used to amplify the fluorescent signal (Harlow and Lane, 1988). The fluorochromes commonly used in immunofluorescence are a UV-excited/blue-emitting coumarin derivative, AMCA; a blue-excited/green-emitting fluorescein derivative, FITC; and either a green- and yellow-excited/orange-red emitting rhodamine derivative, TRITC or an orange-excited/red-emitting sulphorhodamine derivative, Texas red.

7.1.1.2 Confocal microscopy

A common problem encountered with images obtained by conventional microscopy, is that they present information from a specimen that is simultaneously in focus and out of focus. This results in a blurred image in which the out-of-focus information is superimposed on the focused image and hence obscures the final appearance. This problem is more acute when using thick samples, for example when staining tissue samples, where physical sectioning is often employed. When this is inappropriate or when higher resolution of an image is required, the out-of-focus blur is removed by the use of confocal microscopy. This technique is based on the principle of using optical sectioning, which is achieved by the combination of two related effects. Firstly, the illumination is brought to a sharp focus on a single point on the object so that the light above and below the point of focus is defocused and such regions are less illuminated. Second, the light emitted from the focus point is collected and sharply re-focused on an aperture, the confocal pinhole, located before the detector. This means that light which comes from regions of the object above and below the point of focus, is focused behind and in front of the aperture, respectively, and is therefore defocused in the plane of the aperture. Consequently, such emissions fall upon the wall around the pinhole rather than passing through it into the detector. Taken together, these factors mean that not only are points above and below the point of focus less brightly illuminated, but that light from these points is also detected less efficiently. This results in imaging that is completely restricted to the region immediately surrounding the focus point. A two dimensional image can be built up by scanning the point through the lateral plane, and a three dimensional data set obtained by moving the microscope stage axially between the collection of serial images (Entwistle and Noble, 1994).

In this way a very high sensitivity can be achieved because the noise due to the rejection of the out-of-focus blur is removed and the average sum of the emission from many frames is observed. The optical sectioning property of confocal microscopy makes it an excellent tool to examine the distribution of a molecule of interest through a mixed population of cells, or to examine the relative distribution of several molecules of interest within a specimen. The high sensitivity and improved resolution achieved with confocal laser scanning, exceeds that of conventional fluorescence microscopes (Barer and Entwistle, 1991) and was therefore additionally utilised in this study.

7.2 Results

7.2.1 Generation of anti-p150 antibodies

To investigate the subcellular localisation of endogenous p150 in mammalian cells using immunofluorescence microscopy, a p150 specific antibody would be required. The

immunisation of rabbits with peptides corresponding to the N-or C-terminal amino acid sequences of proteins has been demonstrated to generate antisera suitable for immunoblotting and immunoprecipitation (Harlow and Lane, 1988). Therefore, peptides based on the amino acid sequence of p150 were chosen for an immunisation programme in an attempt to generate p150-specific rabbit polyclonal antisera. Amino acid sequences from the N-terminus of p150 were avoided since this region is myristoylated and contains a putative protein kinase domain. Consequently, an antibody binding to the N-terminus of p150 might interfere with possible functional aspects of the protein. As a result, two antigenic peptides deduced using the Peptide Structure program, (UWGCG package) (Devereux *et al.*, 1984) with sequences corresponding to the C-terminal region of the p150 amino acid sequence were chosen:

P1: VTASRDGIVKVWK (amino acids 1346-1358)

P2: KQKVGPSDDTPRRGPESL (amino acids 1307-1324)

Both peptides included an N-terminal cysteine residue to enable coupling to keyhole limpet hemocyanin (section 2.5.1.1), a carrier protein which also enhances peptide antigenicity. Conjugates of peptides P1 and P2 were used to immunise two pairs of rabbits, SK29/SK 30 and SK31/SK32 respectively (Eurogentec). The animals were boosted each month for four months and four serum samples were taken from each rabbit, the immunoreactivity of each sample was tested and compared with pre-immune serum. Serum samples from SK29, SK30, SK31 and SK32 were tested for their ability to react with recombinant p150 by Western blotting, to immunoprecipitate recombinant p150 from baculovirus infected Sf9 cell lysates and to immunoprecipitate endogenous p150 from Jurkat cells (results not shown). These tests demonstrated that the antisera generated were poorly immunoreactive towards p150. Even after affinity purification, the antisera still had negligible ability to detect p150 by Western blotting and immunoprecipitation. In addition, when the antisera were used in an attempt to detect endogenous p150 in COS-7, MDCK and Jurkat cells using indirect immunofluorescence, a diffuse non-specific staining pattern was observed (results not shown). These data indicated that the p150 anti-peptide antibodies generated could not be used to detect endogenous p150 protein and that if the subcellular localisation of this protein was to be investigated by immunofluorescence, an alternative strategy was required.

7.2.2 Immunolocalisation of ectopic p150 expression in COS-7 cells

To investigate the subcellular localisation of p150 in mammalian cells in the absence of specific antisera, an approach was chosen whereby an epitope tagged form of the p150 protein could be introduced into COS-7 cells by transient transfection and detected by indirect immunofluorescence with the aid of an antibody specific for this epitope tag. For these studies a C-terminal epitope (EFMPME) tagged p150, p150(EE) was generated. To

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facilitate transient expression of p150(EE) in COS-7 cells the pSKp150(EE) construct (section 4.2.2.1) was digested with *Not* I restriction enzyme to release the p150(EE) ORF. The p150(EE) ORF was then subcloned into a *Not* I digested mammalian expression vector pMT2SM (sections 2.1.1.3 and 6.2.1) to generate the construct pMT2-p150(EE). To verify the expression of p150(EE), COS-7 cells were transiently transfected using electroporation (section 2.2.1.4) with 2 μg, 5 μg, 10 μg or 20 μg of pMT2-p150(EE) plasmid construct. As a control, COS-7 cells were also transfected using pMT2SM vector alone. After 36 h, the cells were harvested and whole lysates were electrophoresed on a 7.5% SDS-polyacrylamide gel. The separated cellular proteins were then transfered onto PVDF membrane and probed using EE-tag specific mouse monoclonal antibody (EEmAb). A 150 kDa protein was detected in pMT2-p150(EE) transfected cells but not in control cells (figure 7.1). In addition, the highest level of p150(EE) protein expression was observed in lysates from cells transfected with 5 μg or 10 μg of pMT2-p150(EE) construct, demonstrating that 5μg of construct was sufficient to obtain optimal protein expression under these conditions.

To investigate the subcellular distribution of p150, COS-7 cells were transiently transfected with pMT2-p150(EE) construct and seeded onto glass coverslips. After 36 h the cells were fixed and permeabilised with methanol and prepared for immunostaining (section 2.6.3). After incubation with primary EEmAb, bound antibodies were visualised using FITC-conjugated, goat anti-mouse secondary antibody. The staining pattern for p150 protein ectopically expressed in COS-7 cells was primarily perinuclear and punctate in appearance (figure 7.2A). There was some punctate staining in the cytoplasm, however there was little p150 staining at the plasma membrane and more distal portions of the cell. Control cells treated in a similar manner, demonstrated minimal non-specific staining (results not shown) indicating that the staining pattern observed for p150 was attributed to specific binding of the EEmAb to the expressed epitope-tagged p150 protein and not cross-reaction with an endogenous protein.

The presence of C-terminal amino acids 'KDEL' in a protein for example, acts as a signal sequence causing the targeting and retention of that protein in the ER (Munro and Pelham, 1987). To ensure that the perinuclear punctate stain observed for p150(EE) was not due to the C-terminal 'EFMPME' epitope tag behaving as a 'targeting signal' for such localisation, an additional control was included. Cells were transiently transfected to express an unrelated protein, PI 3KC2α(EE) (section 1.3.2), an EE-tagged protein of similar molecular mass to p150 (190 kDa). Immunostaining of these cells (figure 7.2C) showed that unlike p150(EE), transiently expressed PI 3KC2α(EE) was distributed throughout the cytoplasm of the cell. This confirmed that the punctate perinuclear staining pattern observed for p150 was not influenced by the EE-epitope tag.
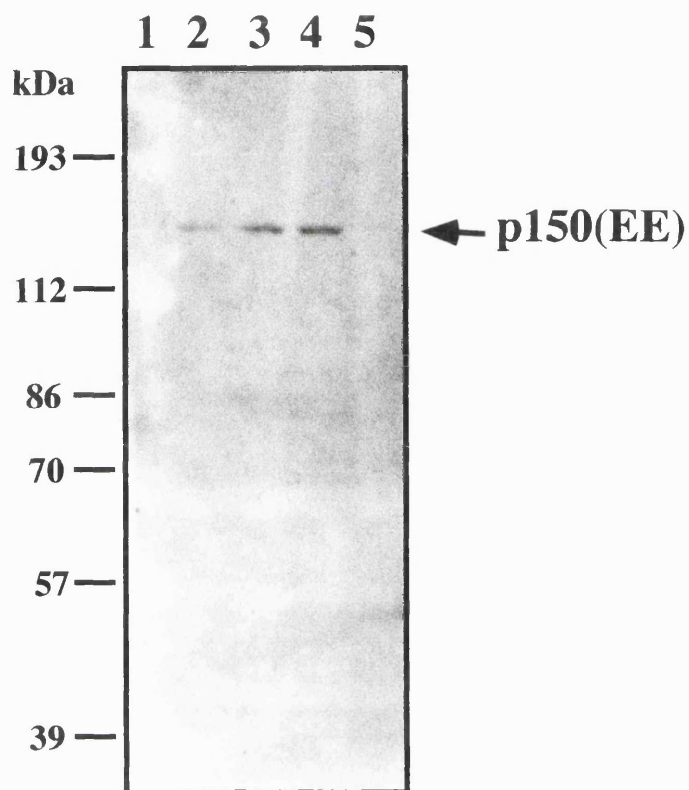
Figure 7.1 Expression of p150(EE) in COS-7 cells

COS-7 cells were transiently transfected with pMT2SM vector alone
(lane 1), or pMT2-p150(EE) expression construct: $2\mu g$ (lane 2),
$5\mu g$ (lane 3), $10\mu g$ (lane 4) and $20\mu g$ (lane 5). Crude lysates were resolved
by 7.5% SDS-PAGE. Expressed p150(EE) was detected by Western
blotting using EEmAb.
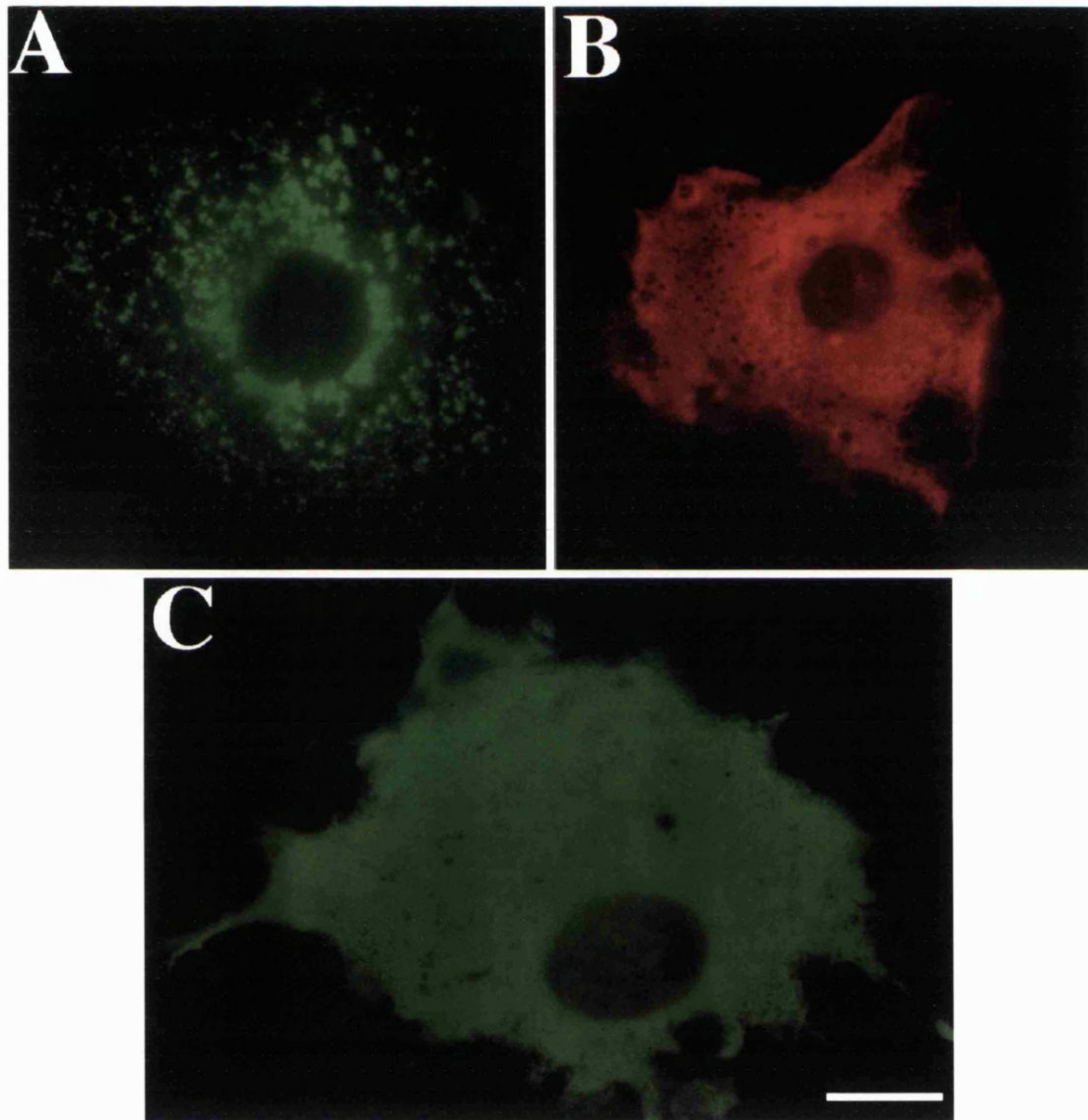
Figure 7.2
Immunolocalisation of transiently expressed p150(EE) and PtdIns 3-kinase in COS-7 cells.

COS-7 cells transiently expressing p150(EE) (Panel A), PtdIns 3-kinase (Panel B) or PI3K-C2α(EE) (Panel C), were seeded on glass coverslips and immunostained with EEmAb (IgG1) (Panels A and C) or rabbit anti-PtdIns 3-kinase antisera (Panel B). Bound antibodies were visualised with anti-mouse FITC-conjugated IgG1 or TRITC-conjugated anti-rabbit secondary antibody. (Bar: 10 μm)

7.2.3 Co-localisation of p150 and PtdIns 3-kinase *in vivo*

Studies were performed next to investigate if p150 and PtdIns 3-kinase co-localised when expressed in COS-7 cells. Initially, the distribution of PtdIns 3-kinase alone was examined. Using the pMT2-PtdIns 3-kinase construct, COS-7 cells were transfected and seeded onto coverslips. After 36 h, the cells were processed for immunostaining using an affinity purified anti-PtdIns 3-kinase rabbit polyclonal primary antibody and a donkey anti-rabbit TRITC-congugated secondary antibody. Surprisingly, PtdIns 3-kinase displayed a diffuse cytoplasmic distribution when expressed in COS-7 cells (figure 7.2B). If transiently expressed PtdIns 3-kinase co-localised with endogenous p150, PtdIns 3-kinase should also have a punctate perinulclear staining pattern, identical to that of transiently expressed p150 (figure 7.2A). The diffuse cytoplasmic stain observed for PtdIns 3-kinase however, could be explained by the fact that all of the endogenous p150 protein may already be in complex with endogenous PtdIns 3-kinase. Consequently, the expressed PtdIns 3-kinase unable to bind p150 protein would remain in the cytoplasm instead. To test this hypothesis, constructs encoding both p150 and PtdIns 3-kinase were used to co-transfect COS-7 cells. Coverslips seeded with co-transfected cells were dual-labelled with EEmAb and rabbit anti-PtdIns 3-kinase primary antibodies and then with FITC-conjugated goat anti-mouse and TRITC-conjugated donkey anti-rabbit secondary antibodies. The staining pattern of co-expressed p150 and PtdIns 3-kinase was examined using confocal microscopy (figure 7.3). The individual staining patterns for p150 (figure 7.3A, green) and PtdIns 3-kinase (figure 7.3B, red) were overlayed to demonstrate the *in vivo* co-localisation of p150 and PtdIns 3-kinase (figure 7.3C, yellow, nb: combining red and green confocal images results in a computer generated yellow image representing co-localisation). It can also be seen that, when co-expressed with p150, PtdIns 3-kinase no longer has a diffuse cytoplasmic staining pattern (figure 7.3B) but now co-localises with p150 and adopts a punctate perinuclear distribution. In addition, the subcellular distribution of endogenous PtdIns 3-kinase in U937 cells was examined. This cell type was chosen since both PtdIns 3-kinase and p150 were cloned from a U937 cDNA library and PtdIns 3-kinase has been detected in these cells (Volinia *et al.*, 1995). The staining pattern of endogenous PtdIns 3-kinase shows a perinuclear punctate stain, corresponding to the type of subcellular distribution observed in p150/PtdIns 3-kinase co-transfected COS-7 cells (figure 7.3D)

7.2.4 Biochemical characterisation of p150 expressed in COS-7 cells

As demonstrated in the results described above (sections 7.2.2 and 7.2.3) p150 transiently expressed in COS-7 cells was able to functionally associate with co-expressed PtdIns 3-kinase. To obtain p150(EE) protein expressed in COS-7 cells for further biochemical analysis, immunoprecipitation was attempted using EEmAb. It had been
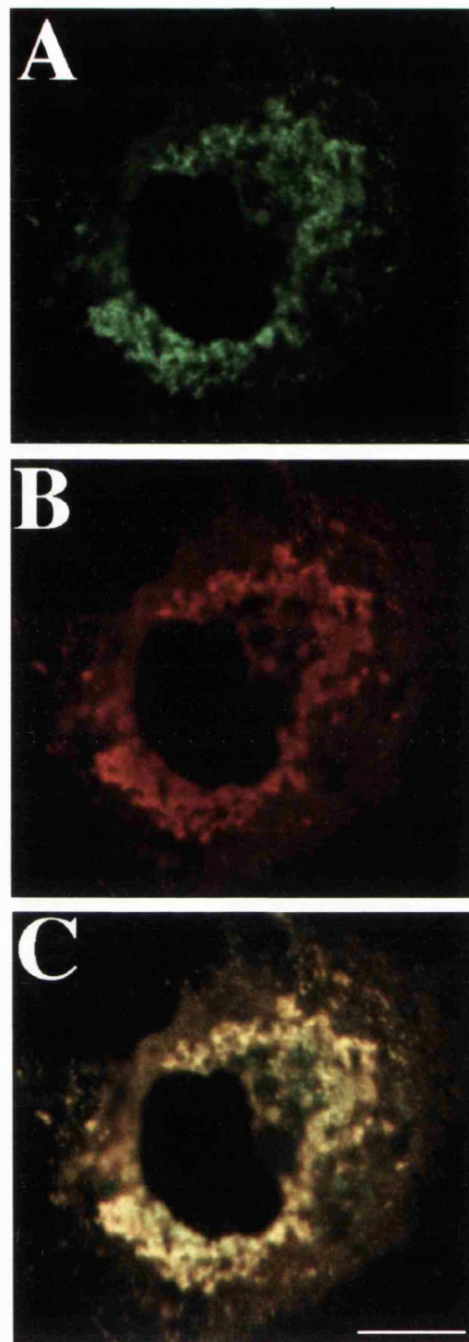
Figure 7.3

Co-localisation of the p150/PtdIns 3-kinase complex.

COS-7 cells transiently co-expressing p150(EE) and PtdIns 3-kinase (Panels A, B and C) or untransfectedU937 cells (Panel D) were seeded on glass coverslips and prepared for immunostaining by methanol fixation.
p150(EE) and PtdIns 3-kinase expressing cells were dual-labelled with EEmAb (IgG1) and PtdIns 3-kinase specific rabbit polyclonal antisera. Bound antibodies were visualised using anti-mouse IgG1 FITC-conjugated antibody and anti-rabbit TRITC-conjugated antibodies respectively. The staining pattern for p150(EE) is shown in Panel A (green) and that for PtdIns 3-kinase in the same cell by Panel B (red). Images from Panels A and B were overlayed (Panel C) to show p150(EE) and PtdIns 3-kinase co-localisation (yellow). Panel D demonstrates endogenous PtdIns 3-kinase distribution examined in untransfected U937 cells. After incubation with PtdIns 3-kinase specific rabbit polyclonal antisera, bound antibodies were visualised using anti-rabbit TRITC-conjugated secondary antibody. (Bar: 10 μm)
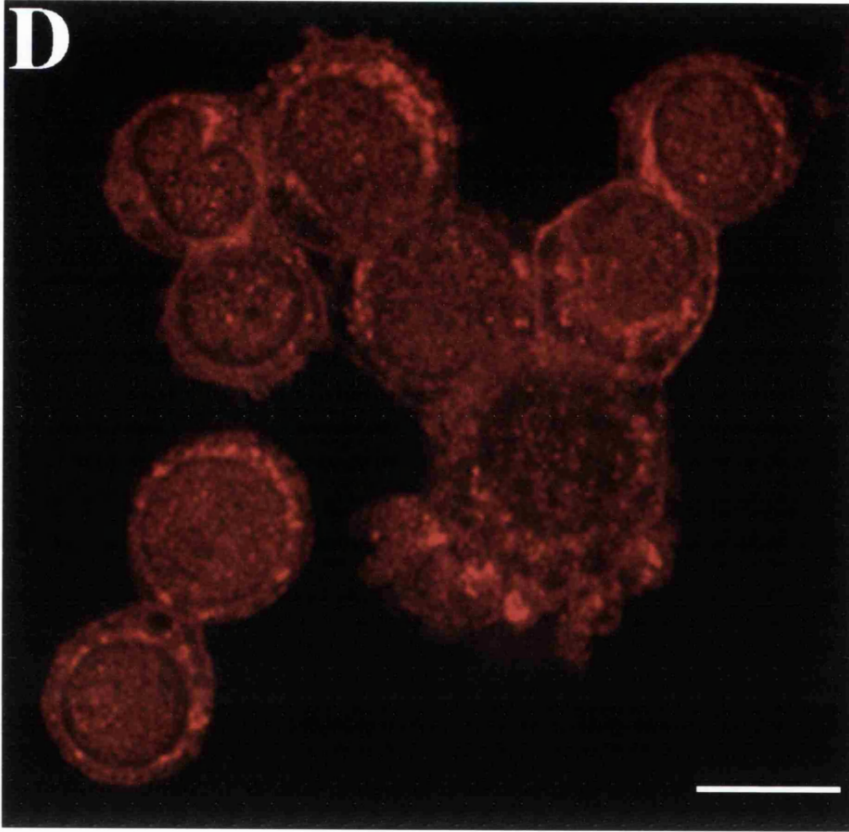
shown previously that p150(EE) could be immunoprecipitated from Sf9 cells infected with baculovirus encoding p150(EE), using EEmAb (section 4.2.2.2). In contrast however, no p150(EE) was immunoprecipitated from the clarified lysates of COS-7 cells that had been transiently transfected with pMT2-p150(EE) and lysed in extraction buffer (EB) containing 1% Triton X-100, 150 mM NaCl, 10 mM Tris pH 7.4 and 50 mM NaF (figure 7.4). p150(EE) could not be immunoprecipitated, but was detected in the Triton X-100 insoluble pellet (figure 7.4A lane 4). This result could not be explained by EEmAb failing to recognise the p150 EE-tag epitope in an immunoprecipitation, since p150(EE) was also undetectable in the supernatant of clarified lysates (figure 7.4A, lane 2). This demonstrated that the p150(EE) protein was absent from the soluble lysate used for immunoprecipitation. In addition, experiments using less pMT2-p150(EE) construct for transfection (1-5 µg) resulted in lower p150(EE) expression levels in COS-7 cells without an increased solubility in p150(EE) (results not shown). This showed that p150(EE) could not be solubilised using EB regardless of its expression level, excluding the possibility that the observed insolubility reflected protein overexpression in these cells. Such protein insolubility was not observed when cells were transfected with pMT2-PtdIns 3-K. When cells expressing PtdIns 3-kinase were lysed in EB buffer and lysates clarified by centrifugation to produce a supernatant (S) fraction and a Triton X-100 insoluble pellet (P), PtdIns 3-kinase was found to be present in the soluble supernatant fraction and also in the Triton-X100 insoluble pellet (figure 7.4B). Significantly, no difference in p150 solubility was observed when p150 and PtdIns 3-kinase were co-expressed (figure 7.4C). p150 did not shift to the supernatant fraction because of its co-expression with the more soluble PtdIns 3-kinase. Similarly, PtdIns 3-kinase did not become more insoluble when co-expressed with p150 (figure 7.4B). This result may initially seem surprising, since when co-expressed with p150, PtdIns 3-kinase co-localised with p150 taking on a punctate perinuclear distribution (figure 7.3). It would be expected therefore that when co-expressed with p150, a greater proportion of PtdIns 3-kinase would be found in the Triton X-100 insoluble pellet. However, the low number of cells (2-5%) co-expressing the p150/PtdIns 3-kinase complex amongst a background of cells expressing PtdIns 3-kinase or p150 alone, might make a possible shift of PtdIns 3-kinase from the supernatant to pellet fraction difficult to detect using this system.

Numerous protein solubilisation agents have been used to extract proteins from the cell and its intracellular structures. The differential release of proteins from the cell following the use of such reagents, may reflect heterogeneity in the association of those proteins with the cell membrane and other cellular compartments (Cutler et al., 1986). For example, buffers containing detergents such as Triton X-100 or NP40 have been used to solubilise integral membrane proteins. Detergents such as Triton X-114 are able to undergo phase separation. At 4°C, Triton X-114 is homogenous, but at temperatures

A.

1 2 3 4

kDa

193 —

← p150(EE)

112 —

86 —

70 —

57 —

39 —
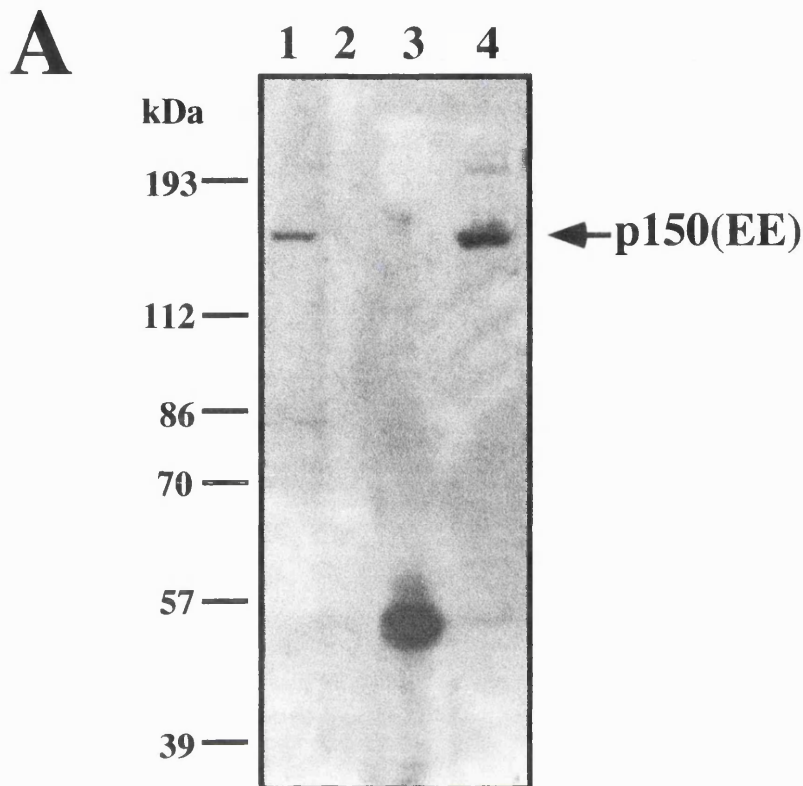
Figure 7.4 p150(EE) transiently expressed in COS-7 cells is found in a Triton X-100 insoluble fraction.

A. COS-7 cells were transiently transfected with pMT2SM(EE) and lysed in EB 36 h post-transfection. Lane 1, whole cell lysate; lane 2, supernatant of clarified cell lysate; lane 3, immunoprecipitate of the supernatant from the clarified cell lysate using EEmAb and protein G-Sepharose; lane 4, Triton X-100 insoluble pellet from the clarified cell lysate. All samples were resolved by 7.5% SDS-PAGE and analysed by Western blotting using EEmAb to detect expressed p150(EE).

B

1   2

S   P   S   P

 ←—PtdIns 3-kinase

C

1   2

S   P   S   P
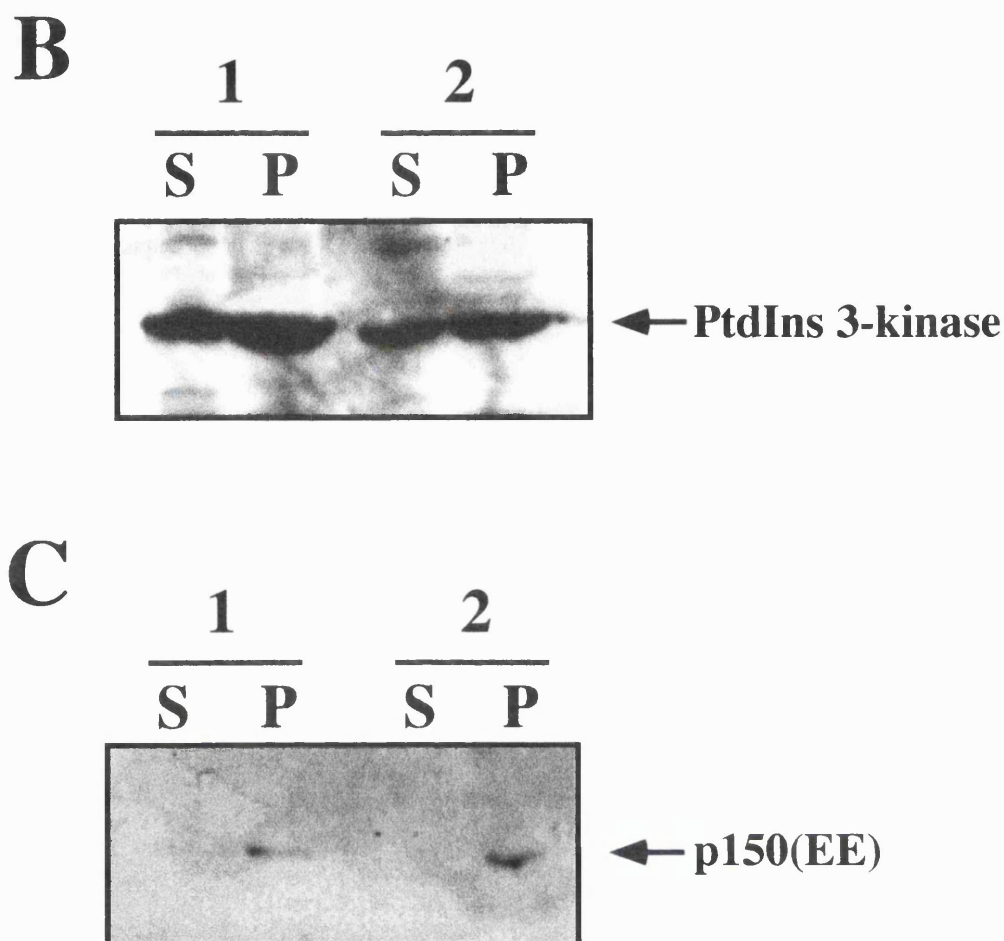
 ←— p150(EE)

Figure 7.4 Co-expression of PtdIns 3-kinase with p150(EE) does not affect the solubility of p150(EE).

B. COS-7 cells were transiently transfected with pMT2-PtdIns 3-kinase, (1), or co-transfected with pMT2-PtdIns 3-kinase and pMT2-p150(EE) (2). After 36 h cells were lysed in EB and clarified by centrifugation at 14,000x*g* to generate supernatant (S) and pellet (P) fractions. Samples were analysed by 7.5% SDS-PAGE and PtdIns 3-kinase was detected by Western blotting using PtdIns 3-kinase specific rabbit polyclonal antisera.

C. COS-7 cells were transiently transfected with pMT2-p150(EE) (1), or co-transfected with pMT2-p150(EE) and pMT2-PtdIns 3-kinase (2). After 36 h cells were lysed in EB and analysed as described in panel B. p150(EE) was detected by Western blotting using EEmAb.

above $20^{\circ}C$, this detergent separates into an aqueous and detergent phase. Using this property, detergent partition of proteins during phase separation in solutions of Triton X-114 allows hydrophilic, soluble proteins to be found in the aqueous phase and integral membrane proteins with an amphiphilic nature or lipid modified (eg myristoylated or palmytoylated) proteins to be recovered in the detergent phase. High or low salt buffers have been used to disrupt protein-protein interactions whilst buffers with a high pH can disturb the protein-membrane interactions of peripheral membrane proteins (Fujiki *et al.*, 1982).

With this information in mind, experiments were conducted in which a variety of extraction buffers were used in an attempt to obtain solubilised p150 from transfected COS-7 cells. In these experiments, COS-7 cells were transfected with pMT2-p150(EE) and lysed in extraction buffer. Lysates were then clarified by centrifugation (as described in section 2.3.1.2) to produce a supernatant (S) and pellet (P) fraction. In some cases the pellet derived from cell lysis by one extraction buffer was subjected to re-extraction by another buffer, which after centrifugation resulted in a second supernatant (S_2) fraction. The extraction buffers used in this experiment were: 1) EB, 2) 0.5% Triton, 100 mM NaCl, 10 mM PIPES pH 6.8 and 300 mM sucrose, for a re-extraction step this buffer was used with an additional 250 mM ammonium sulphate, 3) Low salt buffer (as EB but 150 mM NaCl was ommited), 4) High salt buffer (as EB but the 150 mM NaCl was increased to 0.5 M), 5) RIPA buffer (1% NP40, 0.5% deoxycholate, 0.1% SDS, 150 mM NaCl, 50 mM Tris pH 8.0), 6) Cells were lysed in EB followed by re-extraction of the pellet with a high pH buffer (150 mM $NaCO_3$ pH 11.5) and 7) Triton X-114 buffer (as EB but the 1% Triton X-100 was replaced with 1% Triton X-114). The supernatant and pellet fractions were boiled in 5x sample buffer and electrophoresed using a 7.5% SDS-polyacrylamide gel. The gel was transfered to PVDF membrane and probed with EEmAb to detect p150(EE). In each case, p150(EE) remained in the pellet fraction, indicating that none of the extraction buffers used could solubilise p150 expressed in COS-7 cells (figure 7.5).

The inability of detergents to extract p150 from the pellet fraction indicated that it was unlikely to be an integral membrane protein, similarly, the failure of the other buffers to solubilise p150 suggested that it was not peripherally membrane bound. A more likely explanation was that expression of p150 in COS-7 cells could result in the association of p150 with cytoskeletal structures which are also resistant to extraction by protein solubilisation agents. Alternatively, it is also possible that homo-oligomerisation of p150 may also contribute to its insolubility .
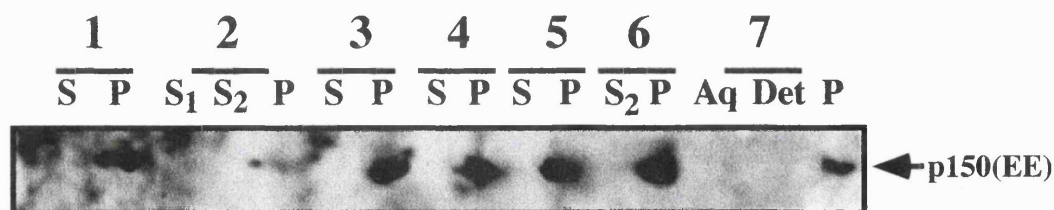
Figure 7.5 Different extraction buffers have no effect on the solubility of p150(EE).

COS-7 cells were transfected with pMT2-p150(EE). After 36 h cells were lysed in extraction buffers 1-7. Lysates were centrifuged at 14,000x*g* to generate supernatant (S) and pellet (P) fractions which were resolved by 7.5% SDS-PAGE. p150(EE) was detected using Western blotting and EEmAb.

Extraction buffers used:

1: EB (1% Triton X-100, 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 50 mM NaF)

2: 0.5% Triton X-100, 100 mM Nacl, 10 mM PIPES pH 6.8, 300 mM sucrose. Exraction with this buffer produced a supernatant fraction (S_1)and a pellet fraction that was re-extracted using 0.5 % Triton X-100, 250 mM ammonium sulphate, 10 mM PIPES pH 6.8 and 300mM sucrose. This generated a supernatant (S_2) and pellet fraction.

3: Low salt buffer (as EB, but 150 mM NaCl was ommited).

4: High salt buffer (as EB but 150 mM NaCl was replaced with 0.5 M NaCl).

5: RIPA buffer (1% NP40, 0.5% deoxycholate, 0.1% SDS, 150 mM NaCl, 50 mM Tris-HCl pH 8.0).

6: Cells were first extracted in EB, followed by re-extraction of the pellet using high pH buffer, (150 mM Na_2CO_3 pH 11.5) to produce S_2 and P fractions.

7: Triton X-114 buffer (as EB but 1% Triton X-100 was replaced with 1% Triton X-114). Cells were lysed in Triton X-114 buffer and centrifuged to generate a pellet fraction (P). The supernatant (kept at 4°C) was then incubated at 37°C to allow phase separation. This produces an aqueous (Aq) and detergent (Det) phase.

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7.2.5 Discussion

The localisation of transiently expressed p150(EE) in COS-7 cells to an insoluble fraction, suggests that p150(EE) is either associated with an insoluble cellular component or is itself an insoluble protein. This however, is difficult to reconcile with the fact that p150 was originally identified as a protein co-immunoprecipitated with PtdIns 3-kinase from Jurkat cell lysates (Volinia *et al.*, 1995). This implies that p150, under some conditions (or in certain cell types) is a soluble protein. It is possible that the insolubility of p150 and the subcellular localisation observed for this protein may be an artefact of transiently expressing p150(EE) in COS-7 cells. Nevertheless, the observation that very low levels of p150(EE) expression in COS-7 cells (section 7.2.4) did not result in p150(EE) shifting to a soluble fraction, suggests that overexpression of p150(EE) is unlikely to be the sole reason for its insolubility. In addition, it should be noted that as a cell line, COS-7 cells are often used in transfection studies as an *in vivo* model system for protein analysis. The expression of p150 in COS-7 cells resulted in a punctate perinuclear staining pattern which was unlike that observed for other proteins (eg PtdIns 3-kinase, PI 3-KC2α) which were also ectopically expressed in COS-7 cells (section 7.2.2). This suggests that the subcellular localisation observed for p150(EE) is not an artefact of protein expression in the COS-7 cell line. However, to totally preclude this possibility, the staining pattern of p150(EE) expressed in some other cell line should also be examined. The aggregation of a protein into an insoluble complex is usually caused by a mis-folded protein exposing hydrophobic surfaces and results in a functionally inactive protein. The expression of p150(EE) in COS-7 cells however, does not appear to functionally alter the p150 protein, since it retains its ability to associate with PtdIns 3-kinase *in vivo* resulting in both proteins displaying a subcellular localisation similar to that of endogenous PtdIns 3-kinase in U937 cells (section 7.2.3). This suggests that the observed insolubility of p150(EE) is unlikely to be due to protein aggregation.

Overexpression of p150(EE) nevertheless, may produce a potential homo-oligomerisation that renders it resistant to solubilisation. It is possible that at endogenous levels *in vivo*, p150 associates with another protein(s) which limits this self-association, possibly functioning by binding a p150-protein interaction surface which if exposed leads to the oligomerisation of p150. The punctate perinuclear staining pattern observed for p150(EE), when expressed in COS-7 cells, suggests a vesicular localisation. This is consistent for a protein whose yeast homologue, Vps15p, is implicated in vesicle trafficking from the TGN. It is interesting to note that when the mammalian ER-to-Golgi vesicle trafficking protein, rbet1 (Hay *et al.*, 1996) is expressed in COS-7 cells, a significant fraction of this protein also remains in the Triton X-100 insoluble pellet after cell lysis. A similar behaviour has also been observed for several other proteins resident in the Golgi and involved in vesicle trafficking. This property has in this case, been

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attributed to protein oligomerisation and may be an important mechanism by which certain proteins can be retained within a specific organelle subcompartment or membrane domain (Schweizer *et al.*, 1994; Weisz *et al.*, 1993).

Caveolin is a 23 kDa integral membrane protein, which like transiently expressed p150, is insoluble in buffers containing Triton X-100, high salt, low salt or sodium carbonate at high pH (Sargiacomo *et al.*, 1993). Caveolin is found in lateral assemblies (rafts) of lipids that form platforms serving to support cellular events in membrane traffic and signal transduction (Harder and Simons, 1997; Simons and Ikonen, 1997). Caveolin is thought to stabilise raft domains at the plasma membrane and in intracellular membranes. Immunolocalisation studies have shown caveolin to be present at the TGN and in TGN-derived transport vesicles (Kurzchalia *et al.*, 1992). A general feature of proteins found in lipid raft domains such as caveolin, is that they undergo a regulated oligomerisation (Harder and Simons, 1997; Parton, 1996). This results in the formation of Triton X-100 insoluble calveolar microdomains. These act as a scaffold upon which other components such as heterotrimeric G proteins and non-receptor tyrosine kinases (such as c-Yes and c-Src) can associate, thereby facilitating the propagation of signalling events (Li *et al.*, 1995c; Sargiacomo *et al.*, 1993).

In an analogous manner, the oligomerisation of p150 on intracellular membranes may result in the clustering of p150/PtdIns 3-kinase complexes. The subsequent production of localised PtdIns3P rich membrane microdomains, may serve a biophysical function, promoting the formation of vesicles (De Camilli *et al.*, 1996) or provide a concentrated region of PtdIns3P substrate for the formation of PtdIns(3,5)P_2 (section 6.1), (Dove *et al.*, 1997). The postulated oligomerisation of p150 may therefore be a functionally significant means through which the p150/PtdIns 3-kinase complex initiates membrane trafficking events.

Another possibility, is that when expressed in COS-7 cells, p150 might interact with an insoluble component of the cell such as a cytoskeletal protein. The possible association of p150 with cytoskeletal sttructures is investigated further in the following section.

7.3 Interaction of p150 with the cytoskeleton

7.3.1 Introduction

The cytoskeleton is made up of a complex network of protein filaments, namely microtubules, actin filaments and intermediate filaments, that all extend throughout the cytoplasm of the cell. The cytoskeleton functions primarily as an integrated network to control and co-ordinate movement of the cell and of organelles within the cytoplasm.

Each type of filament is composed of different protein modules that can be built into a variety of structures according to their associated proteins (Alberts *et al.*, 1989). The cytoskeleton was initially thought to be a static structure, that functioned simply to maintain the cytoplasmic architecture of a cell. In contrast, recent studies have shown that the cytoskeleton plays a much more dynamic role in the control of cell morphology and has been implicated in the regulation of intracellular membrane traffic (Goodson *et al.*, 1997).

Microtubules are formed by α,β-tubulin heterodimers which act as rails for the transport of organelles and other membranous structures throughout the cell. The microtubule is a polar structure with a fast-growing plus end and a minus end. Microtubules are associated with motor proteins such as kinesin and dynein are (Hirokawa, 1998). Localisation and functional assays have shown that kinesin acts as a plus end directed microtubule motor involved in anterograde membrane transport (Hirokawa *et al.*, 1991; Pfister *et al.*, 1989). Kinesin superfamily proteins have been implicated in organelle transport such as the movement of mitochondria and in the transport of synaptic vesicles and lysosomes (Gho *et al.*, 1992; Hirokawa, 1998). Immnocytochemical analysis, *in vitro* motility assays and subcellular fractionation have been used to suggest that the dynein superfamily of proteins are motors that drive the retrograde transport of membranous organelles, such as late endosomes and lysosomes (Hirokawa *et al.*, 1990; Lin and Collins, 1992). It has also been suggested that dynein acts in vesicular transport from the ER to the Golgi (Presley *et al.*, 1997), Golgi derived membranes (Fath *et al.*, 1994) and early to late endosomes (Aniento *et al.*, 1993).

Actin filaments, like microtubules are also associated with motor proteins, namely the myosin superfamily of molecular motors. Actin filaments and myosins have also been implicated in dynamic cellular functions such as cell movement and membrane trafficking (Mermall *et al.*, 1998). A role for the class I myosins (M1s) in endocytic and exocytic membrane traffic has recently been suggested. Two forms of endocytosis macropinocytosis and phagocytosis are impaired in *Dictyostelium* lacking and sometimes overexpressing certain single or multiple M1 genes (Novak and Titus, 1997; Ostap and Pollard, 1996). Similarly, deletion of one of the M1s in budding yeast resulted in defects in receptor-mediated endocytosis (Geli and Riezman, 1996). Localisation studies using antibodies that are specific for M1s suggest that class I myosins associate with Golgi and secretory granules in vertebrate cells (Mermall *et al.*, 1998). Class V myosins (M5s) have also been implicated in organelle movement. M5a has been localised to organelle structures in neurons and the presence of this myosin in preparations of purified synaptic vesicles, suggests a role for M5a in synaptic vesicle movement (Prekeris and Terrian, 1997). Such examples provide evidence for the involvement of actin filaments and

myosins in a variety of membrane-based phenomena, including organelle movement and cellular locomotion.

Intermediate filaments (IFs) together with the filamentous network of microtubules and actin, constitute the eukaryotic cytoskeleton. In contrast to actins and tubulins, which are highly conserved throughout evolution, IF proteins share as little as 20% amino acid sequence identity . However, members of the IF superfamily can still be subdivided into five subclasses (Fuchs and Weber, 1994). IF proteins also exhibit cell-type-specific patterns of expression. Type I and type II IF proteins such as keratins are expressed in epithelial cells, whereas the type III IF protein vimentin, is expressed by cells of mesechymal origin and by a variety of transformed cell lines and tumours (Osborn, 1983). Such tissue specific expression of IFs has allowed their clinical use as a diagnostic marker for the detection of cancer. In some cancers, particularly malignant breast carcinoma, there is a strong indication that vimentin is co-expressed with keratins. It has been demonstrated that overexpression of vimentin in MCF-7 human breast cancer cells results in an augmentation of cancer cell motility and invasiveness which could be transiently downregulated by vimentin antisense nucleotides (Hendrix *et al.*, 1997). Studies have shown that IFs such as vimentin are also capable of interacting with negatively charged macromolecular assemblies such as membrane vesicles (Bauer and Traub, 1995). It has also been reported that vimentin filaments may serve as transient docking sites for membranous compartments such as nuclear membrane vesicles during mitosis (Maison *et al.*, 1993; Takai *et al.*, 1996). These data demonstrate the important role of IFs in cell morphology (Goldman *et al.*, 1996) and vesicle movement.

The involvement of the cellular cytoskeleton in membrane trafficking and the insolubility of p150 when expressed in COS-7 cells (section 7.2.4) suggested the possibility that p150 might interact with a cytoskeletal protein. In this section therefore, experiments were performed to evaluate the interaction of p150 with actin, microtubules and intermediate filaments.

7.4   Results

7.4.1   Studies with actin and microtubule disrupting agents

To investigate whether the intracellular distribution and staining pattern of p150 observed in COS-7 cells is due to its interaction with a cytoskeletal component, the effect of pharmacological disruption of these structures upon the subcellular localisation of p150 was examined. Nocodazole causes the depolymerisation of microtubules while cytochalasin D is a potent inhibitor of actin filament function. The breakdown of microtubule and actin cytoskeletal structure induced by nococdazole and cytochalasin D

respectively, could result in the disruption of the p150 staining pattern in p150(EE) transfected cells. Such an observation would suggest an interaction (directly or indirectly via a cytoskeletal linker protein) between p150 and these microfilaments.

To examine this possibility, COS-7 cells were transfected with pMT2-p150(EE) construct or transfected with pMT2SM vector alone (control cells) and seeded onto coverslips. After 36 h, the transfected cells were incubated in the presence or absence of 10 μM nocodazole or 1.2 μM cytochalasin D for 2 h. The cells were then fixed and stained to detect p150, and either actin, or tubulin. In the presence of cytochalasin D (figure 7.6B), the actin structure of control cells becomes severely perturbed compared with the actin structure of an untreated cell (figure 7.6A). Cytochalasin D however, had no effect on the subcellular distribution of p150(EE) in the cells expressing this protein (figure 7.6 C and D). Similarly, treatment of cells with nocodazole was shown to result in the disruption and depolymerisation of microtubule structure (figure 7.7B) compared to the normal microtubule morphology of untreated cells (figure 7.7A). However, the disruption of microtubules by nocodazole had no effect on the staining pattern of p150(EE), (figure 7.7 C and D). COS-7 cells transfected with pMT2-p150(EE) which were plated onto 15 cm dishes and similarly incubated with nocodazole and cytochalasin D were lysed in EB to produce supernatant and pellet fractions. These fractions were analysed by SDS-PAGE, followed by Western blotting using EEmAb. Although the results showed that nocodazole and cytochalasin D treatment caused the disruption of microtubule and actin filament structure respectively, neither of these drugs had any effect on the solubility of p150(EE), as it still remained in the Triton X-100 insoluble pellet (figure 7.8). Since the disruption of microtubule and actin filament stuctures in COS-7 cells did not effect either the subcelluar localisation or solubility of p150(EE), it can be concluded that p150 does not interact with either of these cytoskeletal components

7.4.2   Co-localisation of p150 with the intermediate filament protein vimentin

Since the distribution of p150(EE) expressed in COS-7 cells did not appear to be dependent upon actin filaments or microtubules (section 7.4.1), the possible interaction of p150 with the third variety of cytoskeletal structure, intermediate filaments, was investigated. Intermediate filaments have a tissue specific distribution and in the case of COS-7 cells, vimentin is the predominant intermediate filament protein expressed. At present, no agents are available which specifically disrupt vimentin intermediate filaments, therefore, anti-vimentin antibodies were used in immunolocalisation studies to examine whether transiently expressed p150(EE) co-localised with the endogenous vimentin filament structure in COS-7 cells. The perinuclear filamentous structure
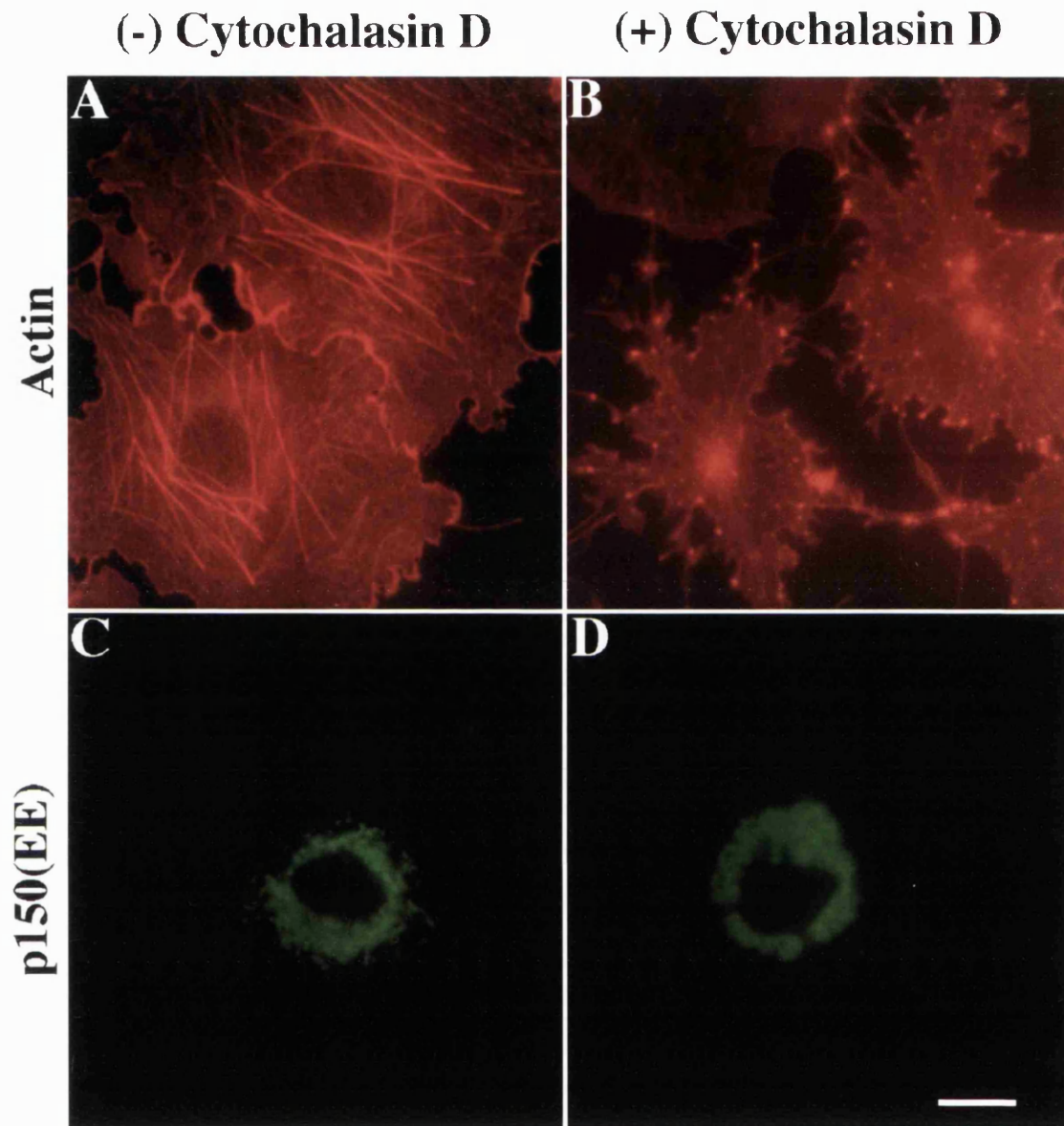
Figure 7.6

The actin disrupting agent cytochalasin D does not effect p150(EE) localisation.

COS-7 cells were either transiently transfected with pMT2-p150(EE) (Panels C and D), or pMT2SM vector alone (Panels A and B). Cells seeded on glass coverslips were treated in the absence (Panels A and C) or presence (Panels B and D) of 1.2μM cytochalasin D for 2 h at 37ºC. Cells were then fixed with formaldehyde and immunostained. p150(EE) was detected using EEmAb (IgG1) and visualised with anti-mouse IgG1 FITC-conjugated antibody (Panels C and D). Actin was detected using rhodamine-conjugated phalloidin (Panels A and B). (Bar: 10 μm)

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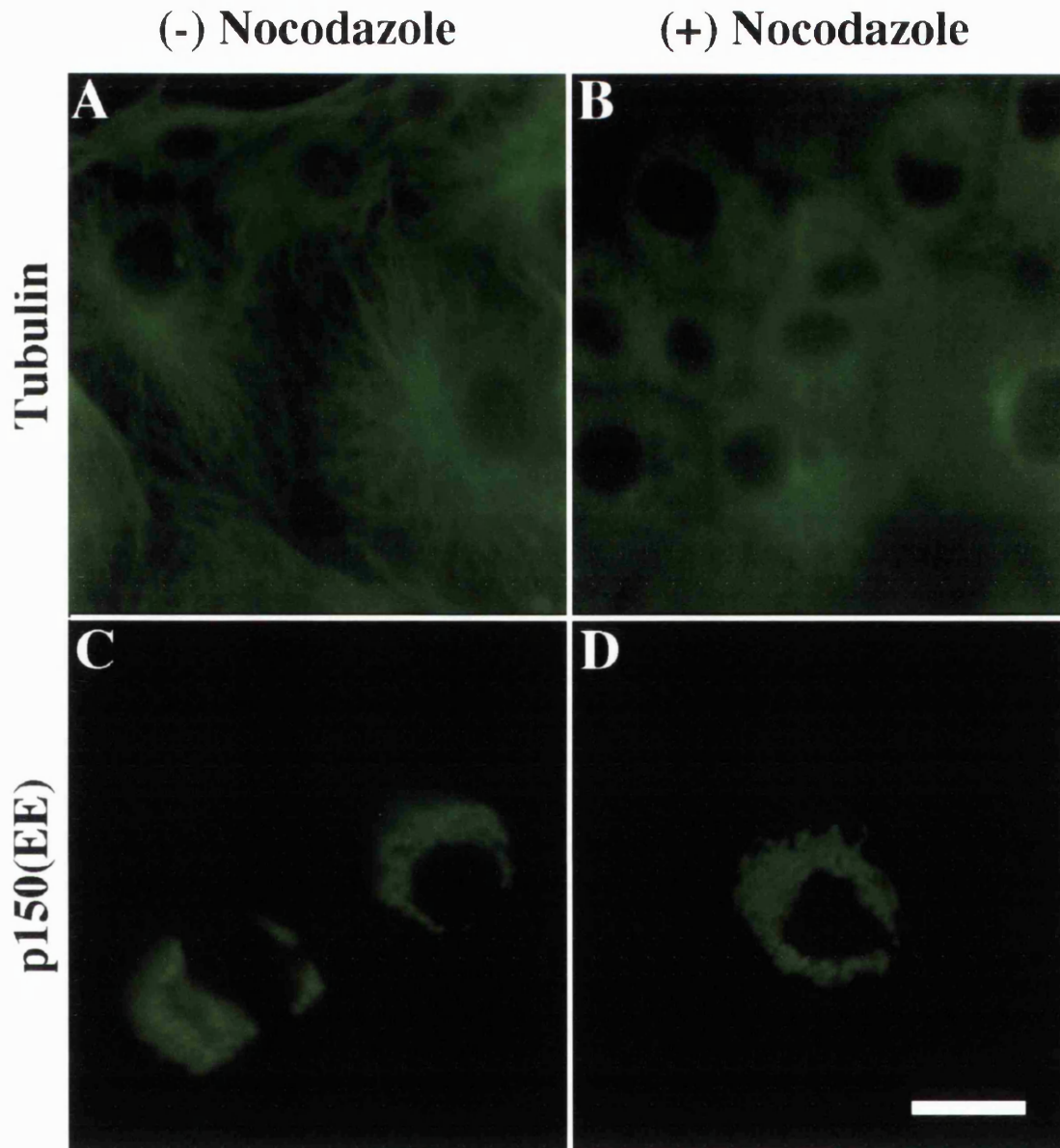
Figure 7.7
The microtubule disrupting agent nocodazole, does not effect p150(EE) localisation.

COS-7 cells were transiently transfected with pMT2-p150(EE) (Panels C and D), or pMT2SM vector alone (Panels A and B). Cells seeded on glass coverslips were treated in the absence (Panels A and C) or presence (Panels B and D) of 10 μM nocodazole for 2 h at 37°C. Cells were then prepared by methanol fixation for immunostaining. p150(EE) was detected using EEmAb and visualised with anti-mouse IgG1 FITC-conjugated antibody (Panels C and D). Tubulin was detected using tubulin specific mouse IgG1 monoclonal antibody and visualised using anti-mouseIgG1 FITC-conjugated antibody (Panels A and B). (Bar:10 μm)
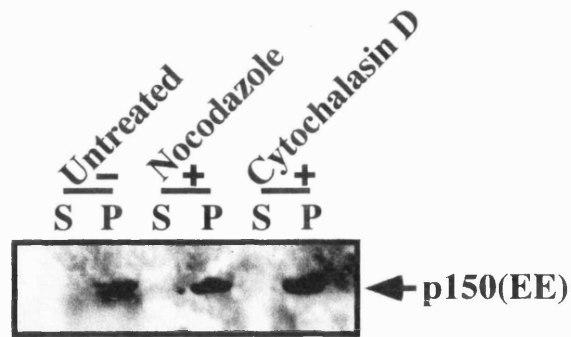
Figure 7.8 Tubulin and actin disrupting agents have no effect on p150(EE) solubility.

COS-7 cells were transiently transfected with pMT2-p150(EE).
After 36 h cells were incubated in the presence or absence of
10 μM nocodazole or 1.2 μM cytochalasin D for 2 h. Cells were
then lysed in EB and centrifuged at 14000xg to generate
supernatant (S) and pellet (P) fractions. Samples were resolved
by 7.5% SDS-PAGE and p150(EE) was detected by Western
blotting using EEmAb.

observed for vimentin filaments in COS-7 cells is demonstrated in figure 7.9 (panels A and B)

To examine the possible co-localisation between vimentin and p150(EE), COS-7 cells were transfected to express p150(EE) and dual-labelled for immunofluorescence. Although both the primary anti-vimentin antibody and EEmAb are mouse monoclonals, they belong to different sub-classes (EEmAb and anti-vimentin antibodies are IgG1 and IgM subclasses respectively), this allowed the use of specific second antibodies. Therefore, to visualise vimentin filaments and p150(EE), anti-IgM TRITC and anti-IgG1 FITC antibodies were used respectively. The immunofluorescence observed for both p150(EE) expressed in COS-7 cells and endogenous vimentin is shown in figure 7.10 (panels A and B respectively). The predominantly yellow staining pattern indicates that there is extensive co-localisation between p150(EE) and vimentin (figure 7.10C). In addition, upon expression of p150(EE), the filamentous nature of the vimentin cytoskeletal structure disappeared and adopted the punctate perinuclear distribution of p150(EE) (figure 7.10A and B). This result indicated that expression of p150(EE) caused severe disruption to vimentin filaments which also resulted in the co-localisation of vimentin with p150(EE).

Western blot analysis of supernatant and pellet fractions from p150(EE) expressing COS-7 cells indicated that both vimentin and p150(EE) are present in the same Triton X-100 insoluble pellet (figure 7.11). Vimentin has been shown to be an extremely insoluble protein, which can only be solubilised using 8M urea where it exists as a soluble tetramer (Fuchs and Weber, 1994). The observed co-localisation of p150(EE) with vimentin may account for the difficulty in solubilising p150(EE) expressed in COS-7 cells.

It is interesting to note that the disruption of intermediate filament structure by p150(EE) may explain an observed alteration in COS-7 cell morphology. When COS-7 cells express p150(EE) a change in cell shape was observed, from a fully spread configuration to a more rounded one, exhibiting varying degrees of retraction of the cell border. For example, the distance between the basal surface (the cell surface nearest the substratum of the coverslip) and apical region of a COS-7 cell transfected with pMT2SM vector alone was 6-8 microns, whereas the equivalent distance in a cell expressing p150(EE) was 16-18 microns. The disruption of vimentin structure due to the expression of p150(EE) may explain the morphological changes seen, such as cell rounding and the increase in basal-apical height. These are alterations that could be attributed to an interference in cytoskeletal architecture.

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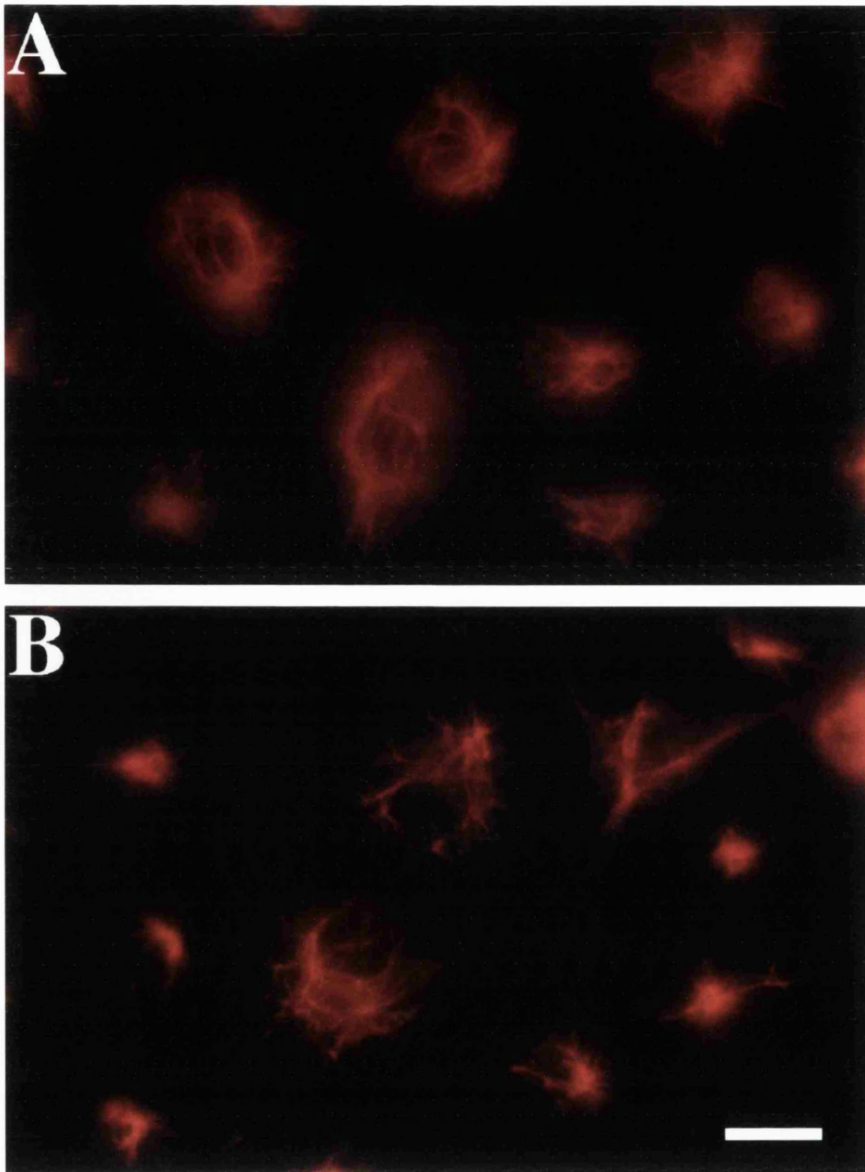
Figure 7.9

Immunolocalisation of vimentin intermediate filaments.

Untransfected COS-7 cells were seeded on glass coverslips and prepared by methanol fixation for immunostaining. Endogenous vimentin filaments (Panels A and B), were detected using a vimentin specific IgM monoclonal primary antibody and visualised with an anti-mouse IgM TRITC-conjugated secondary antibody. (Bar:10 μm)
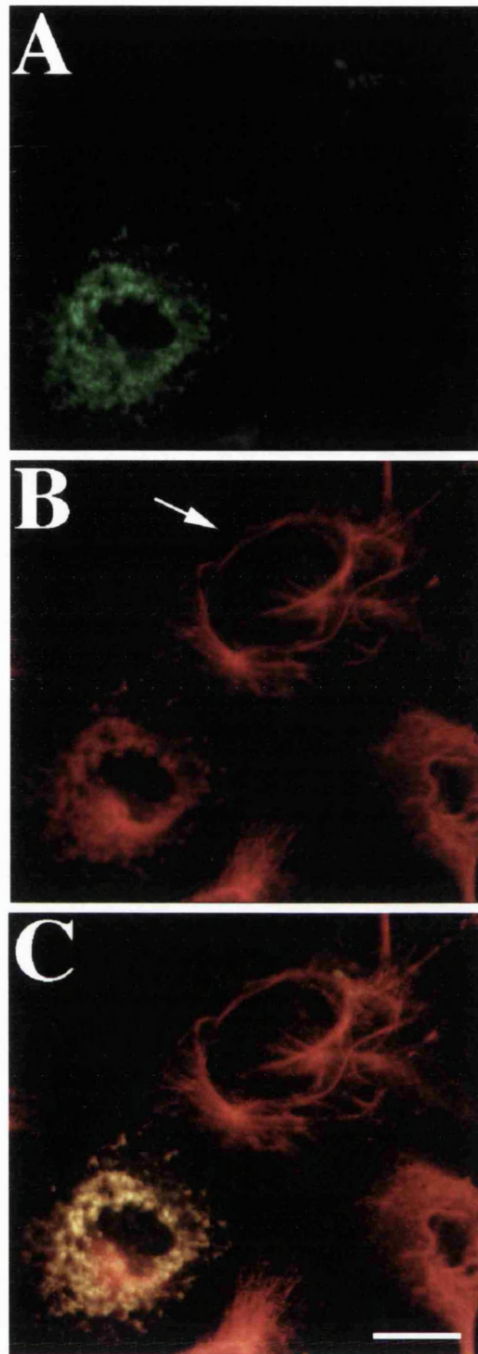
Figure 7.10

p150(EE) co-localises with and disrupts vimentin filaments.

COS-7 cells were transiently transfected to express p150(EE), seeded
on glass coverslips and prepared by methanol fixation for immunostaining.
Cells were dual-labelled with EEmAb (IgG1) and vimentin specific mouse
IgM mouse monoclonal antibody. Bound antibodies were visualised using
anti-mouse IgG1 FITC- and anti-mouse IgM TRITC-conjugated secondary
antibodies respectively. The staining pattern for p150(EE) is shown in
Panel A and that for vimentin in the same cell by Panel B. An arrow indicates
normal vimentin structure in an untransfected cell. Panel A (green) and
Panel B (red) images were overlayed (Panel C) to show p150 and vimentin
co-localisation (yellow). (Bar:10 μm)
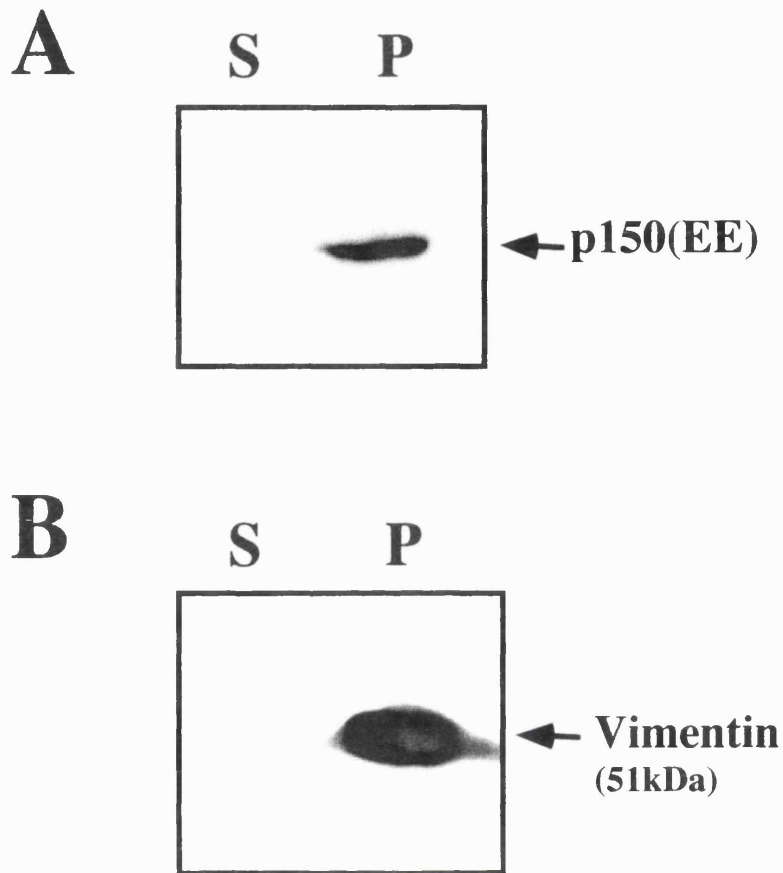
Figure 7.11 p150(EE) and the intermediate filament protein
vimentin can be found in the same Triton X-100 insoluble fraction.

COS-7 cells were transfected with pMT2-p150(EE), after 36h, cells were
lysed in EB and centrifuged at 14,000x*g* to generate supernatant (S) and
pellet (P) fractions. Samples were resolved by 7.5% SDS-PAGE and
analysed using Western blotting. p150(EE) was detected using EEmAb
(Panel A), vimentin was detected in samples run in parallel to those in
panel A, using a vimentin specific mouse monclonal antibody (Panel B).

It is possible that the disruption of vimentin filaments by p150(EE) and the co-localisation of p150(EE) with vimentin, may be due to protein overexpression. However, examination of actin and microtubule structure in cells overexpressing p150(EE), using dual immunofluorescence (figure 7.12A and 7.12B), showed that p150(EE) does not perturb actin or tubulin structure or co-localise with these proteins. This demonstrated that the disruption of vimentin reflects a specific association between p150(EE) and this intermediate filament protein, rather than a non-specific binding or aggregation of overexpressed protein.

The effect of PtdIns 3-kinase expression on vimentin structure was also examined. COS-7 cells were co-transfected to express p150(EE) and PtdIns 3-kinase and then triple-labelled in order to detect the p150/PtdIns 3-kinase complex and vimentin. Anti-mouse IgG1 FITC and anti-mouse IgM-TRITC antibodies were used to visualise p150(EE) (green fluorescence) and vimentin (red fluorescence), respectively. An anti-rabbit biotinylated antibody together with a streptavidin conjugated coumarin derivative (AMCA), was used to visualise PtdIns 3-kinase, which appeared as a blue fluorescence (figure 7.13). In cells expressing p150(EE) or the p150(EE)/PtdIns 3-kinase complex, a disruption in vimentin filaments can be observed, however, in cells expressing only PtdIns 3-kinase, vimentin retained its normal perinuclear filamentous stucture. This result therefore, indicates that the overexpression of PtdIns 3-kinase does not promote the disassembly of vimentin filaments, suggesting that disruption of vimentin structure is specifically induced by the expression of p150(EE).

7.4.3 Discussion

The results presented in section 7.4.1 demonstrate that an actin and microtubule cytoskeleton was not required for the staining pattern observed for p150(EE) expressed in COS-7 cells. The actin and tubulin depolymerizing agents, cytochalasin D and nocodazole respectively, had no effect on p150(EE) subcellular distribution or solubility, although they were very effective in disrupting actin and microtubule structure. Disruption of the microtubule cytoskeleton by nocodazole can also lead to the fragmentation and perturbation of the Golgi apparatus (Turner and Tartakoff, 1989) and blocks retrograde membrane traffic between the ER and the Golgi complex (Lippincott Schwartz et al., 1990). Therefore, the failure of nocodazole to disrupt the staining pattern of p150(EE) suggests that p150(EE) may not be associated with the Golgi complex. In addition, since neither nocodazole nor cytochalasin D effect p150(EE) distribution, it is unlikely that the movement of possible p150(EE) containing vesicles require an actin or microtubule cytoskeleton.
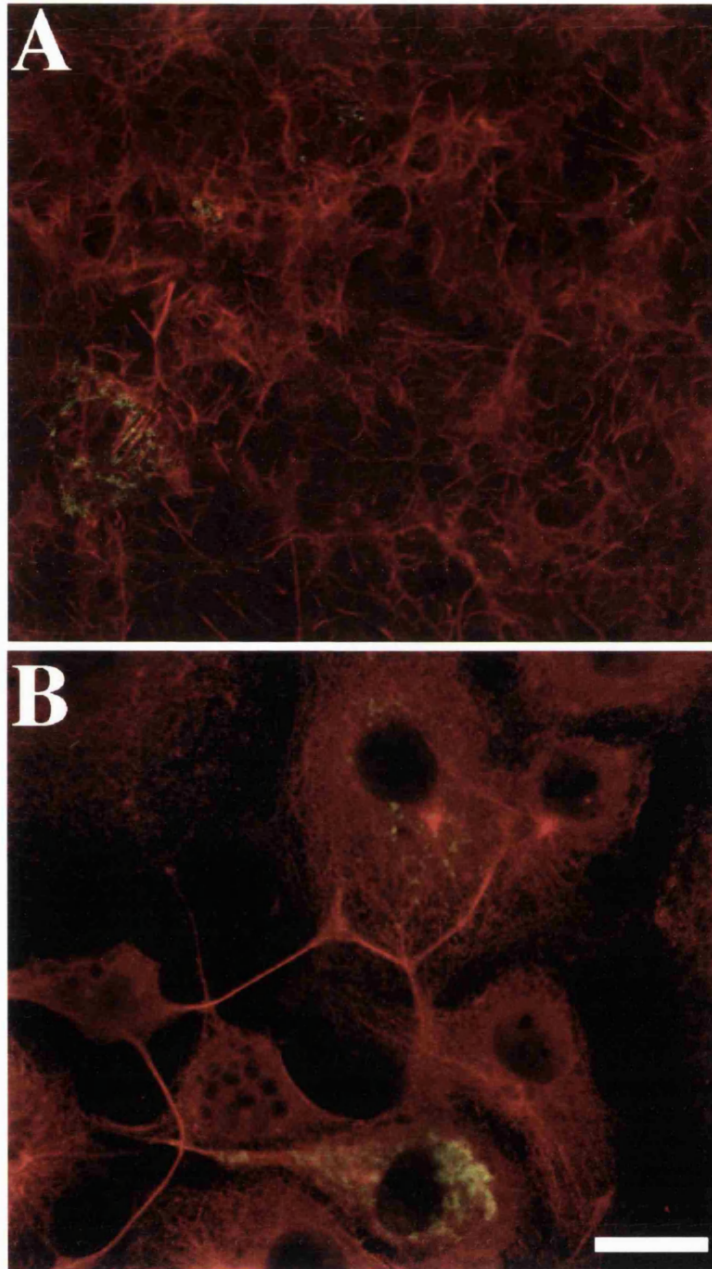
Figure 7.12

p150(EE) expression does not disrupt actin or microtubule cytoskeletal structure.

COS-7 cells were transiently transfected to express p150(EE) (Panels A and B). Cells were seeded on glass coverslips and prepared by formaldehyde (Panel A), or methanol (Panel B) fixation for immunostaining. Cells were dual-labelled with EEmAb (IgG1) to detect p150(EE) and either rhodamine-conjugated phalloidin to detect actin (Panel A) or specific rabbit polyclonal antisera to detect tubulin (Panel B). Bound antibodies were visualised with anti-mouse IgG1 FITC- and anti-rabbit TRITC-conjugated secondary antibodies. (Bar:10 μm)
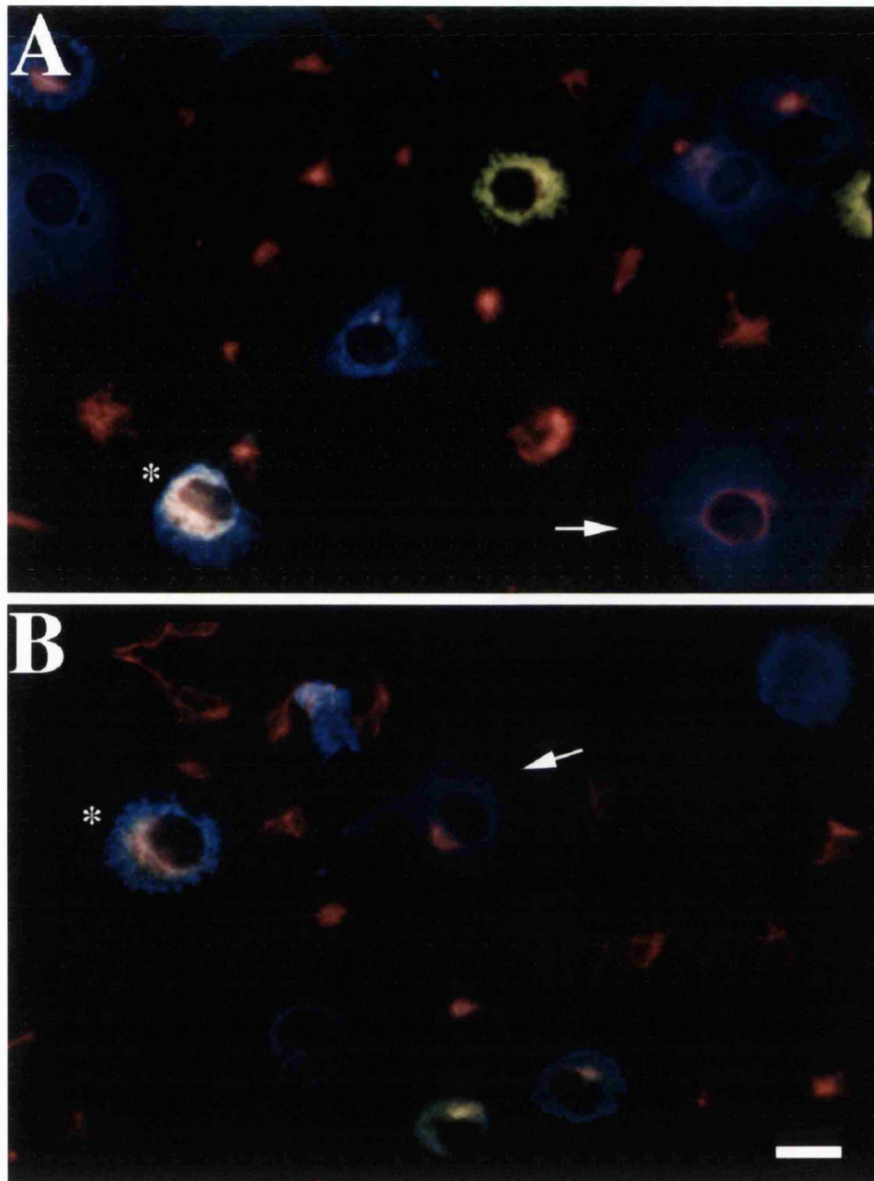
Figure 7.13

Expression of PtdIns 3-kinase does not effect vimentin distribution.

COS-7 cells were co-transfected to express the p150/PtdIns 3-kinase complex (Panels A and B). Cells were seeded on glass coverslips and methanol fixed for triple-labelling. EEmAb (IgG1) was used to detect p150(EE) and was visualised with an anti-mouse IgG1 FITC-conjugated antibody (green fluorescence). PtdIns 3-kinase specific rabbit polyclonal antisera were used to detect PtdIns 3-kinase and was visualised using a biotinylated anti-rabbit antibody with streptavidin-conjugated AMCA (blue fluorescence). Vimentin was detected using vimentin specific mouse IgM monoclonal antibody and visualised with anti-mouse IgM TRITC-conjugated antibody (red fluorescence). An asterix indicates a cell co-expressing the p150/PtdIns 3-kinase complex with endogenous vimentin. Arrows indicate a cell with endogenous vimentin, expressing PtdIns 3-kinase only. (Bar:10 μm)

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The immunofluorescence studies described in section 7.4.2 however, indicate that there is an interaction between p150 and the vimentin intermediate filament cytoskeleton. Dual immunofluorescence experiments have showed that p150 co-localises with vimentin. The use of confocal microscopy to demonstrate this co-localisation however, is not definitive proof of a direct interaction between p150 and vimentin as it is possible that p150 might interact with a vimentin binding protein. Therefore, the ability of p150 to bind vimentin *in vitro* should be investigated, however, since vimentin is only soluble in 8M urea, the examination of protein-protein interactions in such an environment would be prohibitive. The expression of p150(EE) in COS-7 cells also resulted in the disruption of vimentin filament organisation. The disruptive effect of p150(EE) on vimentin was not due to its overexpression, since no perturbation in actin or microtubule structure was observed in COS-7 cells overexpressing p150(EE). In addition, overexpression of another protein such as PtdIns 3-kinase, had no effect on vimentin structure.

Unlike actin and tubulin, the role of vimentin in membrane trafficking has not yet been fully defined. Consequently, the functional significance of the interaction of p150(EE) and the intermediate filament cytoskeleton is not particularly clear. It is perhaps, relevant here to note that vimentin has the capacity to bind negatively charged macromolecular assemblies such as membrane vesicles (Bauer and Traub, 1995). Furthermore, it has been demonstrated that vimentin filaments can act as transient docking sites for nuclear membrane vesicles during mitosis (Maison *et al.*, 1993; Takai *et al.*, 1996). Studies examining the mechanism of histamine release from mast cells, have reported a role for intermediate filaments in this process (Tasaka, 1994). When permeabilised, mast cells were stimulated with Ca^{2+}, a translocation of PKC from the cytosol to the membrane fraction was observed, leading to the phosphorylation of vimentin. Phosphorylation of vimentin resulted in the disruption of vimentin filament structure, this in turn correlated with an increased mobility of granules, initiating degranulation and histamine release (Tasaka, 1994). Since the disruption of vimentin structure is required for the movement of secretory granules within the cell, it is possible that p150 may function to facilitate the disruption of vimentin, allowing an increased movement of membrane vesicles. This could be achieved by the phosphorylation of vimentin by the putative N-terminal protein kinase domain of p150, or by a protein-protein interaction that results in vimentin filament perturbation. The function of p150 in this context could be examined by mutational analysis in which deletions of p150 protein domains can be used to investigate the ability of p150 to disrupt or co-localise with vimentin (section 7.7). These types of experiments would yield more information as to the functional significance of the p150 protein and its domain structure. Nevertheless, the exact subcellular membrane compartments to which p150 localises, are at present unknown. Identification of these

cytoplasmic structures may provide further insight into the specific biological role of p150.

7.5 Examination of p150 subcellular localisation

7.5.1 Introduction

Following the characterisation of p150 and PtdIns 3-kinase (Panaretou *et al.*, 1997; Volinia *et al.*, 1995) it has been suggested that their biological role is analogous to that of their yeast homologues Vps15p and Vps34p which regulate vesicle traffic from the TGN to the vacuole (Stack *et al.*, 1995c). Treatment of rat and human cell lines with the PI 3-kinase inhibitors wortmannin (Arcaro and Wymann, 1993) and LY294002 (Vlahos *et al.*, 1994), leads to the secretion of an incorrectly processed form of the mammalian hydrolase, cathepsin D (Brown *et al.*, 1995b; Davidson, 1995). Secretion of the cathepsin D precursor, instead of its accurate targeting and processing to the lysosome, is comparable to the abberant secretion of procarboxypeptidase Y in yeast cells expressing mutant Vps34p. The effect of wortmannin on the localisation of lysosomal type I integral membrane glycoproteins to swollen compartments, also suggested a role for PI 3-kinase activity in regulating membrane traffic late in the endocytic pathway (Reaves *et al.*, 1996). The lipid kinase activity of the human Vps34p homologue, PtdIns 3-kinase, is inhibited by wortmannin with an IC_{50} in the low nM range, which correlates well with the effects of wortmannin on lysosomal protein targeting in mammalian cells. Consequently, such data supports the idea that p150/PtdIns 3-kinase could play a role in membrane trafficking. However, since wortmannin inhibits other PI 3-kinase family members (Domin and Waterfield, 1997; Vanhaesebroeck *et al.*, 1997a) and even other lipid kinases such as PtdIns 4-kinase (Nakanishi *et al.*, 1995) and the protein kinase myosin light chain kinase (Nakanishi *et al.*, 1992), its disruption of vesicle trafficking events cannot be attributed solely to PtdIns 3-kinase activity. At present, the identity of the PI 3-kinase involved in vesicle mediated membrane trafficking in the late endocytic pathway remains to be conclusively established.

The intracellular targeting of a protein to a particular organelle often provides valuable insight into its specific biological role. Overexpression of p150 in COS-7 cells resulted in a punctate perinuclear type staining pattern (section 7.2.2). This is unlike the tubular reticular pattern characteristic of the ER (Schweizer *et al.*, 1994). Instead, the distribution observed for p150 is one that more closely resembles intracellular membranes involved in the endocytic pathway, such as the Golgi, early/late endosomes and lysosomes (Chapman and Munro, 1994). Results from section 7.4.2, demonstrated that when expressed in COS-7 cells, p150 co-localised with vimentin and resulted in the disruption

of intermediate filament structure. In this section, however, using Golgi and endocytic markers, the subcellular distribution of p150 is further examined.

7.6 Results

7.6.1 Analysis of p150 localisation using immunofluorescence

To examine the localisation of p150(EE) expressed in COS-7 cells, an affinity purified rabbit polyclonal antibody that recognises the *cis*-Golgi protein GM130 (Dr Graham Warren ICRF), was used as a marker for the Golgi complex (Nakamura *et al.*, 1995). Using confocal microscopy, the subcellular localisation of p150(EE) transiently expressed in COS-7 cells was compared to that of GM130, using EEmAb and the anti-GM130 antibodies. p150(EE) and the endogenous GM130 protein were visualised using FITC-conjugated anti-mouse and TRITC-conjugated anti-rabbit antisera respectively. Figure 7.14A shows a confocal image of p150(EE) and GM130 distribution in the same cell. GM130 is localised to a compact juxta-nuclear reticulum, characteristic of the Golgi apparatus. There was very little co-localisation observed between p150(EE) and GM130, which suggests that p150(EE) is not localised to the Golgi complex.

However, since GM130 is a *cis*-Golgi protein, it remained possible that p150 may still be localised to the *trans*-Golgi region of the organelle. To investigate this possibility, experiments were conducted with Brefeldin A (BFA). BFA is a fungal metabolite that inhibits a guanine nucleotide-exchange factor for a small GTPase ARF (section 1.5.4.1.3) (Peyroche *et al.*, 1996). The inhibition of ARF function by BFA blocks protein transport into the Golgi apparatus, resulting in the rapid re-distribution of the Golgi into the ER (Klausner *et al.*, 1992; Lippincott Schwartz *et al.*, 1991). Consequently, when cells are treated with BFA, a morphological affect can be observed, in which the *cis*- and *medial*-Golgi breaks down into numerous vesicle and tubule processes and the *trans*-Golgi becomes concentrated at the Microtubule-Organising Centre (MTOC) (Reaves and Banting, 1992). COS-7 cells expressing p150(EE) were treated with 36 μM BFA for 2 h, and then processed for immunofluorescence. The BFA treated cells were dual labelled to visualise p150(EE) and GM130. BFA caused a marked re-distribution of GM130, demonstrating that it effectively disrupted the Golgi apparatus (figure 7.14B). BFA treatment had little effect on the p150(EE) staining pattern which remained relatively unchanged. Re-distribution of p150(EE) to the MTOC or to anywhere else in the cell was not observed, suggesting that p150(EE) is not localised at the Golgi complex. This agrees with the results observed when p150(EE) expressing cells were treated with nocodazole (section 7.4.1). Disruption of the microtubule cytoskeleton by nocodazole also leads to the fragmentation and perturbation of the Golgi apparatus.
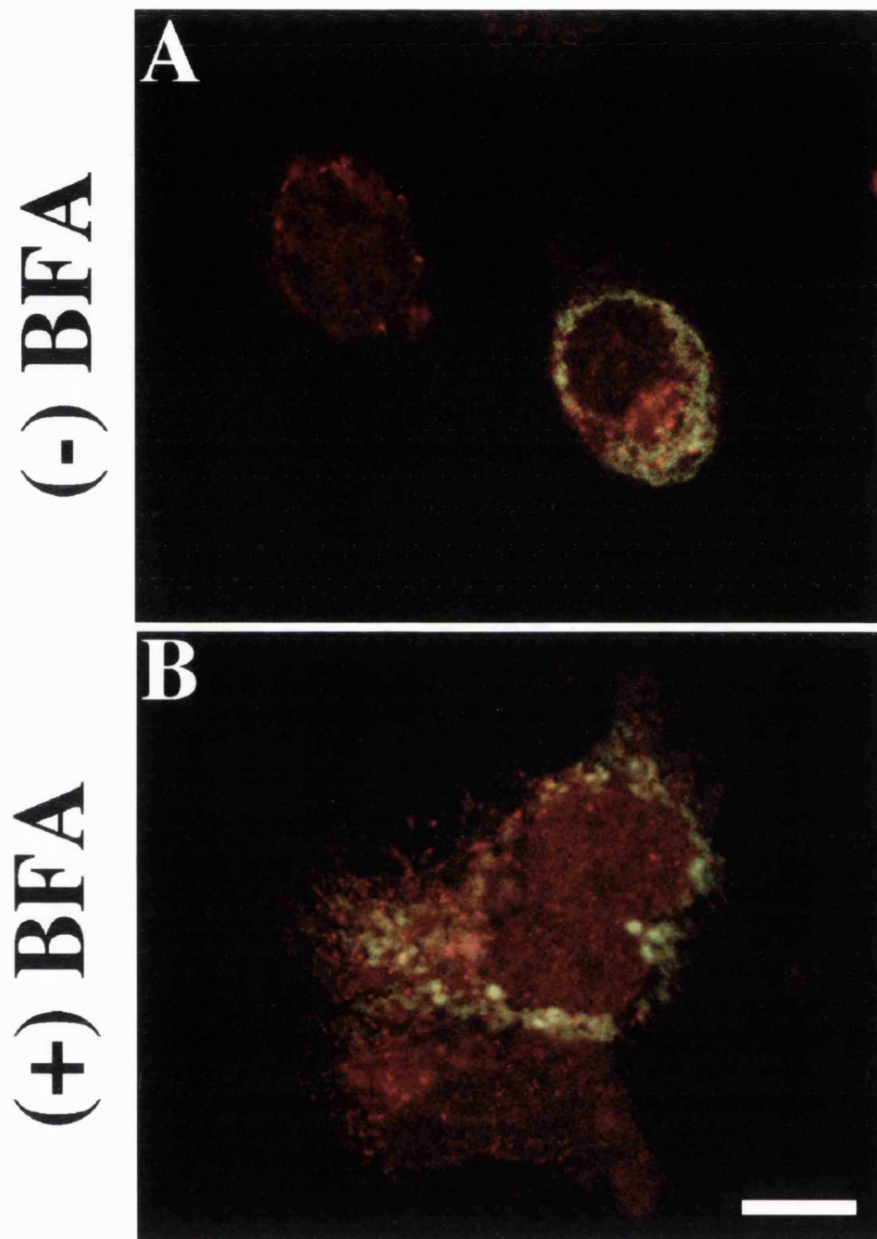
Figure 7.14

p150(EE) does not co-localise with the *cis*-Golgi protein GM130 and is not disrupted by BFA.

COS-7 cells were transfected to express p150(EE), seeded on glass coverslips and treated in the absence (Panel A) or presence (Panel B) of 36 μM BFA for 2 h at 37°C. Cells were then methanol fixed for immunostaining. Cells were dual-labelled (Panels A and B) with EEmAb (IgG1) and GM130 specific rabbit polyclonal antisera. Bound antibodies were visualised using anti-mouse IgG1 FITC- and anti-rabbit TRITC-cojugated secondary antibodies respectively. (Bar:10 μm)

However, p150(EE) localisation remained unchanged in the presence of nocadazole, confirming that p150 expressed in COS-7 cells is not confined to the Golgi complex.

Since p150(EE) did not appear to be resident at the Golgi, the localisation of p150 to other subcellular compartments on the endocytic pathway, such as endosomes and lysosomes was examined. Rabbit polyclonal antibodies were obtained that recognised late endosomal/lysosomal proteins such as Cathepsin D (Dr Howard Davidson, University of Cambridge) and LAMP-1 (Dr Mark Marsh LMCB MRC). Cathepsin D is a major mammalian aspartyl protease which is synthesised as a 53 kDa proenzyme called procathepsin D. After export from the TGN and delivery to endosomal compartments, the propeptide is removed to form a 47 kDa intermediate, which is subsequently cleaved in lysosomes to form the mature hydrolase comprising noncovalently associated 31 kDa and 14 kDa polypepetides (Delbruck *et al.*, 1994). LAMP-1 (lysosome-associated membrane protein) is a highly glycosylated integral membrane protein, the precise function of which is unknown, but is postulated to have a function in lysosome stability (Hunziker and Geuze, 1996). Antibodies against both LAMP-1 and cathepsin D will detect late endosomal and lysosomal compartments.

COS-7 cells transiently expressing p150(EE) were dual labelled to visualise p150(EE) and either i) Cathepsin D or ii) LAMP-1. Figure 7.15 shows the staining pattern observed in COS-7 cells of endogenous Cathepsin D (panel A) and LAMP-1 (panel B). Figure 7.16 shows cells which were dual labelled to visualise p150(EE) with i) cathepsin D (panel A) and ii) LAMP-1 (panel B). Little co-localisation was seen for p150(EE) with either marker, indicating that p150(EE) does not reside on the same endosomal/lysosmal compartments as cathepsin D or LAMP-1.

So far, a number of drugs, including nocodazole, cytochalasin D and BFA have failed to alter the subcellular distribution of p150. In the following experiments, wortmannin was used to see if the inhibition of PI 3-kinase activity had any effect on the localisation of p150 or PtdIns 3-kinase. COS-7 cells expressing p150(EE) were treated in the presence and absence of wortmannin (50nM) for 30 minutes and then examined by immunofluorescence. No change in p150 distribution was observed in cells treated with wortmannin (figure 7.17B) compared with untreated cells (figure 7.17 A).

The effect of wortmannin on PtdIns 3-kinase distribution was also examined. COS-7 cells expressing PtdIns 3-kinase were similarly treated in the presence or absence of the PI 3-kinase inhibitor. Although wortmannin did not effect PtdIns 3-kinase localisation (figure 7.18A and B) cells incubated with the drug appeared to contain a greater number of vacuolar structures (figure 7.18B) compared with untreated cells (figure 7.18A).

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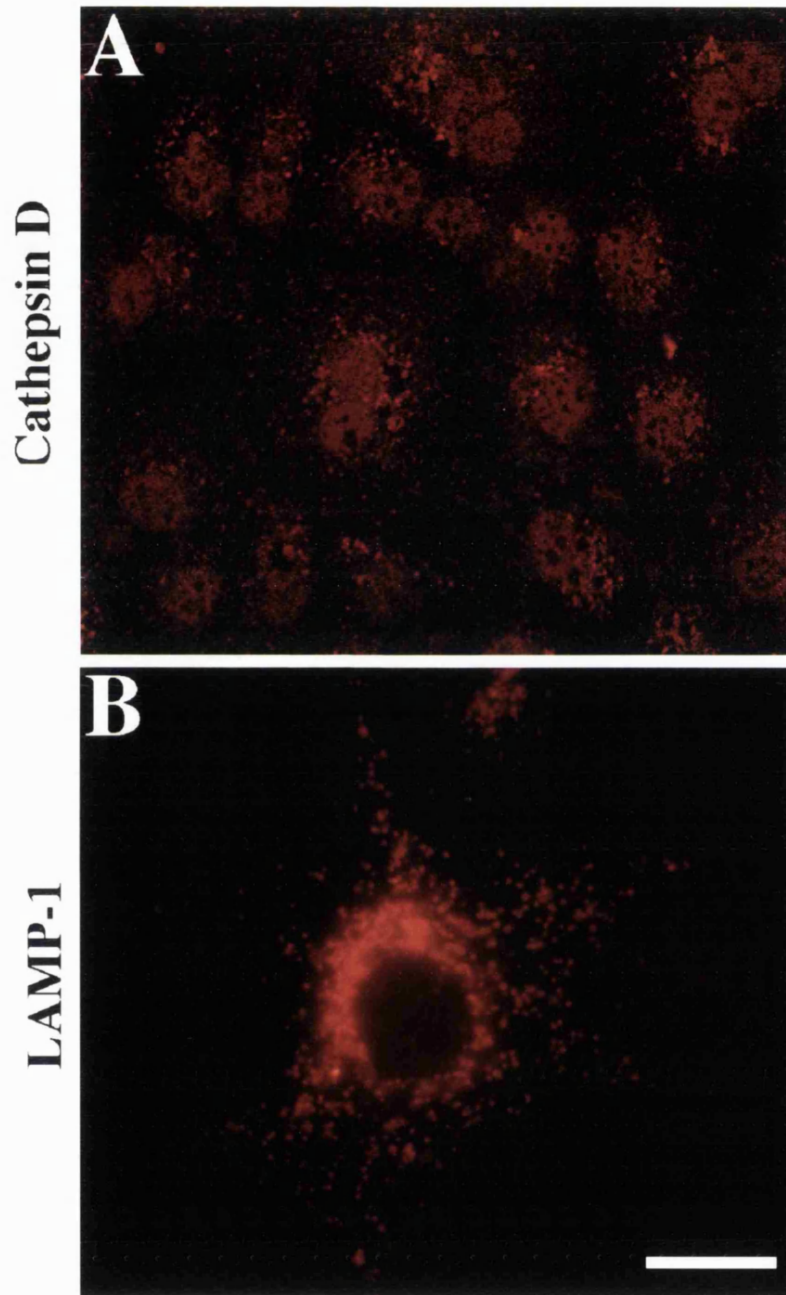
Figure 7.15

Immunolocalisation of late endosomal/lysosmal proteins, cathepsin D and LAMP-1

Untransfected COS-7 cells were seeded on glass coverslips and prepared by methanol fixation for immunostaining. After incubation with primary antibodies, cathepsin D specific rabbit polyclonal antisera (Panel A) or LAMP-1 specific rabbit polyclonal antisera (Panel B), bound antibodies were visualised using TRITC-conjugated anti-rabbit secondary antibody. (Bar: 10 μm)
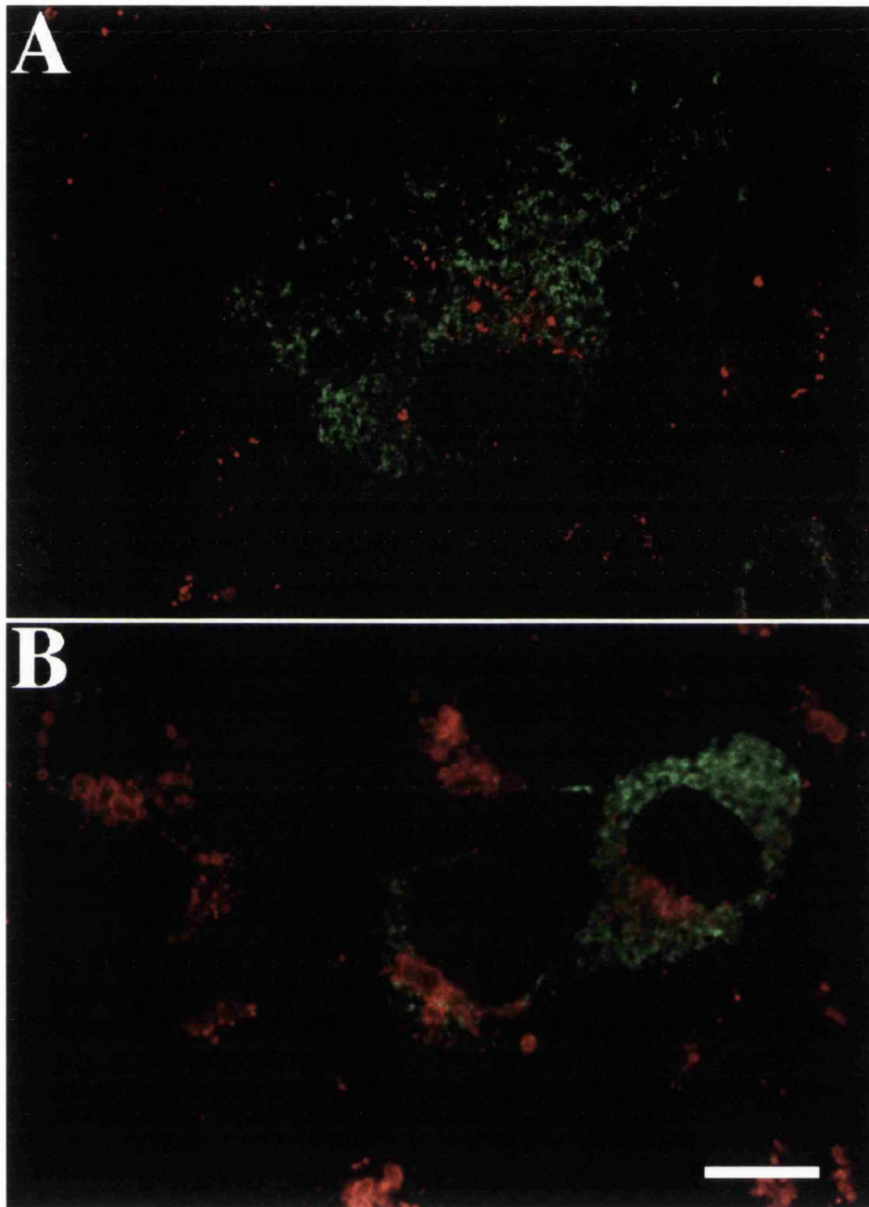
Figure 7.16
p150(EE) does not co-localise with cathepsin D or LAMP-1.

COS-7 cells were transfected to express p150(EE), seeded on glass coverslips and prepared by methanol fixation for immunostaining. Cells were dual-labelled with EEmAb (IgG1) to detect p150(EE) and either cathepsin D specific rabbit polyclonal antisera (Panel A) or LAMP-1 specific rabbit polyclonal antisera (Panel B). Bound antibodies were visualised using anti-mouse IgG1 FITC- and anti-rabbit TRITC-conjugated secondary antibodies. (Bar:10 μm)
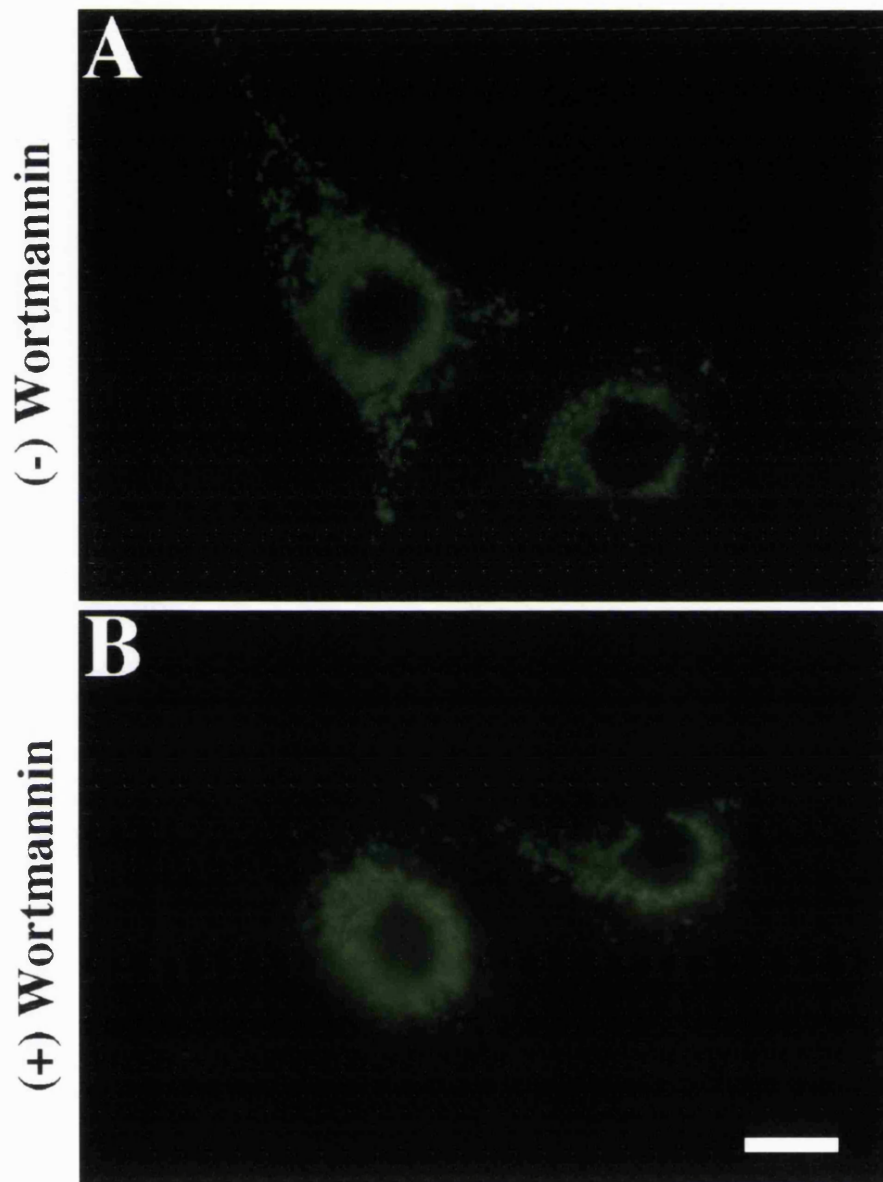
Figure 7.17
Wortmannin has no effect on p150(EE) localisation.

COS-7 cells were transfected to express p150(EE), seeded on glass coverslips and treated in the absence (Panel A) or presence (Panel B) of 50 nM wortmannin for 30 mins at 37°C. Cells were prepared by methanol fixation for immunostaining. p150(EE) was detected using EEmAb (IgG1) and visualised using anti-mouse IgG1 FITC-conjugated secondary antibody. (Bar:10 μm)
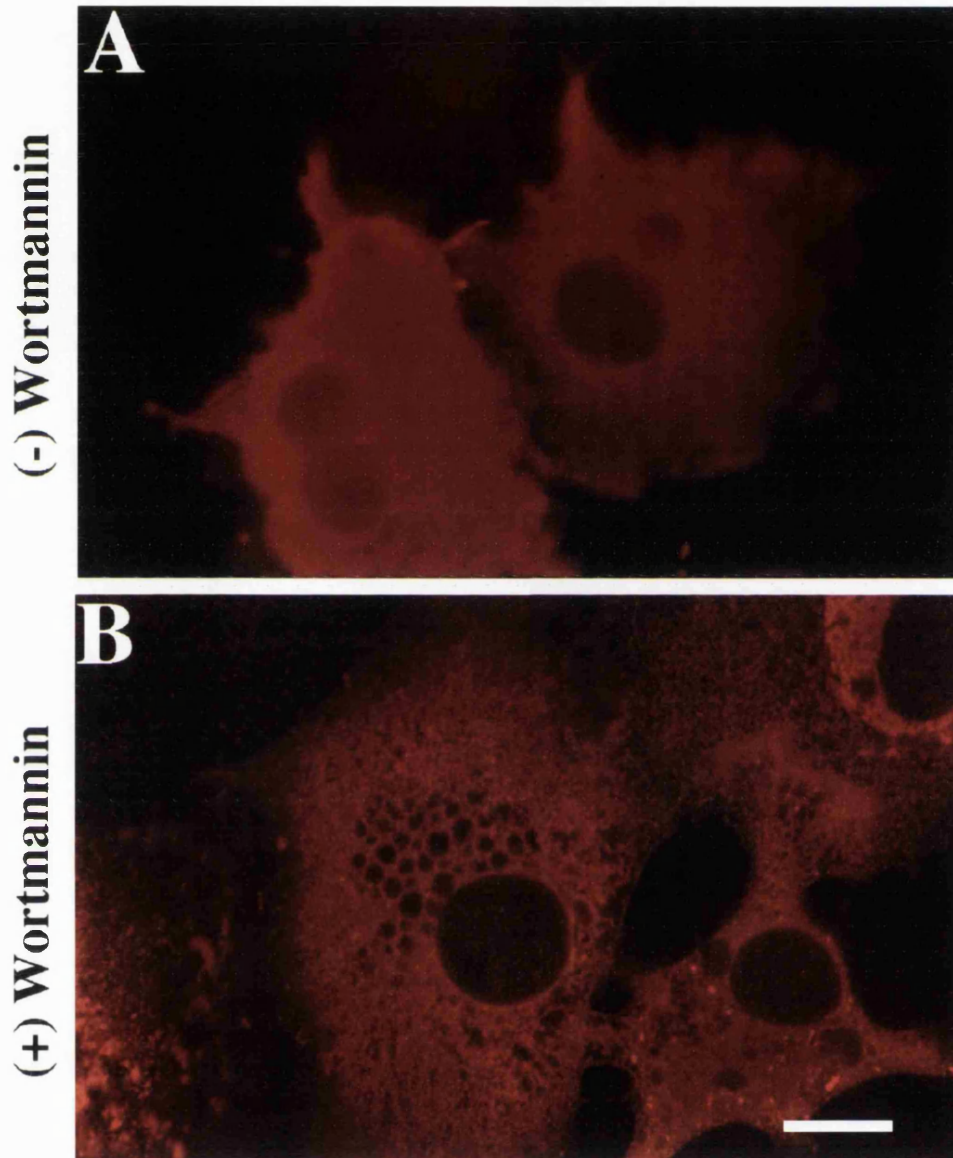
Figure 7.18
The effect of wortmannin on PtdIns 3-kinase localisation.

COS-7 cells were transfected to express PtdIns 3-kinase, seeded on glass
coverslips and treated in the absence (Panel A) or presence (Panel B),
of 50 nM wortmannin for 30 mins at 37°C. Cells were prepared by methanol
fixation for immunostaining. PtdIns 3-kinase was detected using PtdIns 3-kinase
specific rabbit polyclonal antisera and visualised with anti-rabbit TRITC-
conjugated secondary antibody. (Bar:10μm)

A similar result was observed in COS-7 cells expressing a mutant form of PtdIns 3-kinase in which the lipid kinase activity had been abrogated. This mutant PtdIns 3-kinase was made (Jeff Linacre LICR) using the pMT2-PtdIns 3-K construct as a template in a procedure employing site-directed mutagenesis by overlap extension using PCR (Ho et al., 1989). Using this PCR technique, essential residues required for lipid kinase activity were mutated (D^{743} to A and N^{748} to I).

The mutations were checked both by DNA sequencing and biochemically, by expressing wild type (WT) and mutant ($D^{743}A$, $N^{748}I$) PtdIns 3-kinase in COS-7 cells. Immunoprecipitation from transfected cell lysates using a PtdIns 3-kinase specific antibody and assaying for lipid kinase activity showed how the mutated PtdIns 3-kinase had no lipid kinase activity compared to the wild type protein (figure 7.19). Immunofluorescence studies on COS-7 cells expressing the mutant PtdIns 3-kinase demonstrated that although this enzymatically inactive protein had a cytosolic distribution similar to WT-PtdIns 3-kinase (figure 7.20) these cells also had the vacuolar type structures observed in the wortmannin treated cells which expressed WT-PtdIns 3-kinase (figure 7.18, panel B). These results suggest that the vacuolar structures observed in wortmannin treated cells expressing WT-PtdIns 3-kinase are attributable to the inhibition of its lipid kinase activity.

7.6.2 Discussion

The results reported in section 7.6.1 showed that p150(EE) transiently expressed in COS-7 cells did not localise to either the Golgi apparatus or to endosomal/lysosomal compartments which contain cathepsin D or LAMP-1 proteins. Since the yeast Vps15p/Vps34p complex has been postulated to be located between the TGN and the vacuole, it was surprising that p150/PtdIns 3-kinase complex did not co-localise to the TGN or endosome/lysosome when expressed in mammalian cells. It is possible that p150 may be present on a population of vesicles that cathepsin D or LAMP-1 antibody markers do not recognise. This suggests that p150 could be functioning on a separate and distinct pathway, involving vesicles that do not contain cathepsin D or LAMP-1.

The AP-3 adaptin (section 1.5.4.2.1) is an example of a protein complex that has a punctate perinuclear distribution which does not co-localise with endosomal markers and has been suggested to reside in a post-TGN compartment (Simpson et al., 1997). The postulated function of the post-TGN compartment is to receive endocytosed proteins, but does not appear to represent a conventional endosome. It is possible that p150 is localised to analogous vesicular structures. Recent studies have provided evidence for a less conventional trafficking pathway involving caveolin-1, in a cycling pathway between the cell surface and the Golgi apparatus in human fibroblasts (Conrad et al., 1995).

Caveolin-1 has been shown to be internalised via a microtubule-independent, but as yet, otherwise undefined pathway back to the ER/Golgi compartment, from where it returns to the surface via a microtubule dependent transport step (Parton, 1996). Consequently it would be interesting to examine the co-localisation of p150 with other subcellular organelles such as secretory granules, clathrin coated transport vesicles or the early endosome.

The early endosomal antigen (EEA) 1, is a 170 kDa protein which has amino acid homology to yeast proteins that have been genetically implicated in membrane trafficking such as Vps27p (Patki et al., 1997; Piper et al., 1995). The precise function of EEA 1 in mammalian cells is currently unknown, however, experiments using wortmannin have been shown to cause a pronounced dissociation of EEA 1 from peripheral endosomes in intact cells. EEA 1 binds specifically to liposomes containing PtdIns3P in vitro, suggesting that the effect of wortmannin on EEA 1 is due to an inhibition of PtdIns3P production (Patki et al., 1997). Data from these studies therefore, suggest that EEA 1 is regulated by an enzyme with PI 3 kinase activity such as PtdIns 3-kinase, which generates PtdIns 3P as its major product in vivo (section 6.2.2), although the possible role of other PtdIns-specific PI 3-kinases (Stephens et al., 1994a) cannot not be ruled out.

The range of available markers for subcellular organelles did not allow the identification of a subcellular structure to which p150 is localised. It is clear that p150 does not reside on Golgi or late endosomal/lysosomal compartments, nevertheless, as described above, a number of other trafficking pathways and vesicle intermediates exist within the cell. It remains possible that the punctate staining pattern and insolubility observed for p150 is due to its oligomerisation within the cytoplasm of the cell, however, its co-localisation with the intermediate filament vimentin, suggests p150 is associated with a specific cytoskeletal component. If p150 is localised in a specialised vesicle associated with intermediate-filaments, the identification of the nature and role of this vesicle would aid in elucidating the biological function of the p150/PtdIns 3-kinase complex. To examine the subcellular localisation of p150/PtdIns 3-kinase more closely, immunoelectron-microscopical studies utilising immunogold labelling techniques could be used to investigate whether transiently expressed p150/PtdIns 3-kinase is in fact localised to vesicle structures in COS-7 cells.

A

Mg^{2+} Mn^{2+}

1   2   3   4

PtdIns3P →

B

1   2

kDa

112 —   ← PtdIns 3-K

86 —

70 —

57 —
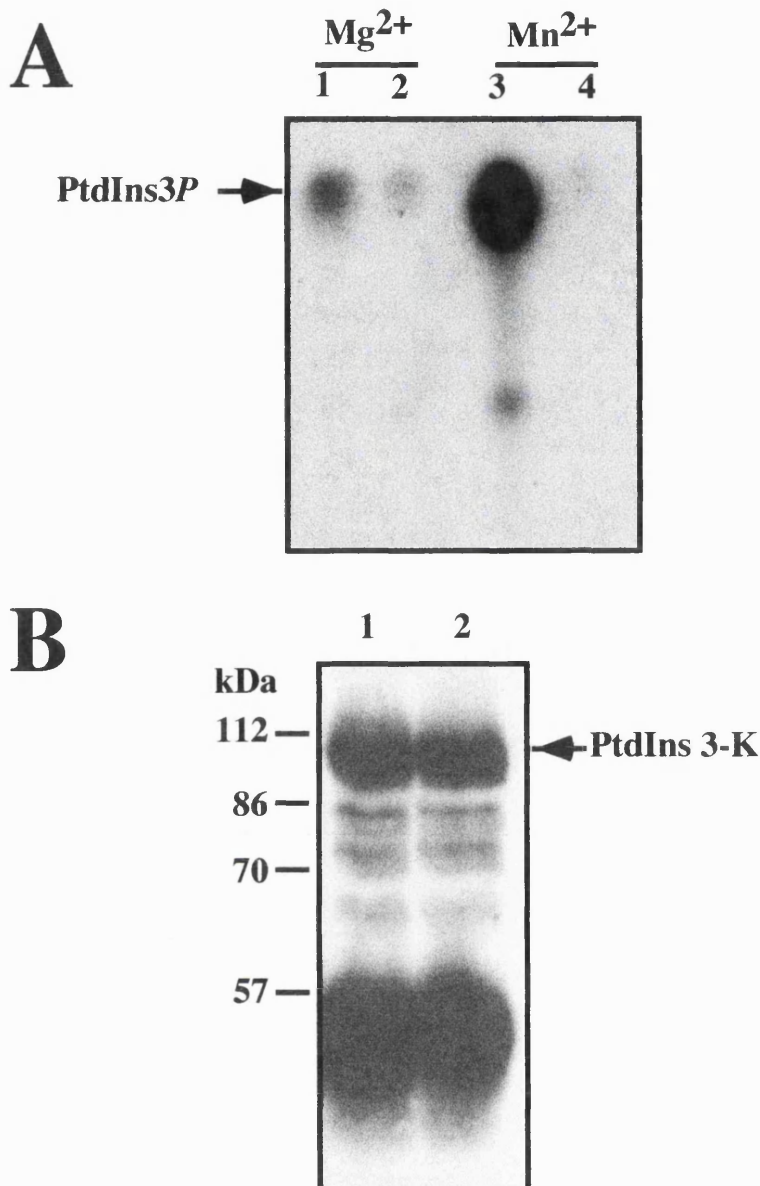
Figure 7.19 Mutation of two amino acids in the lipid kinase domain of PtdIns 3-kinase (D^{743}A and N^{748}I) results in an enzyme with no lipid kinase activity.

COS-7 cells were transiently transfected to express wild type PtdIns 3-kinase or PtdIns 3-kinase (D^{743}A : N^{748}I) After 36 h, PtdIns 3-kinase was immuno-precipitated from cell lysates using anti-PtdIns 3-kinase antisera. Immunoprecipitated proteins were collected on protein A-Sepharose beads washed and used in PI 3-kinase assays (Panel A), or resolved by SDS-PAGE (Panel B).

A. Immunoprecipitates were subjected to *in vitro* PI 3-kinase assays in the presence of Mg^{2+} or Mn^{2+} using PtdIns as substrate on wild type PtdIns 3-kinase, (lanes 1 and 3), or PtdIns 3-kinase (D^{743}A:N^{748}I) (lanes 2 and 4).

B. Immunoprecipitates produced in parallel to those used in A were resolved by 7.5% SDS-PAGE. Wild type PtdIns 3-kinase (lane 1) and PtdIns 3-kinase (D^{743}A:N^{748}I) (lane 2) were detected by Western blotting using PtdIns 3-kinase specific antisera.
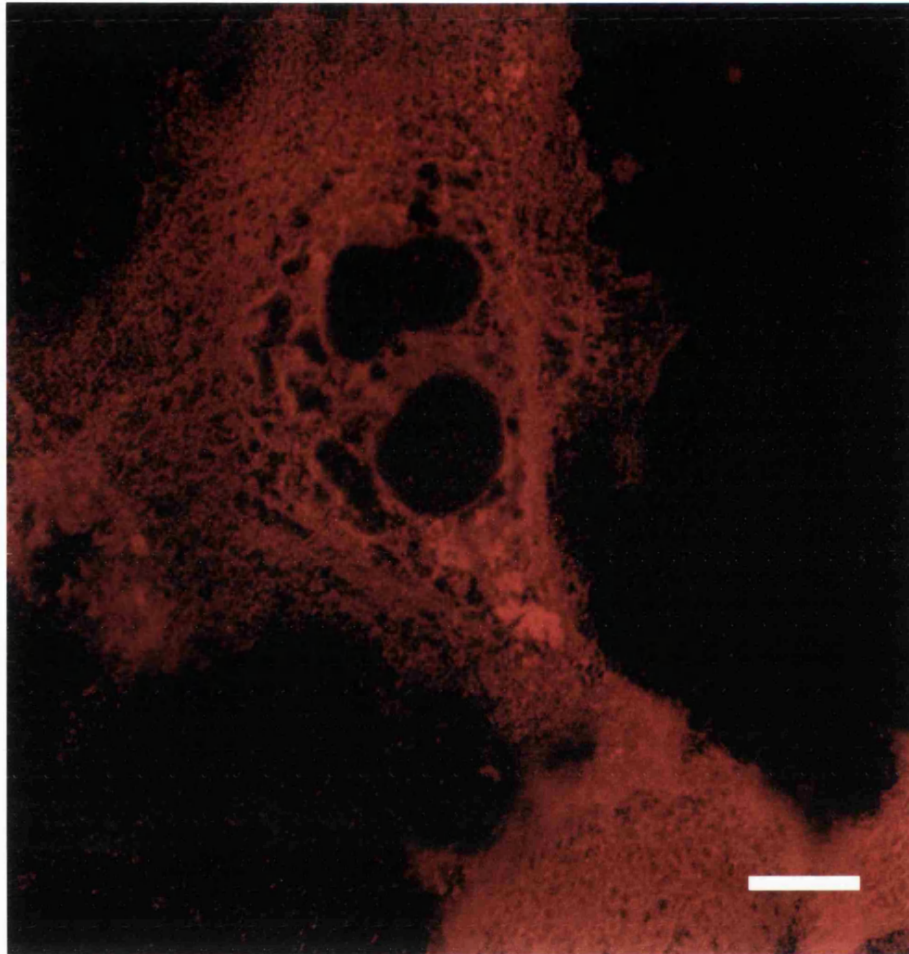
Figure 7.20
Immunolocalisation of PtdIns 3-kinase ($D^{743}A : N^{748}I$).

COS-7 cells were transfected to express PtdIns 3-kinase ($D^{743}A : N^{748}I$), a mutant that lacks lipid kinase activity. Transfected cells were seeded on glass coverslips and prepared by methanol fixation for immunostaining. PtdIns 3-kinase ($D^{743}A : N^{748}I$) was detected using PtdIns 3-kinase specific rabbit polyclonal antisera and visualised with anti-rabbit TRITC-conjugated secondary antibody. (Bar:10 μm)

Swollen vacuoles, containing lysosomal integral membrane proteins, have been observed in wortmannin treated NRK cells and are thought to be caused by the inhibition of a PI 3-kinase regulated membrane traffic event that occurs late in the endocytic pathway (Reaves *et al.*, 1996). The swollen compartments observed in wortmannin treated cells expressing PtdIns 3-kinase, or in untreated cells expressing a mutated PtdIns 3-kinase in which lipid kinase activity was attenuated (section 7.6.1) are as yet uncharacterised. Whether these structures are a consequence of an altered PtdIns 3-kinase activity in membrane trafficking or possibly reflect a role for PtdIns 3-kinase in osmoregulation (Dove *et al.*, 1997) is at present unclear.

Experiments using the PI 3-kinase inhibitors wortmannin and LY294002 have implicated a role for PI 3-kinase activity in membrane trafficking (Shepherd *et al.*, 1996). Many of these investigations have alluded to the human Vps34p homologue, PtdIns 3-kinase, as being one of the most likely candidates for a lipid kinase activity involved in vesicle transport events (particularly form the TGN). Recent data, however, has suggested that other PI 3-kinases might also play a role in such intracellular membrane trafficking events.

A PI 3-kinase activity has been found associated with TGN46, the human homologue of the rat protein TGN38. TGN46 is an integral membrane glycoprotein that cycles between the TGN and the cell surface and is involved in the recruitment of cytosolic factors which function in the regulation of at least one type of vesicle formation at the mammalian TGN (Hickinson *et al.*, 1997). Immunoprecipitates from HeLa and U937 cells using anti-TGN46 polyclonal antisera, identified a lipid kinase activity with the TGN46 complex. Further analysis showed that PtdIns3P was the lipid produced by this TGN46-associated lipid kinase and that like human PtdIns 3-kinase, it was also inhibited by low levels of wortmannin (IC_{50} value: 2.5 nM). However, unlike human PtdIns 3-kinase, which has a cation requirement for Mn^{2+}, the TGN46-associated lipid kinase requires Mg^{2+}. In addition, using [^{3}H]wortmannin, the TGN46-associated lipid kinase activity has been attributed to a 250 kDa protein (Hickinson *et al.*, 1997). This data suggests that a 250 kDa PtdIns 3-kinase, instead of the 100 kDa human Vps34p homologue, is likely to be the lipid kinase activity responsible for regulating exocytic vesicle traffic events from the TGN. In the absence of any amino acid sequence data, this 250 kDa PtdIns 3-kinase could be a member of a totally new class of PI 3-kinase, although the class II PI 3-kinases are similar in terms of their high molecular mass (section 1.3.2). Similarly, it is tempting to suggest that other large proteins such as the ~245 kDa FRAP/RAFT molecules which have putative lipid kinase domains (Brown *et al.*, 1994; Sabatini *et al.*, 1994) might also represent possible candidates for this 250 kDa TGN46-associated PtdIns 3-kinase.

In contrast, the rat protein TGN38 has been shown to associate with p62^{cplx} a 85 kDa complex, composed of a 62 kDa protein (p62) and Rab6 a 25 kDa small GTP-binding protein. The recruitment of p62^{cplx} to TGN38 is required for the formation of plasma membrane-directed exocytic transport vesicles (Jones et al., 1993). Amino acid sequence data for p62, identified a 24 amino acid peptide which was identical to amino acids 82-105 found within the BCR domain of the class I$_A$ PI 3-kinase regulatory subunit p85α (Jones and Howell, 1997). The BCR domain has been suggested to bind GTPases and is found in many GTPase activating proteins (Diekmann et al., 1991). The amino acid sequence identified derived from p62 suggests that it might be a p85α-like protein, however, considering the homology of the p62 peptide with the BCR domain and the observation that p62 is found associated with the GTPase Rab6, the possibility that p62 might be a GTPase activating protein should not be precluded.

A lipid kinase activity has been found associated with p62^{cplx}, which has a cation preference for Mn^{2+}, a substrate specificity for PtdIns and is sensitive to high concentrations of wortmannin (IC$_{50}$ value: 3.5 μM) (Jones et al., 1998b). Furthermore, the PtdIns 3-kinase identified in this study was shown to act synergistically with PI-TP in the formation of constitutive transport vesicles from the TGN (Jones et al., 1998b). This TGN38/p62^{cplx}-associated PtdIns 3-kinase was attributed to a 100 kDa protein, which contrasts with the 250 kDa lipid kinase activity associated with TGN46 (Hickinson et al., 1997). Intriguingly, even though p62 appears to have homology with p85α, the 100 kDa (p100) PtdIns 3-kinase does not appear to be the p110α, β, or δ catalytic subunits which are known to associate with p85α and its truncated variants. Therefore, although p100 has several biochemical characteristics in common with human PtdIns 3-kinase, its sensitivity to wortmannin (IC$_{50}$: 3.5 μM) is more reminiscent of a mammalian PtdIns 3-kinase activity that has been identified but not fully characterised (Stephens et al., 1994a). Furthermore, PtdIns 3-kinase (the human Vps34p homologue) has been found associated in vivo with the 150 kDa Vps15p homologue p150 and not a 62 kDa protein displaying homology to the BCR domain of p85α.

Although TGN38 and TGN46 are highly homologous, the studies mentioned previously, have shown that potentially different PtdIns 3-kinases have been found associated with these proteins. Despite the fact that the TGN38 and TGN46 associated PtdIns 3-kinase activities have been assigned to 100 kDa and 250 kDa proteins respectively, one common feature is that both these lipid kinase activities have a substrate specificity for PtdIns. This suggests that PtdIns3P plays a role in vesicle biogenesis and budding. As yet, it is not clear which PtdIns 3-kinase is involved in the TGN38/46 associated formation of exocytic vesicles. Nevertheless, the apparent requirement of PtdIns3P in the formation of secretory or constitutive transport mechanisms, still supports a role for PtdIns 3-kinases,

including the human Vps34p homologue, PtdIns 3-kinase and its associated protein, p150 in vesicle mediated events.

7.7 Mutational analysis of p150

7.7.1 Introduction

The observation that p150(EE) transiently expressed in COS-7 localises to perinuclear puncate structures, co-localises with PtdIns 3-kinase and vimentin, and forms an insoluble complex, raised questions as to which regions of the p150 protein were responsible for these events. To examine this experimentally, the systematic deletion of defined protein domains and mutagenesis of essential amino acids required for i) putative serine threonine protein kinase activity and ii) N-terminal myristoylation was undertaken.

7.8 Results

7.8.1 Generation of p150 deletion constructs

The amino acid sequence of human p150 contains four main areas of co-linear homology with yeast Vps15p (section 3.2.3). Homology region (HR) 1 (amino acids 1-10) contains a consensus sequence for the attachment of myristic acid. HR 2 (amino acids 11-300) is a putative ser/thr protein kinase domain (PKD), HR 3 (amino acids 400-700) contains repeating units found in a number of proteins known as HEAT repeats and finally, HR 4 (amino acids 1000-1350) which contains WD-40 repeats. In addition to these four homology domains, a further two regions have been assigned in work presented in this chapter (figure 7.21). The region between the protein kinase domain and the HEAT repeats (amino acids ~300-400), was designated as the 'inter- protein kinase domain-HEAT' region (iPKD-Ht), while the region between the HEAT repeats and the WD-40 domain (amino acids ~700-1000), was designated as the 'inter-HEAT-WD-40' region (iHt-WD). To generate constructs in which these domains have been sequentially deleted, a PCR based approach was employed in which convenient restriction sites in the p150(EE) ORF were used. The ORF from the pSKp150(EE) construct was used as a template for PCR. Figure 7.21 (panel 1) is a schematic representation of the p150(EE) ORF (as a Not I-Not I cassette) indicating the position of the restriction sites and primers (1-8) used to generate the deletion constructs. Panel 2 illustrates the protein domain regions that the PCR primers will generate. The p150(EE) deletion (Δ) and site-directed mutagenised constructs were made using the following methods:
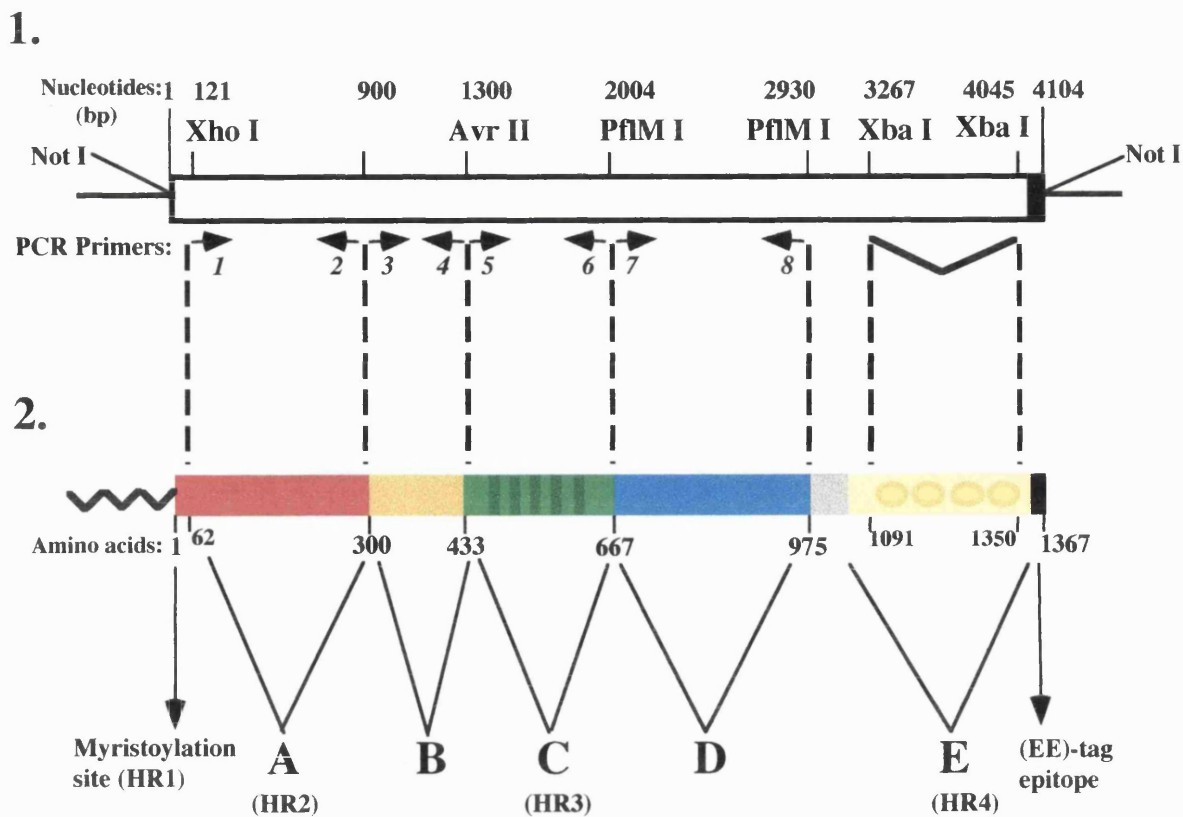
Figure 7.21 Schematic representation of the p150 cDNA open reading frame and the p150 protein domain structure.

1. Schematic representation of the p150(EE) open reading frame as a Not I-Not I cassette of 4104bp. PCR primers and restriction sites used to make the p150(EE) deletion constructs are shown.

2. Schematic representation of the p150(EE) protein domain structure:

   Domains: A. Protein kinase domain (PKD).
             B. Inter-Protein kinase-HEAT region (iPKD-Ht).
             C. HEAT domain.
             D. Inter-HEAT-WD-40 region (iHt-WD).
             E. WD-40 domain

7.8.1.1 HR 1 site directed mutagenesis of the p150 myristic acid attachment site (Myr⁻):

The N-terminal sequence of p150, $(M)^1G^2N^3Q^4L^5G^6L^7$, is highly homologous to the proposed consensus sequence that signals myristic acid addition (section 3.2.3.1). Experiments using [^{3}H]myristic acid (section 5.2.1) showed that p150 expressed in Sf9 cells is N-terminally myristoylated. To prevent N-terminal myristoylation of p150, the critical glycine residue at position 2 was changed to an alanine (codon GGA changed to GCC) and an asparagine residue at position 3 changed to aspartate (codon AAT changed to GAC). These amino acid substitutions were generated using a PCR procedure (described in section 5.2.1) resulting in the pVL-Myr⁻/p150(EE) construct. Biochemically, this construct has been shown to express p150 protein (in Sf9 cells) that has no myristic acid modification (section 5.2.1).

7.8.1.2 HR 2 site directed mutagenesis of the putative p150 ser/thr protein kinase domain (Kin⁻):

The N-terminal regions of both p150 and Vps15p contain extensive amino acid homology with the ser/thr protein kinase superfamily (section 3.2.3.2). Both proteins contain the amino acid motifs, DFA which is implicated in ATP binding (Brenner, 1987) and APE, a conserved sequence element often referred to as an indicator of protein catalytic domains (Hanks and Quinn, 1991; Hunter, 1991). Two amino acid substitutions in the Vps15p protein kinase domain ($D^{165}FA$ to RFA and APE^{200} to APR) have been demonstrated to be sufficient in abolishing any Vps15p autophosphorylation activity (Herman *et al.*, 1991a). No p150 autophosphorylation has been detected and no substrates for p150 kinase activity have yet been identified (section 5.2.2.2). Nevertheless, residues in p150 that are analogous to those shown to abolish Vps15p protein kinase activity were changed ($D^{166}FA$ to RFA, codon change: GAT to AGA and APE^{200} to APR, codon change: GCT to AGA). It is possible that the mutations used to abolish Vps15p protein kinase activity may also abolish any protein kinase activty p150 might have *in vivo*. In order to introduce these two amino acid substitutions, pSKp150(EE) was used as template with PCR primer pair 1 (figure 7.22B) and the QuikChange™ site-directed mutagenesis kit procedure (Stratagene, section 2.1.1.6). This generated a construct in which D^{166} had been changed to R, the mutation was confirmed by DNA sequencing. Using the p150(EE)D^{166}R construct and primer pair 2 (figure 7.22 panel B), the second mutation, E^{200} to R was introduced. The construct containing the double mutation, pSKp150-Kin⁻ was verified by DNA sequencing.

7.8.1.3 HR 2 protein kinase domain deletion (ΔPKD):

Using primers 3 and 4 (figures 7.21 and 7.22) and the p150(EE) ORF as template, a 400 bp fragment was amplified by PCR that corresponded to nucleotides 900-1300 of p150(EE).

A. PCR primers used to make p150(EE) deletion mutants.

Primers 1-8 (5' → 3')	Restriction site (underlined).
1. CCGG<u>CTCGA</u>GCCAAGCACCGAGAAGGCCTGG	*Xho* I
2. CATCG<u>CCTAGG</u>TTTATCTGGCTGACGGTGAAT	*Avr* II
3. CCG<u>CTCGA</u>GGTTTAGAGGCAGAAGATTAC	*Xho* I
4. CCTCAC<u>CCTAGG</u>AACAGAGTCATTGG	*Avr* II
5. TCTGTT<u>CCTAGG</u>GTGAGGGCTGAAGC	*Avr* II
6. GGAG<u>CCAGGTGGTGG</u>ATGACACAGGAAGG	*PflM* I
7. TACGAA<u>CCTAGG</u>TTATGGATACGTTATGGTGC	*Avr* II
8. ACGCCAT<u>CCAGGTGGTGG</u>TGGTTTACTC	*PflM* I

B. Primers used for site directed mutagenesis to make p150(EE) Kin⁻ (D^{166}R and E^{200}R)

Primer pair:	Primers (5' → 3')	Codon change
1.	TTCTTCTAACTAGATTTGCCAGTTTT AAACTGGCAAATCTAGTTAGAAGAAC	GAT→ AGA, D^{166}→ R
2.	TATATTGCTCCTAGACGTTTTGTTGAT ATCAACAAAACGTCTAGGAGCAATATA	GCT→ AGA, E^{200}→ R

Figure 7.22 PCR primers used to make p150(EE) deletion and Kin⁻ mutants.

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This fragment was digested with *Xho* I and *Avr* II and ligated into a similarly digested pSKp150(EE) construct. This generated the pSKΔPKD construct, which could be digested with *Not* I to give a fragment containing the p150(EE) ORF, but lacking nucleotides 121-900, which encode most of the putative ser/thr protein kinase domain (the generation of this and the following constructs are summarised in figures 7.23 and 7.24).

7.8.1.4 The 'inter-protein kinase domain-Heat repeat' deletion

(ΔiPKD-Ht): Using primers 1 and 2 (figures 7.21 and 7.22), nucleotides 121-900 of the p150(EE) ORF were used as template to generate a 779bp PCR fragment. This PCR fragment was digested with Xho I and *Avr* II and ligated into the pSKp150(EE) construct which was also digested with the same enzymes. This procedure generated pSKΔiPKD-Ht, a construct containing a the p150(EE) ORF as a *Not* I-*Not* I cassette in which nucleotides 900-1300 encoding the region between the putative protein kinase domain and HEAT repeats were deleted.

7.8.1.5 HR 3 HEAT repeat deletion (ΔHEAT): Using primers 7 and 8

(figures 7.21 and 7.22), nucleotides 2004-2930 of the p150(EE) ORF are used as template to generate a 926bp PCR fragment which was digested with *Avr* II and *PflM* I and ligated into a similarly digested pSKp150(EE) construct. This generated the pSKΔHEAT construct which contained the p150(EE) ORF as a *Not* I-*Not* I cassette in which nucleotides 1300-2004 encoding the HEAT domain was deleted.

7.8.1.6 The 'inter-HEAT-WD-40 domain deletion (ΔiHt-WD):

Using primers 5 and 6 (figures 7.21 and 7.22), nucleotides 1300-2004 of the p150(EE) ORF were used as template to generate a 704bp PCR fragment which was digested with *Avr* II and *PflM* I and then ligated into a similarly digested pSKp150(EE) construct. This generated pSKΔiHt-WD, a construct in which the p150(EE) ORF was a *Not* I-*Not* I cassette in which nucleotides 2004-2930 encoding the domain between the HEAT and WD-40 repeats were deleted.

7.8.1.7 HR 4 WD-40 domain deletion (ΔWD): To make this construct,

pSKp150(EE) was digested with the restriction enzyme *Xba* I. This removed a 778bp region (nucleotides 3267-4045, figure 7.21) which encoded the four WD-40 repeats. The *Xba* I restriction sites were filled in with Klenow polymerase (section 2.1.1.4) and the construct re-ligated so that the remaining coding region would still be in frame. This generated the pSKΔWD construct containing the p150(EE) ORF as a *Not* I-*Not* I cassette in which the region encoding the four p150 WD-40 repeats had been removed.
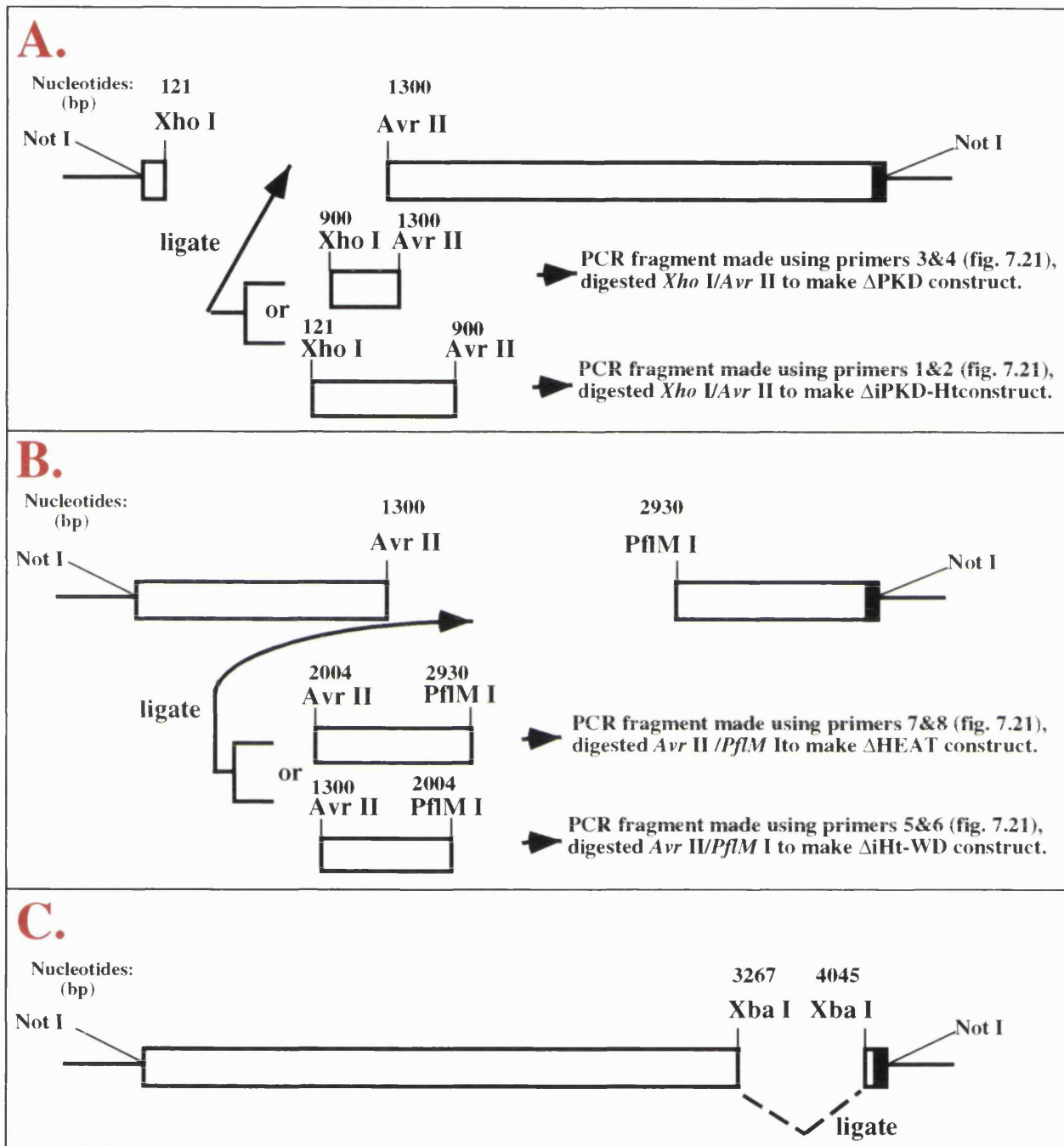
Figure 7.23 Generation of p150(EE) deletion mutants.

A. The construct pSK-p150(EE) was digested with *Xho* I and *Avr* II to remove nucleotides 121-1300. Primer pairs 3&4 or 1&2 were used to generate PCR fragments which were ligated into the digested pSK-p150(EE) construct to make ΔPKD and ΔiPKD-Ht constructs respectively.

B. The construct pSK-p150(EE) was digested with *Avr* II and *PflM* I to remove nucleotides 1300-2930. Primer pairs 7&8 or 5&6 were used to generate PCR fragments which were ligated into the digested pSK-p150(EE) construct to make ΔHEAT and ΔiHt-WD constructs respectively.

C. The construct pSK-p150(EE) was digested with *Xba* I to remove nucleotides 3267-4045. Restriction site were filled in using Klenow and re-ligated to generate the ΔWD-40 construct.

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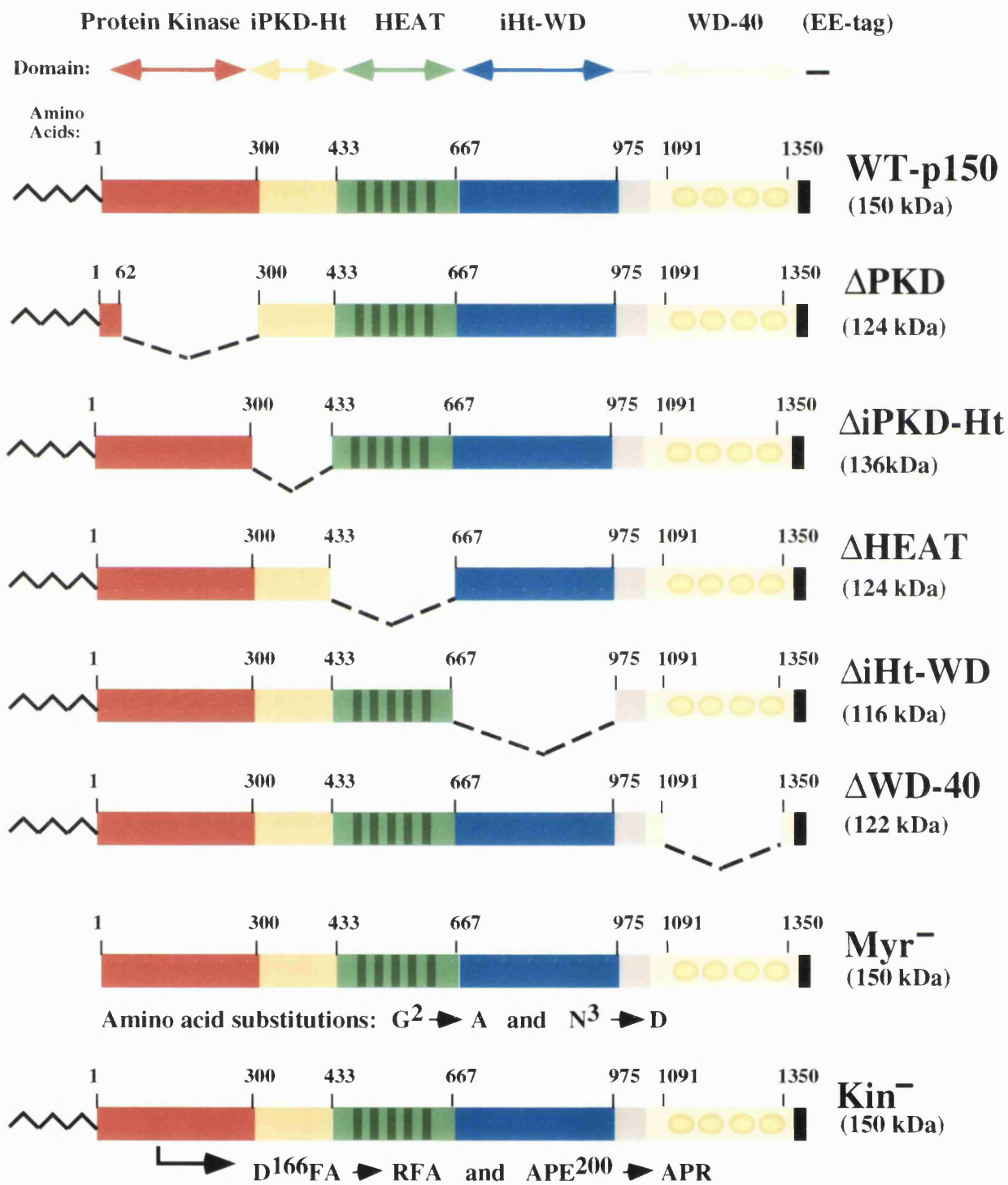
Figure 7.24 Summary diagram of p150 deletion mutants generated.

7.8.1.8 Verification, subcloning and expression of p150(EE) mutants

The nucleotide sequences of the domain deletion and mutagenesis constructs described above were verified by DNA sequencing (section 2.1.4). Each construct generated, retained the EE-epitope tag at the C-terminus and is represented schematically in figure 7.24. Each mutant was then subcloned into expression vectors for expression in Sf9 cells or COS-7 cells. The pSK-mutant constructs were digested with *Not* I to obtain the ORF and ligated into *Not* I digested pMT2SM or pVL1393 vectors. The pMT2- constructs were transiently transfected into COS-7 cells whereas the pVL-constructs were co-transfected into Sf9 cells with baculovirus genomic DNA (AcMNPV), where they undergo homologous recombination to generate baculoviruses which can be used to express p150(EE) mutants in Sf9 cells.

7.8.2 Subcellular localisation of p150(EE) mutants

To verify protein expression from the pMT2-p150(EE) domain deletion and mutagenesis constructs, 5μg of each construct [pMT2-Myr⁻, pMT2-Kin⁻, pMT2-ΔPKD, pMT2-iΔPKD-Ht, pMT2-ΔHEAT, pMT2-ΔiHt-WD and pMT2-ΔWD) including wild type pMT2-p150(EE) {WT-pMT2-p150(EE)}] were transiently transfected into COS-7 cells. After 36 h, the cells were harvested, lysed in EB and centrifuged to produce supernatant (S) and pellet (P) fractions which were separated by SDS-PAGE, transferred to PVDF and Western blotted using EEmAb. Proteins of the predicted molecular mass (see figure 7.24) were expressed from each transiently transfected construct (figure 7.25). The results also demonstrated that neither the domains deleted nor the amino acid changes (abolishing N-terminal myristoylation or any putative protein kinase activity) had any effect on the solubility of p150, since all the mutated constructs expressed protein which appeared in the Triton X-100 insoluble pellet.

The subcellular localisation of the p150(EE) mutants transiently expressed in COS-7 cells were also examined using immunofluorescence (section 2.6). Compared to wild type p150(EE) (figure 7.26A), there was no significant change in subcellular distribution shown by any of the p150(EE) mutants, as they all appeared to have a punctate perinulear localisation (figure 7.26 panels C, E, G, I, K, M and O). To investigate whether any of the domains of p150 were responsible for the disruption of vimentin intermediate filaments, the same cells were double labelled with an antibody specific for vimentin (figure 7.26 panels B, D, F, H, J, L, N and P). Each p150(EE) mutant except one, appeared to have the ability to disrupt vimentin filaments in a way comparable to the wild type p150(EE) protein (figure 7.26B). The p150(EE) mutant ΔiHt-WD (figure 7.26J), however, had an apparently normal perinuclear filamentous vimentin structure, indicating that the ΔiHt-WD p150 protein was unable to disrupt vimentin cytoskeletal organisation.

2 1 6
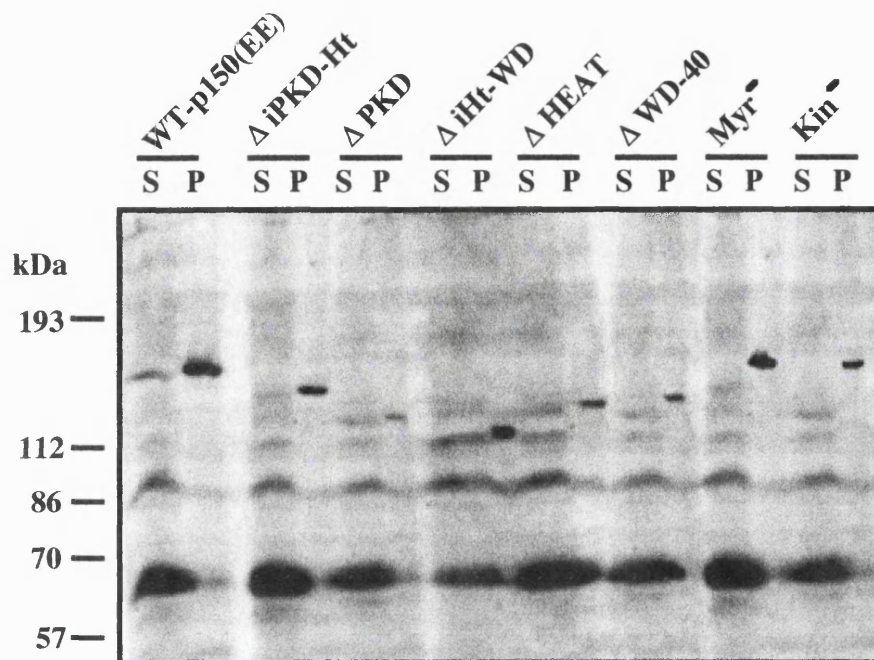
Figure 7.25 Expression of p150(EE) mutants in COS-7 cells
COS-7 cells were transiently transfected to express wild type p150(EE)
or the p150(EE) mutants:

ΔiPKD-Ht, ΔPKD, ΔiHt-WD, ΔHEAT, ΔWD-40, Myr⁻ and Kin⁻.
After 36 h cells were lysed in EB and centrifuged at 14,000x*g* to generate
supernatant (S) and pellet (P) fractions which were resolved
using 7.5% SDS-PAGE. p150(EE) mutants were detected by Western
blotting, using EEmAb.
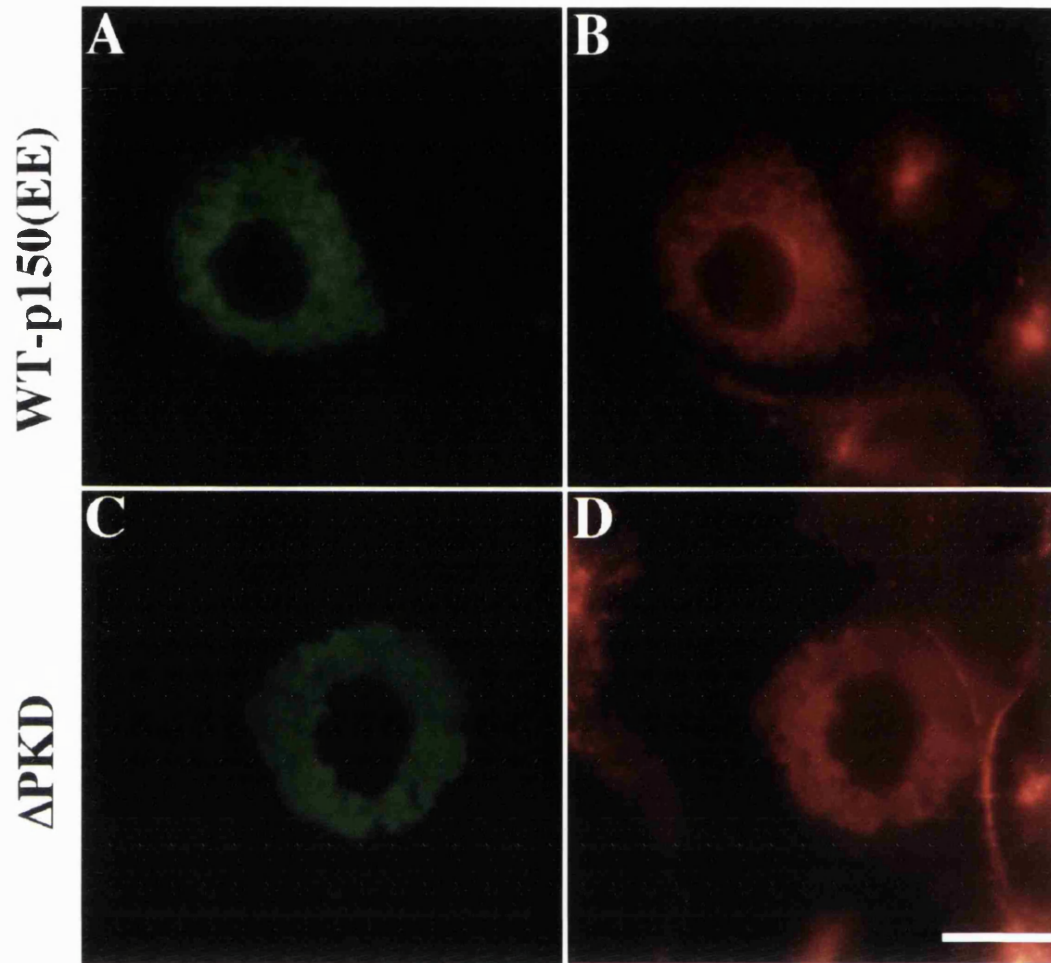
Figure 7.26

Immunolocalisation of p150(EE) mutants and vimentin in COS-7 cells.

COS-7 cells were transfected to express wild type p150(EE) (Panels A and B) or the following p150(EE) mutants: ΔPKD (Panels C and D), ΔiPKD-Ht (Panels E and F), ΔHEAT (Panels G and H), ΔiHt-WD (Panels I and J), ΔWD-40 (Panels K and L), Myr⁻ (Panels M and N) and Kin⁻ (Panels O and P). Cells were seeded on glass coverslips and prepared by methanol fixation for immunostaining. Cells were dual-labelled with EEmAb (IgG1) to detect wild type p150(EE) or mutants (Panels A, C, E, G, I, K, M and O), and vimentin in the same cells using vimentin specific mouse IgM monoclonal antibody (Panels B, D, F, H, J L, N and P). Bound antibodies were visualised using anti-mouse IgG1 FITC- and anti-mouse IgM TRITC-conjugated secondary antibody. (Bar:10 μm)

ΔiHt-WD   ΔHEAT   ΔiPKD-Ht

Kin- Myr- ΔWD-40
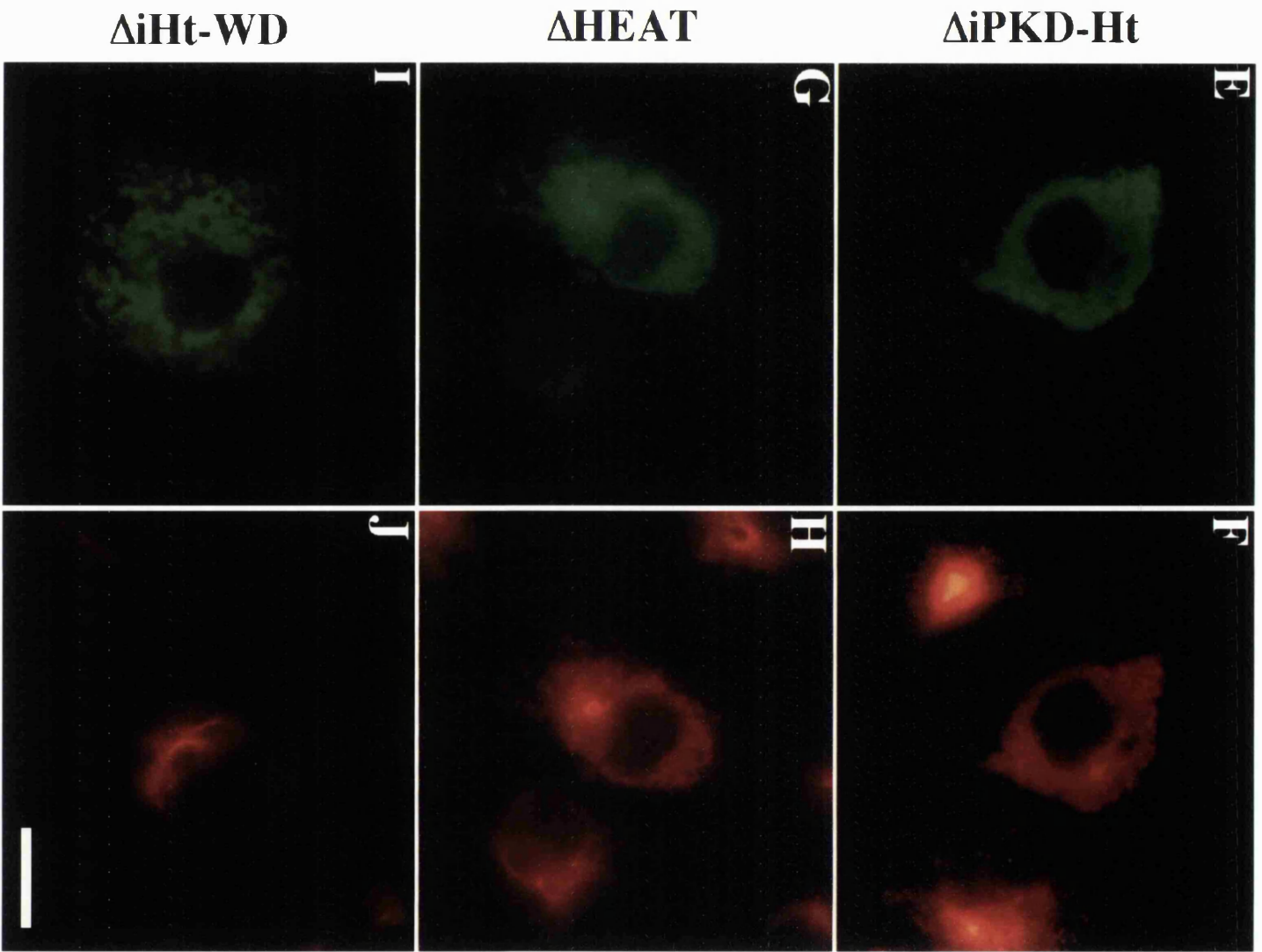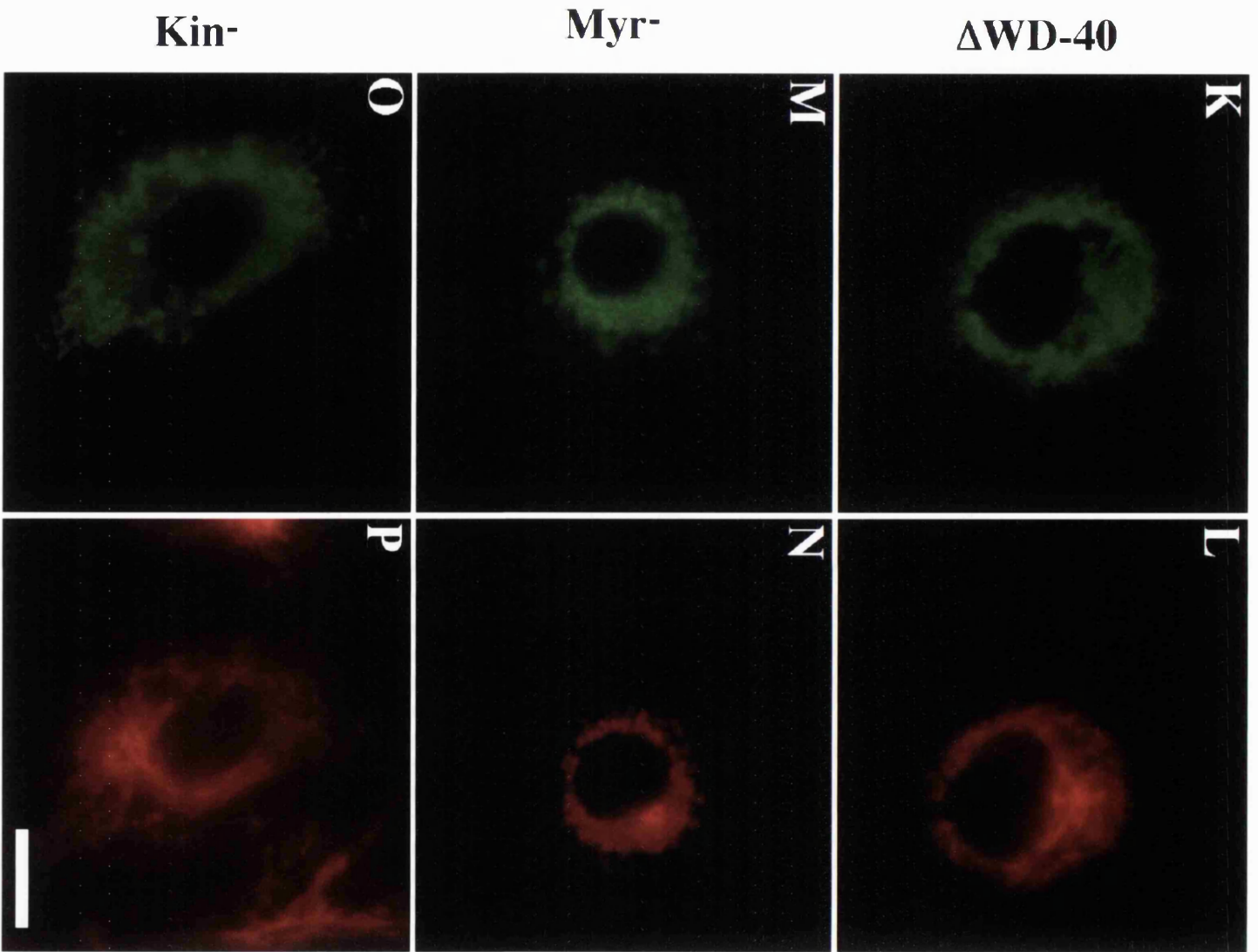
This suggested that the region located between the HEAT repeats and the WD-40 domain of p150 was primarily responsible for vimentin filament disruption. However, further experiments are required to ensure that this result is statistically meaningful.

7.8.3 Association of PtdIns 3-kinase with p150(EE) mutants

To investigate which regions of p150 are involved in binding PtdIns 3-kinase, co-infection studies were undertaken in Sf9 cells using baculoviruses expressing PtdIns 3-kinase and the truncated/amino acid mutated forms of p150 protein. To achieve this, the p150(EE) mutants were subcloned into the baculoviral transfer vector pVL1393 (as described in section 7.8.1.8). Each pVL-construct was then co-transfected into Sf9 cells with AcMNPV, baculoviral genomic DNA (section 2.2.2.2) to generate recombinant baculoviruses. Medium from co-transfected Sf9 cells was harvested six days post-infection and the recombinant viruses amplified several times to make virus stocks which were tested for protein production.

Recombinant baculoviruses that had been amplified three times were used to infect Sf9 cells to test the expression of each p150(EE) mutant. Sf9 cells were infected with varying amounts of recombinant baculovirus and harvested 2.5 days post-infection. Cells were lysed in EB and clarified supernatants incubated with EEmAb and protein G Sepharose beads. Immunoprecipitated proteins were separated using SDS-PAGE and visualised using Coomassie blue stain. Each recombinant baculovirus expressed a protein which corresponded to its predicted molecular mass (figures 7.27 and 7.24). The amount of recombinant baculovirus required for maximal protein expression was also assessed and a 1:500 dilution into media was found to be optimal (figure 7.27).

To investigate whether the p150(EE) mutants could associate with PtdIns 3-kinase *in vivo*, Sf9 cells were co-infected with baculovirus expressing PtdIns 3-kinase and each of the p150(EE) mutants. The 100 kDa PtdIns 3-kinase could be co-immunoprecipitated with each of the EE-tagged p150 mutants (figure 7.28), indicating that none of the domain deletions or amino acid mutations disrupted the interaction of p150(EE) with PtdIns 3-kinase. To examine whether this was still the case in an *in vivo* mammalian environment, COS-7 cells were co-transfected with pMT2-PtdIns 3-K and each of the pMT2-p150(EE) mutant constructs. The co-localisation of p150(EE) mutants and PtdIns 3-kinase was examined by immunofluorescence and confocal microscopy (figure 7.29). Areas of the cell where p150(EE) mutants and PtdIns 3-kinase are co-localised are represented in yellow (generated by the computerised merging of confocal images from FITC and TRITC dual labelled cells). WT-p150(EE) and PtdIns 3-kinase co-localised in COS-7 cells (figure 7.29A) as previously demonstrated (figure 7.3C). A high degree of co-localisation was also observed between PtdIns 3-kinase and most of the p150(EE)
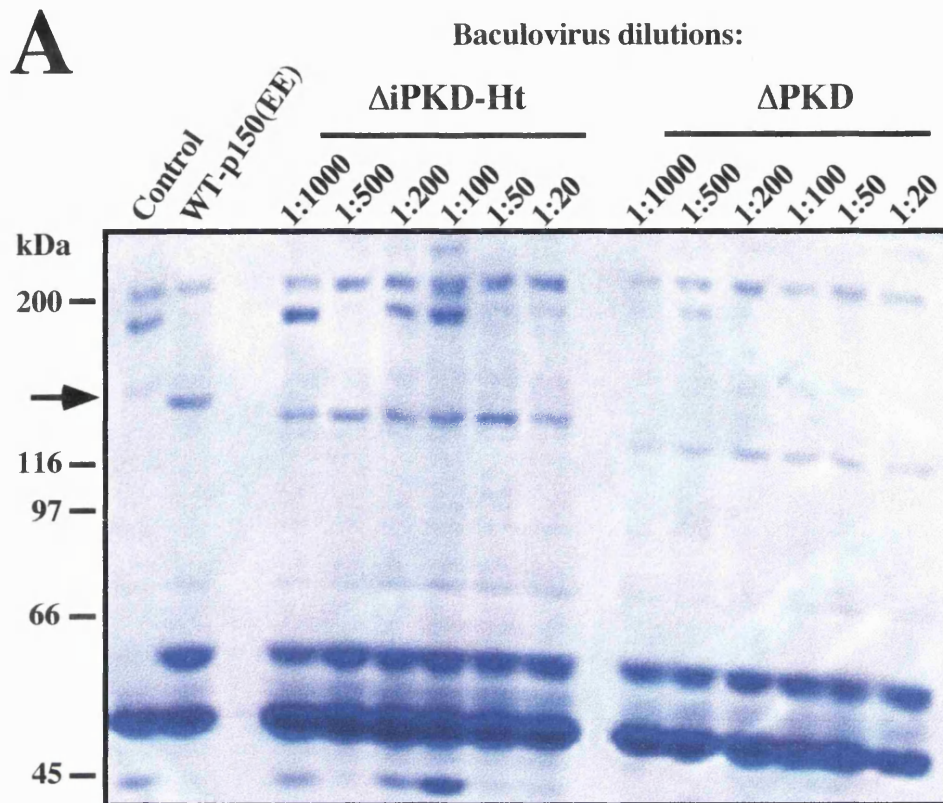
Figure 7.27 Expression of p150(EE) mutants in insect Sf9 cells.

Sf9 cells were infected with recombinant baculovirus (dilution range 1:20-1:1000).
60 h post-infection, cells were lysed in EB and EE-tagged proteins immunoprecipitated
EEmAb and collected on protein G-Sepharose. Immobilised proteins
were washed and analysed using SDS-PAGE.

A. Sf9 cells were infected with recombinant baculovirus to express WT-p150(EE),
or the mutants ΔiPKD-Ht and ΔPKD. Uninfected Sf9 cell lysate was also analysed
as a control. EE-tagged proteins were resolved by 7.5% SDS-PAGE and
visualised using Coomassie Blue stain. An arrow indicates the position of
WT-p150(EE) (150 kDa).
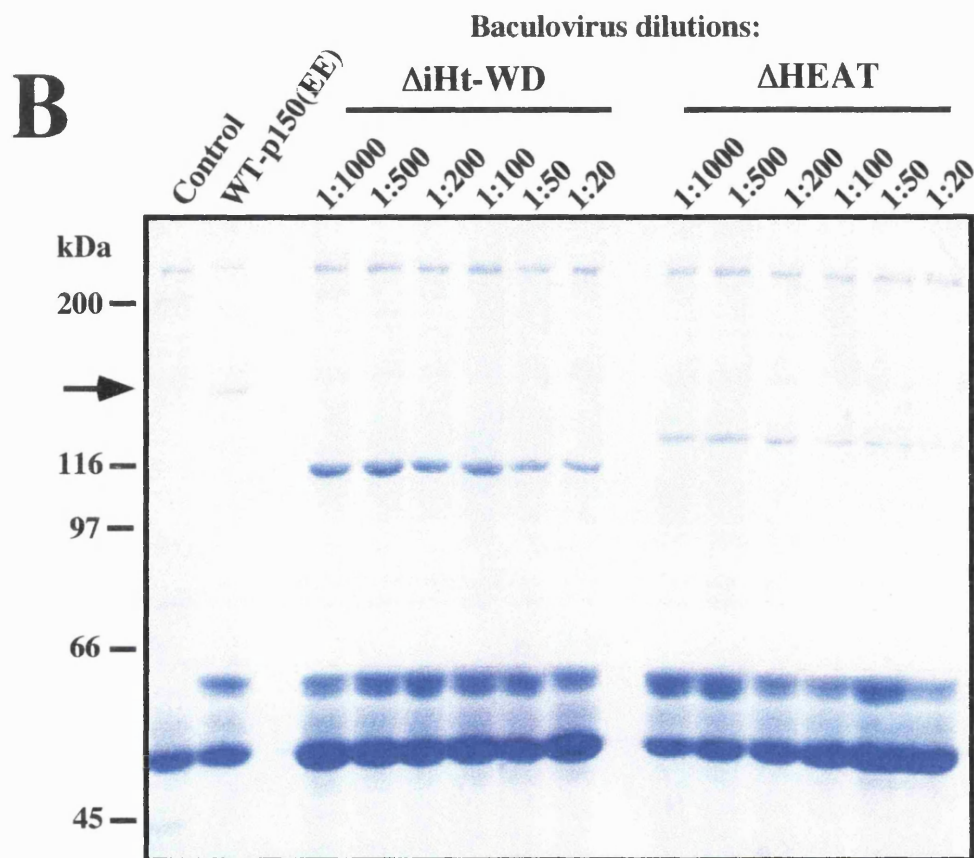
Figure 7.27 Expression of p150(EE) mutants in insect Sf9 cells.

B. Sf9 cells were infected with recombinant baculovirus to express WT-p150(EE), or the mutants ΔiHt-WD and ΔHEAT. Uninfected Sf9 cell lysate was also analysed as a control. EE-tagged proteins were resolved by 7.5% SDS-PAGE and visualised using Coomassie Blue stain. An arrow indicates the position of WT-p150(EE) (150 kDa).
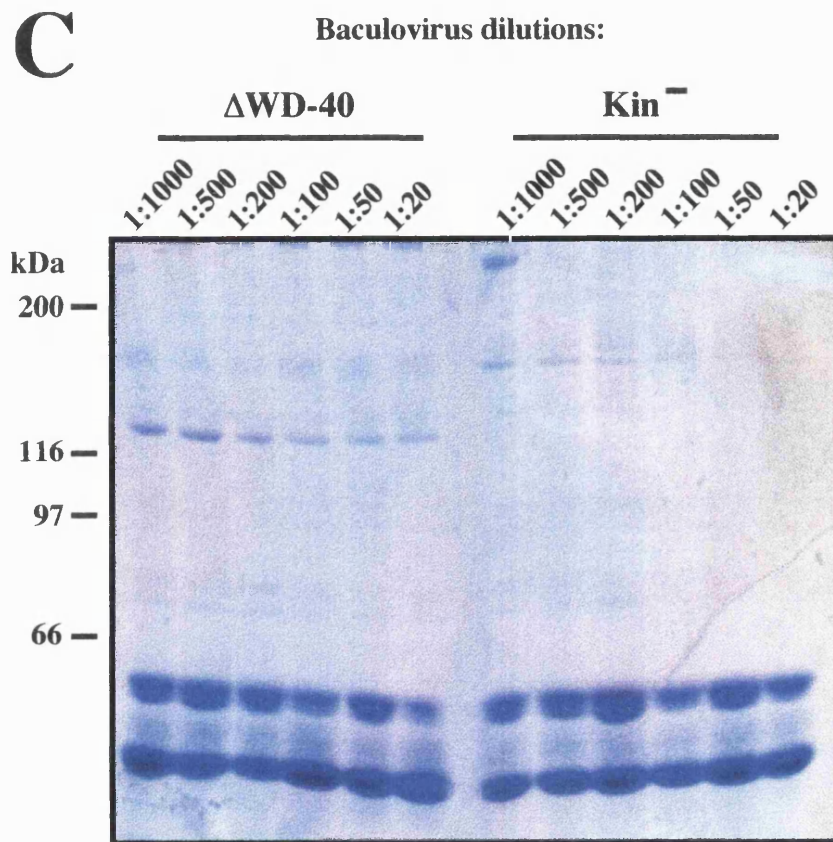
Figure 7.27 Expression of p150(EE) mutants in insect Sf9 cells.

C. Sf9 cells were infected with recombinant baculovirus to express the mutants ΔWD-40 and Kin⁻. EE-tagged proteins were resolved using 7.5% SDS-PAGE and visualised using Coomassie Blue stain.
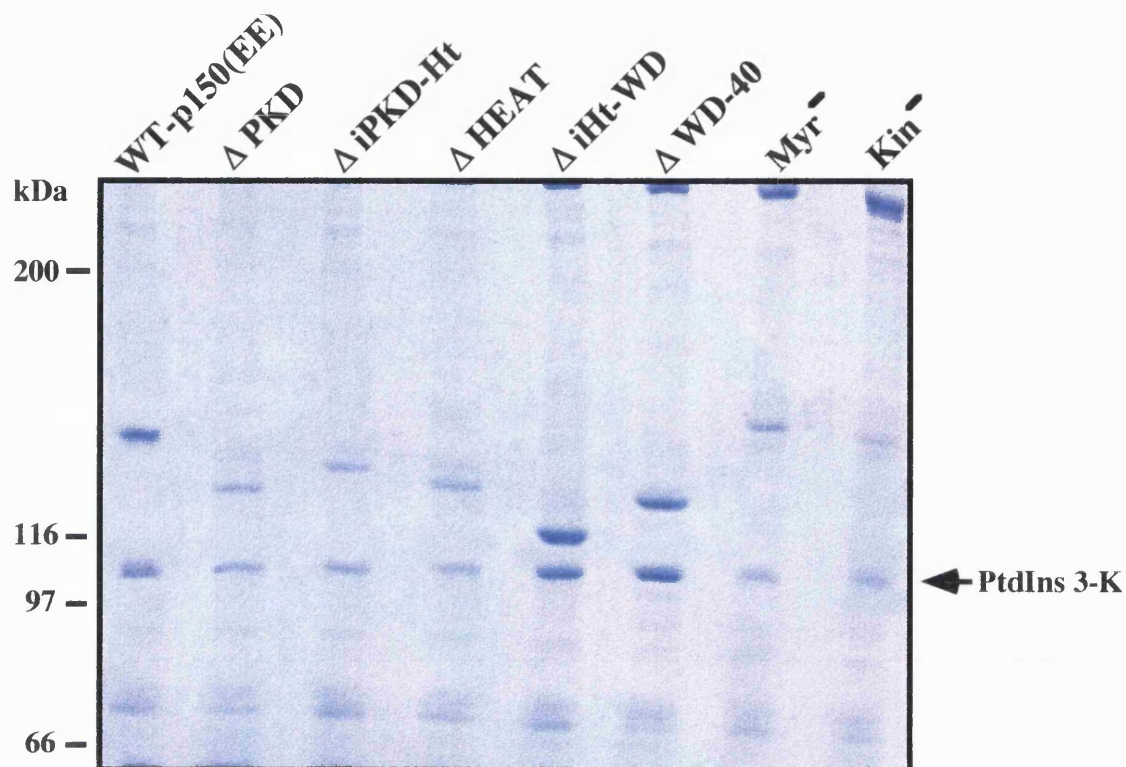
Figure 7.28 Association of p150(EE) mutants with PtdIns 3-kinase.

Sf9 cells were co-infected with recombinant baculoviruses to express PtdIns 3-kinase and WT-p150(EE) or the following p150(EE) mutants: ΔPKD, ΔiPKD-Ht, ΔHEAT, ΔiHt-WD, ΔWD-40, Myr⁻, and Kin⁻. After 60h, EE-tagged protein complexes were immunoprecipitated with EEmAb and collected on protein G-Sepharose beads. Immobilised proteins were washed and analysed by 6.0% SDS-PAGE and Coomassie Blue staining.
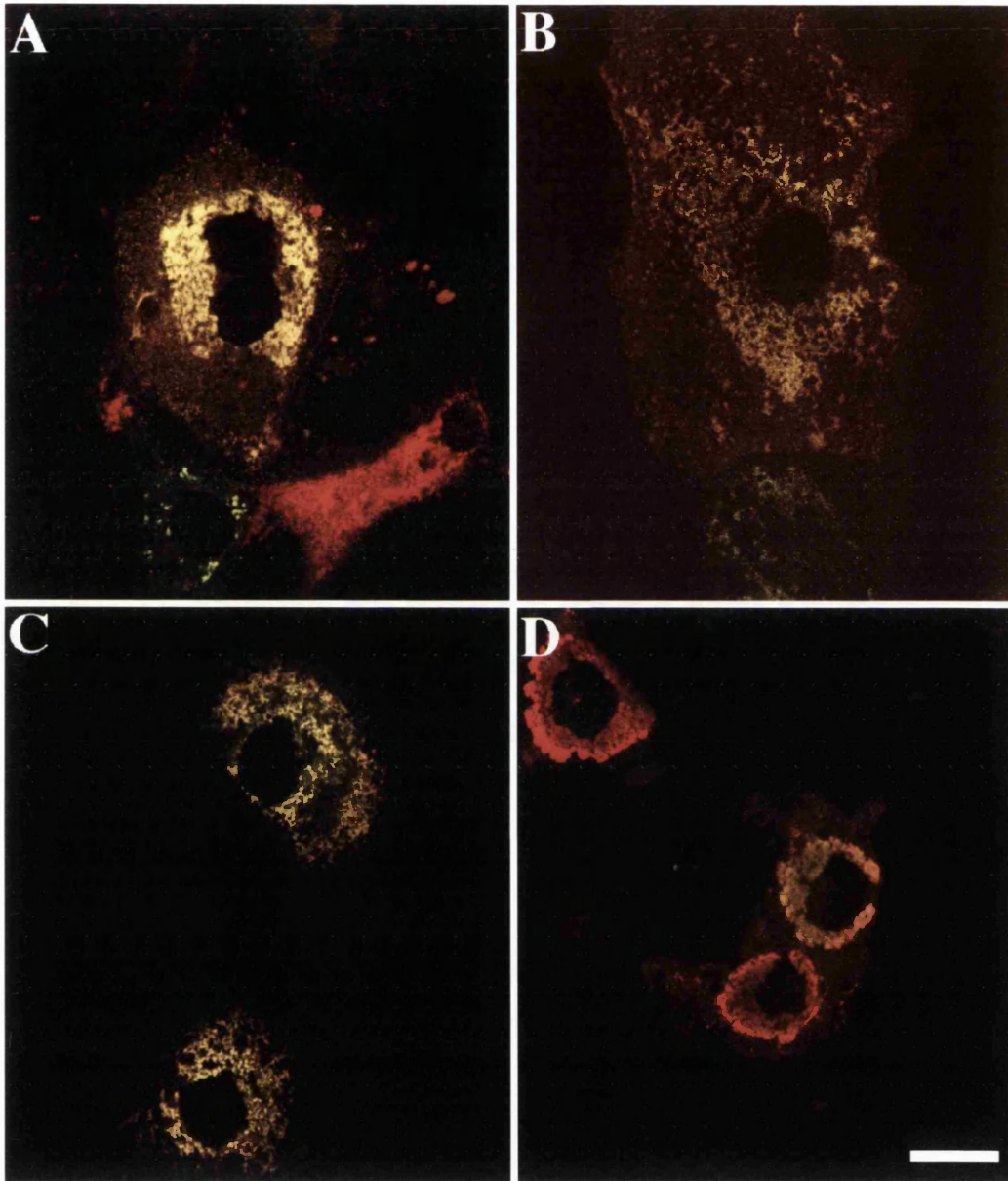
Figure 7.29

Co-localisation of p150(EE) mutants with PtdIns 3-kinase.

COS-7 cells were co-transfected with PtdIns 3-kinase and wild type p150(EE)
(Panel A), or PtdIns 3-kinase was co-transfected with the following p150 (EE)
mutants : ΔPKD (Panel B), ΔiPKD-Ht (Panel C), ΔHEAT (Panel D), ΔiHt-WD
(Panel E), ΔWD-40 (Panel F), Myr⁻ (Panel G) and Kin⁻ (Panel H). Cells were
seeded on glass coverslips and prepared by methanol fixation for immunostaining.
Cells were dual-labelled with EEmAb (IgG1) and PtdIns 3-kinase specific rabbit
polyclonal antisera. Bound antibodies were visualised using anti-mouse IgG1 FITC-
and anti-rabbit TRITC-conjugated second antibodies. (Bar:10 μm)
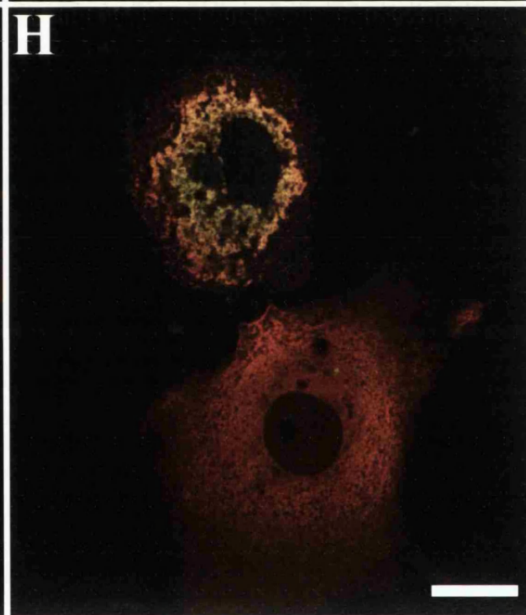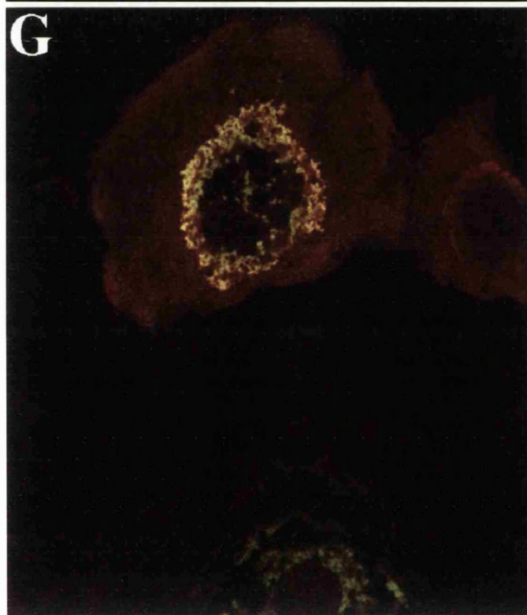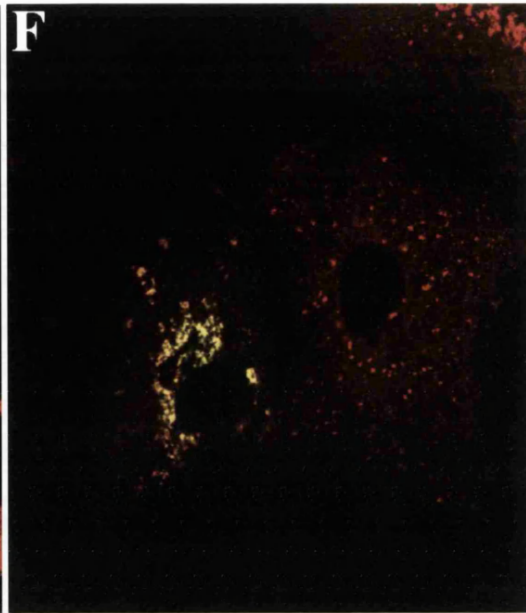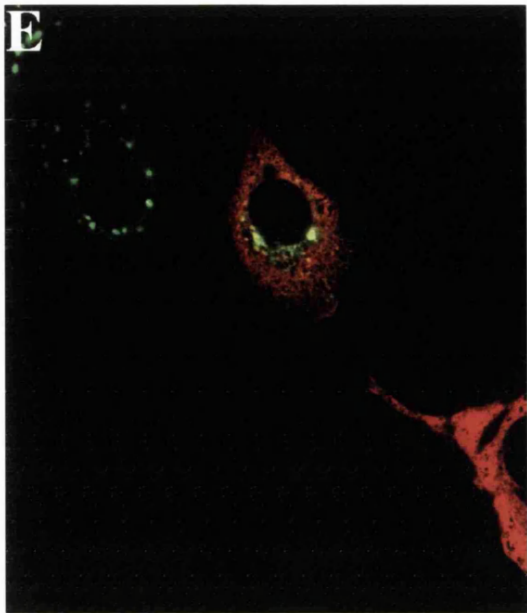
mutants (figure 7.29 panels B, C, F, G, and H), except for ΔHEAT and ΔiHt-WD where a lower level of yellow was observed. This suggests that ΔHEAT and ΔiHt-WD p150(EE) proteins might not associate with PtdIns 3-kinase as efficiently as WT-p150(EE) or the other p150(EE) mutants. Consequently, this may indicate that a region in p150, between the beginning of the HEAT repeats and the start of the WD-40 domain (amino acids 433-975, see figures 7.21 and 7.24) might serve as a binding site for PtdIns 3-kinase.

7.9 Discussion

The results described in section 7.8.2 (summarised in figure 7.30) show that none of the deletions or amino acid mutations made to p150(EE) dramatically affected its subcellular localisation. This however, does not preclude the possiblity that these mutants may in some way alter intracellular signalling mechanisms or the trafficking of certain membranous compartments which cannot be detected using the experimental techniques employed. To address this possibility, the use of reagents such as FITC-conjugated dextran which can be internalised by cells via fluid phase endocytosis or FITC-labelled transferrin which can be endocytosed by cell surface transferrin receptors, would demonstrate the effect of p150 mutants on endocytic events. Alternatively the possible mislocalisation of proteins such as cathepsin D due to the expression of p150 deletion mutants could also be examined.

Included in the panel of p150 mutants was one in which the N-terminal myristoylation moiety was abolished. One function ascribed to covalently attached lipids is to increase protein hydrophobicity thereby increasing the propensity of the protein for association with cellular membranes (Towler et al., 1988b). It has been postulated that myristic acid modification of Vps15p (Herman et al., 1991b) and p150 is responsible for the targeting of these proteins to vesicle membranes. The localisation of p150 to vesicle membranes would correlate with its punctate, perinuclear distribution when transiently expressed in COS-7 cells. It was surprising, therefore, that a p150 mutant (Myr⁻), which could not be N-terminally myristoylated had a staining pattern identical to WT-p150(EE). This result suggested that the myristic acid moiety alone, may not be entirely responsible for the possible membrane localisation of p150. It has been shown that myristoylation in itself does not provide sufficient hydrophobicity to result in a stable membrane association (Peitzsch and McLaughlin, 1993). Instead, a cluster of positively charged residues acts cooperatively with the myristoyl moiety to promote stable association with negatively charged membranes (Bhatnagar and Gordon, 1997). It is possible therefore, that another region of p150(EE) may both co-operate with the myristic acid moiety, or alone be sufficient to confer p150(EE) localisation.
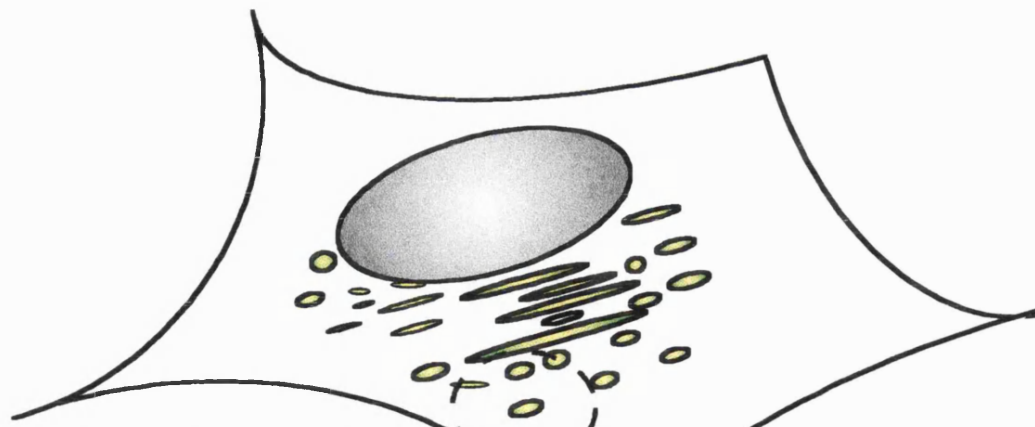
Expression of p150 in COS-7 cells gives a punctate perinuclear vesicular type stain that is unaffected by BFA, nocodazole and cytochalasin D drug treatment and does not co-localise with late endosomal/lysosomal markers (sections 7.2.2, 7.6.1, and 7.4.1).

Expression of p150 in COS-7 cells results in a Triton X-100 insoluble complex which may be due to the formation of p150 homo-oligomers. These may function in microdomains allowing formation of protein complexes facilitating vesicle formation and membrane trafficking (section 7.2.4).

PtdIns3P → PtdIns(3,5)P_2

PtdIns 3-K

The iHt-WD domain of p150 is a region required for interaction and subsequent disruption of vimentin intermediate filaments. (sections 7.4.2 and 7.8.2).

Vimentin

The HEAT and iHt-WD domain region might be implicated in binding PtdIns 3-kinase (section 7.8.3). PtdIns 3-kinase co-localises with p150 (section 7.2.3). A membrane bound p150 will allow PtdIns 3-kinase to be in closer proximity to its PtdIns substrate. Formation of p150/PtdIns 3-kinase complexes would facilitate a local increase in PtdIns3P, this in turn could have a biophysical role in promoting membrane curvature, generating a site for the interaction of proteins involved in vesicle trafficking and/or provide a source of substrate for PtdIns(3,5)P_2 production

p150 domain structure:

Myr   iPKD   iHt-WD

PKD   HEAT   WD-40

Figure 7.30 Schematic summary of Chapter 7 results.

(abbreviations: Myr, myristoylation site; PKD, protein kinase domain; iPKD, inter-protein kinase domain; HEAT, HEAT repeat domain; iHt-WD, inter-HEAT-WD-40 domain; WD-40, WD-40 repeat domain)

pp60^{c-src} (Buser *et al.*, 1994) and the myristoylated alanine-rich C kinase substrate (MARCKS) (McLaughlin and Aderem, 1995) are examples of proteins that require more than one region, in addition to their myristic acid modification for efficient membrane localisation. MARCKS is a protein which exhibits a 'myristoyl-electrostatic switch'. MARCKS associates with the plasma membrane through co-operative interactions of its N-terminal myristoyl group and a positively charged domain, with the hydrophobic core and negatively charged phospholipid groups, respectively, of the membrane. When PKC is stimulated, serine residues within the MARCKS positively charged domain become phosphorylated reducing the total positive charge of the protein. The resulting weakened electrostatic attraction is not sufficient to support membrane association in conjunction with the myristoyl moiety and the protein translocates to the cytosol (Bhatnagar and Gordon, 1997; McLaughlin and Aderem, 1995). It is possible that p150 is localised to vesicle membranes in a similar manner, whereby regions of positive charge on p150, together with the N-terminal myristic acid moiety are required for membrane interaction. This may explain why the deletion of any single region of p150, is not sufficient to disrupt it from its potential intracellular membrane site

To test this hypothesis, p150 mutants could be made where the consensus sequence for myristic acid attachment is abolished in conjunction with domain deletions or substitution of amino acid residues with positive charge. These constructs could be transiently transfected and the subcellular localisation of the proteins expressed, examined using immunofluorescence. It would be tempting to speculate that p150 might also have a 'myristoyl-electrostatic switch'. In response to a particular signal, p150 could become phosphorylated resulting in a concomitant neutralisation of net positive charge. This would cause a weakened electrostatic attraction with the membrane and result in the translocation of p150 (or more likely the p150/PtdIns 3-kinase complex) into the cytosol.

Data from section 7.4.2 (summerised in figure 7.30) has shown that expression of p150(EE) in COS-7 cells causes disruption of vimentin filament assemblies. In addition, the use of immunofluorescence and confocal microscopy had demonstrated that p150(EE) was also able to co-localise with vimentin. Whether this represented a direct association between the two proteins or was facilitated by an interaction with a 'linker' protein, could not be determined. It has been reported that vimentin filaments become disrupted when serine-phosphorylated (Fuchs and Weber, 1994). The vimentin cytoskeleton undergoes dynamic changes in its organisation during mitosis. This is achieved by the phosphorylation of vimentin by the cell cycle dependent kinase p34^{cdc2}, resulting in the disassembly of vimentin filaments which is required before mitosis can occur (Tsujimura *et al.*, 1994). Vimentin filaments also become phosphorylated by PKC and their

consequent disruption correlates with a movement and release of histamine containing granules from mast cells (Tasaka, 1994).

Since p150 has a putative ser/thr protein kinase domain, it is possible that the disruption of vimentin filaments caused by the expression of p150(EE) in COS-7 cells could be due to the *in vivo* phosphorylation of vimentin by p150(EE). The results from section 7.8.2 however, suggest that this is not the case. Immunofluorescence studies on p150(EE) mutants in which i) the protein kinase domain was deleted (ΔPKD) or ii) residues reported to be essential for catalytic activity (Herman *et al.*, 1991a) were substituted .(Kin⁻), demonstrated that ΔPKD or Kin⁻ p150(EE) mutants were still able to disrupt vimentin filament structure. An alternative way by which p150(EE) could cause vimentin disruption was by a protein-protein interaction. The immunofluorescence results presented in section 7.8.2 showed that the following p150(EE) mutants: Myr⁻, ΔiPKD-Ht, ΔHEAT and ΔWD, were still able to disrupt vimentin, suggesting that the domains deleted were not responsible for vimentin disassembly. Nevertheless, one domain deletion, ΔiHt-WD, did not induce disruption of vimentin filaments when this mutant was expressed in COS-7 cells. This suggested that the region between the HEAT repeats and WD-40 domain (amino acids 667-975), could be responsible for the disruption of vimentin by p150.

Interestingly, this iHt-WD region has significant amino acid homology (20.5% identity and 42% similarity) with vimentin itself. Vimentin is a 51 kDa protein (Fuchs and Weber, 1994), composed of four α helices (1A, 1B, 2A and 2B). The formation of vimentin filaments is accomplished by the lateral association of the α-helical rod domains via hydrophobic interactions to form dimeric coiled-coils. The external surface of the dimeric coiled-coil exhibits alternate zones of positive and negative charges which are thought to be a major driving force for the formation of tetramers and/or higher order structures (Meng *et al.*, 1994; Meng *et al.*, 1996). Studies have shown that a synthetic peptide representing the C-terminal end of the vimentin rod domain, inhibits intermediate filament assembly and disassembles preformed filaments (Hatzfeld and Weber, 1992; Kouklis *et al.*, 1991). It is possible that the iHt-WD region of p150 with its homology to vimentin, may function in an analogous manner, whereby the iHt-WD region of overexpressed p150 serves to compete with vimentin binding sites. This would interfere with filament formation and result in vimentin filament disruption and the co-localisation of p150 with insoluble vimentin protein. To investigate this possibility further, it would be interesting to express the 208 amino acid iHt-WD region in COS-7 cells to see if this protein module alone functions by disrupting vinentin filament organisation.

The deletion of this iHt-WD domain from p150 however, did not appear to affect the insolubility initially observed for WT-p150(EE) (section 7.8.2). It is feasible that in addition to the potential vimentin-binding iHt-WD region, the presence of other domains such as the HEAT repeats which also have a predicted α helical structure and hydrophobic nature (Andrade and Bork, 1995) may result in the formation of p150 oligomers and insoluble complexes.

Co-infection studies in Sf9 cells were used to examine the ability of the p150(EE) mutants to associate with PtdIns 3-kinase (section 7.8.3). Studies on yeast Vps15p, showed that amnio acid substitutions (D^{165} to R and E^{200} to R), were sufficient to abolish Vps15p protein kinase activity (Herman $et\ al.$, 1991a). In turn, it was also demonstrated that a Vps15p which had an inactive protein kinase activity, was unable to associate with the PtdIns 3-kinase, Vps34p, implying that the Vps15p protein kinase activity was required for interaction with Vps34p (Stack $et\ al.$, 1995b). The results presented in section 7.8.3 indicated that all the p150(EE) mutant proteins, including ΔPKD and Kin$^-$ (where D^{166} and E^{200} were changed to R) were still able to associate with PtdIns 3-kinase. Analogous studies were performed, where the truncated p150(EE) proteins were co-expressed with PtdIns 3-kinase in COS-7 cells and co-localising proteins analysed using immunofluorescence and confocal microscopy. This approach was adopted as a way in which the interaction of proteins could be examined within a physiological environment. The results from these investigations showed that two truncated proteins in particular, ΔHEAT and ΔiHt-WD had a reduced ability to interact with PtdIns 3-kinase $in\ vivo$. The experiments were conducted in a qualitative manner and further investigations would be required to investigate precise affinities between PtdIns 3-kinase and the truncated p150 proteins. At present however, the results from section 7.8.3 suggest that a region of p150, between amino acids 433 and 975 could represent a potential binding site for PtdIns 3-kinase. The inability of any of the p150(EE) deletions to abolish PtdIns 3-kinase binding also suggests that the primary amino acid sequence of p150 could contain more than one region responsible for the binding of PtdIns 3-kinase. When the p150 polypeptide folds to form a tertiary protein structure these different regions could form one single binding site for PtdIns 3-kinase. Further analysis would be required to determine whether additional deletions including those in the 433-975 amino acid region of p150 would be sufficient to abrogate its binding to PtdIns 3-kinase.

CHAPTER 8

General discussion

CHAPTER 8

8.0 General discussion

The viability of a eukaryotic cell depends on the spatial and temporal organisation of its phospholipid membranes. The distinct composition of the different intracellular compartments is maintained despite continuous intercompartmental transport of membrane and lipid components. This homeostasis depends on vesicular carriers that orchestrate traffic by means of vectorial transfer of selected membrane and luminal cargoes (De Camilli *et al.*, 1996; Seaman *et al.*, 1996). In addition, eukaryotic cells contain numerous membrane-bound compartments whose specialised functions require distinct protein compositions. These proteins originate predominantly in the ER and must then be sorted and distributed by transport vesicles to their correct destinations. The mechanism by which compartments acquire the correct set of proteins is now becoming understood. This is based largely on recently identified interactions responsible for formation, targeting and fusion of transport vesicles (Rothman and Wieland, 1996).

Phosphorylated products of PtdIns such as 3'-phosphoinositides have been demonstrated to play critical roles in the regulation of membrane traffic in addition to their classical role as second messengers in signal transduction from the cell surface (sections 1.7 and 1.2.2.) (Roth and Sternweis, 1997). Biochemical and cDNA cloning strategies have been used to identify a wide range of PI 3-kinase isoforms which can be placed into three classes based on their primary structure and probable mechanism of regulation (section 1.3), (Domin and Waterfield, 1997; Vanhaesebroeck *et al.*, 1997a). For these PI 3-kinase isoforms, genetic and biochemical studies have shown that the class III PtdIns specific PI 3-kinase encoded by the yeast *VPS34* gene, in conjunction with an associated ser/thr protein kinase, Vps15p, is required for the efficient sorting and delivery of soluble hydrolases to the vacuole (section 1.5.3.4) (Stack *et al.*, 1995c). A human homologue (PtdIns 3-kinase), of the yeast *VPS34* gene product has been characterised as part of a complex with a cellular protein of 150 kDa (Volinia *et al.*, 1995). This 150kDa protein was predicted to be the human homologue of the yeast Vps34p adaptor protein Vps15p (Volinia *et al.*, 1995). In this thesis, I have presented data to show that p150 is the human homologue of Vps15p and that together with PtdIns 3-kinase is likely to be a complex required for vesicle trafficking in mammalian cells

The cDNA cloning techniques described in chapter 3 enabled the isolation of a human cDNA clone which encoded a protein (termed p150) with a predicted molecular mass of 150kDa and an amino acid sequence that was highly homologous (29.6% identical and 53% similar) to that of the yeast Vps15p protein. Expression of recombinant p150

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showed that it had a molecular mass of 150kDa and was able to associate specifically with human PtdIns 3-kinase (chapter 4). Results presented in this thesis also showed that human p150 displayed similar biochemical characteristics to those of yeast Vps15p. Thus, p150 was shown to be N-terminally myristoylated like Vps15p. In addition, association of PtdIns 3-kinase with p150 was found to result in an increase in lipid kinase activity which was comparable to the requirement of Vps15p for the stimulation of Vps34p PtdIns 3-kinase activity (Stack *et al.,* 1995c). p150 and PtdIns 3-kinase appear to be phosphorylated *in vivo*, yet my studies showed that neither protein, individually or in complex appeared to autophosphorylate *in vitro* (chapter 5). A protein kinase activity however, could only be observed when p150 and PtdIns 3-kinase were associated, resulting in the phosphorylation of exogenous substrate such as MBP. This is in contrast to previous studies (Stack and Emr, 1994), which showed that both Vps15p and Vps34p appear to have intrinsic autocatalytic activity. It is possible that either an unidentified protein kinase phosphorylates p150/PtdIns 3-kinase *in vivo*, or a particular environment (e.g. the presence of accessory proteins or cofactors) is required to facilitate p150/PtdIns 3-kinase phosphorylation *in vitro*. The generation of Kin$^-$ (as described in chapter 7), a potentially catalytically inactive p150 protein, maybe a useful reagent for future experiments designed to answer some of these questions, particularly whether the p150/PtdIns 3-kinase phosphorylation state has any functional consequence.

Data presented in this thesis have shown that when PtdIns 3-kinase is transiently expressed in COS-7 cells, PtdIns3*P* is the main 3'-phosphorylated lipid produced (chapter 6). PtdIns3*P* has been shown to bind specifically to an early endosomal protein, EEA1 (Patki *et al.*, 1997). This interaction is considered to mediate its localisation since EEA1 is rapidly released from cell membranes following wortmannin treatment. This suggests that the effect of wortmannin is due to the inhibition of PtdIns3*P* production. Consequently, EEA1 might be a downstream target of a PtdIns 3-kinase which may mediate vesicle transport at the early endosome. 3'-phosphoinositides such as PtdIns3*P*, may also cooperate with membrane proteins in the recruitment of vesicle trafficking proteins such as coatamers and adaptins (Roth and Sternweis, 1997). The binding of these proteins to the head group of a phospholipid could help correctly orientate the coat proteins and facilitate vesicle coat formation. Alteration of the lipid composition of vesicular carriers via the generation of PtdIns3*P* may also play a role in the biophysical formation of a vesicle bud or function at the fusion step of the transport reaction. Here again, the head group of PtdIns3*P* might serve to recruit, activate or modulate the activity of factors necessary for membrane fusion.

Results presented in this thesis have also shown that in cells over expressing PtdIns 3-kinase, elevated levels of PtdIns(3,5)P_2, in addition to PtdIns3*P* can be generated *in*

vivo. Dove *et al*, have demonstrated that Vps34p is required to produce PtdIns(3,5)P_2, a 3'-phosphorylated lipid generated in response to osmotic stress (Dove *et al.*, 1997). It is possible that PtdIns(3,5)P_2 may function as a signalling molecule, the downstream target(s) of which might be implicated in osmotically regulated vesicle trafficking events. The observation that the expression of a lipid kinase deficient PtdIns 3-kinase in COS-7 cells (chapter 7) results in the formation of swollen vacuolar structures, suggests that PtdIns 3-kinase could be implicated in maintaining vesicle osmotic integrity. There is evidence that the phospholipid PtdIns(4,5)P_2 is involved in the regulation of Na$^+$ and K$^+$ ion channels (Hilgemann and Ball, 1996), it is possible that PtdIns(3,5)P_2 might play a similar role in regulating volume gated channels resulting in osmoregulation. Alternatively, PtdIns 3-kinase could be implicated in facilitating membrane flow. Studies by Reaves *et al*, have shown that treatment of NRK cells with wortmannin resulted in the formation of swollen structures containing lysosomal glycoproteins (Reaves *et al.*, 1996). The uptake of acridine orange into these compartments was unaffected by wortmannin suggesting that their swelling was not due to the dissipation of a pH gradient but due to a perturbation in membrane flow.

Results presented in this thesis have also shown that the PtdIns/PtdCho transfer protein, PI-TP, can increase the lipid kinase activity of p150/PtdIns 3-kinase in a dose dependent manner. Furthermore, the identification of a Mn^{2+} dependent PtdIns specific PI 3-kinase activity associated with PI-TP immunoprecipitates, suggests that PI-TP may function with p150/PtdIns 3-kinase *in vivo* (chapter 6). PI-TP is a factor required for vesicle exocytosis and has been implicated in membrane trafficking events involving a p62^{cplx} associated PtdIns 3-kinase activity (Jones *et al.*, 1998b). In a similar fashion, PI-TP could function with p150/PtdIns 3-kinase at vesicle membranes presenting PtdIns substrate to p150/PtdIns 3-kinase, facilitating the production of PtdIns3P. Indeed, p150/PtdIns 3-kinase might be part of a multiprotein complex which could also include a PtdIns3P 5-kinase required to generate PtdIns(3,5)P_2 from PtdIns3P. Such a complex could also contain other associated proteins needed for vesicle formation and trafficking events. These may include as yet unidentified mammalian homologues of VPS proteins (section 1.5) such as Vps10p and Vps21p. Both proteins function on the same pathway as Vps15p/Vps34p and encode a CPY receptor and Rab5 homologue respectively. Initial experiments which identified p150 as a PtdIns 3-kinase associated protein, also showed that a 200 kDa and 120 kDa protein co-immunoprecipitated with endogenous PtdIns 3-kinase form Jurkat cells (Volinia *et al.*, 1995). This supports the presence of other p150/PtdIns 3-kinase binding partners. The identification of supplementary p150/PtdIns 3-kinase binding proteins may provide further clues as to the precise function of p150/PtdIns 3-kinase in the mammalian cell.

The molecular machinery underlying membrane trafficking events appears to be highly conserved among all eukaryotes (Ferro Novick and Jahn, 1994). The yeast Vps15p/Vps34p complex functions between the late Golgi compartment and the yeast vacuole correctly targeting soluble yeast hydrolases. Therefore, using yeast as a model, the human Vps15p/Vps34p homologues, p150/PtdIns 3-kinase would be thought to function between the TGN and the mammalian equivalent of the yeast vacuole, the late endosome/lysosome. Studies involving the treatment of mammalian cells with the potent PI 3-kinase inhibitors, wortmannin and LY294002 have provided evidence to support the role of human PtdIns 3-kinase in vesicle transport (sections 1.7.1.3.1 and 1.7.1.3.2). For example, treatment of mammalian K-562 and NRK cells with wortmannin results in the missorting and secretion of the soluble hydrolase cathepsin D (Brown et al., 1995b; Davidson, 1995), in much the same way as Vps34p deficient yeast missort the soluble hydrolase CPY (section 1.5.3.2). This suggests a requirement for PI 3-kinase activity to correctly target cathepsin D to mammalian lysosomes. Similarly, wortmannin/LY294002 inhibits membrane trafficking events, including transcytosis, endocytosis and alterations in endosome morphology. Consequently, the requirement of PI 3-kinase activity in vesicle mediated events has been well documented. However, assigning biological activity through the use of pharmacological agents is frought with difficulty, since their specificity always remains an issue. For example, at low nanomolar concentrations, wortmannin also inhibits the activity of phopholipase A2 (Cross et al., 1995b). In addition, since human PtdIns 3-kinase is not the only PI 3-kinase sensitive to wortmannin/LY294002, it still remains unclear which of the many PI 3-kinase family members is actually responsible for particular membrane trafficking events. As a result, it could be asked, where does the p150/PtdIns 3-kinase complex function in the vast array of vesicle trafficking pathways?

To investigate the role of p150/PtdIns 3-kinase in vesicle trafficking, immunofluorescence techniques and confocal microscopy were employed to examine the subcellular localisation of p150 and p150/PtdIns 3-kinase. Using these methods, studies presented in this thesis have shown, that p150 has a punctate perinuclear staining pattern when expressed in COS-7 cells (chapter 7) and that like the Vps15p/Vps34p complex, p150 functions in associating with PtdIns 3-kinase and localising the lipid kinase to punctate perinuclear structures (chapter 7). The subcellular localisation observed for p150/PtdIns 3-kinase is consistent with a potential TGN, late endosomal/lysosomal function. However, experiments using the Golgi disrupting agent BFA (chapter 7) indicated that p150 was unlikely to be present at the TGN. Similarly, co-localisation experiments with the late endosomal/lysosomal markers, LAMP-1 and cathepsin D, indicated that p150 did not co-localise with these compartments. This suggests a possible functional divergence from yeast, implicating p150/PtdIns 3-kinase in a potential

trafficking role distinct from that of yeast Vps15p/Vps34p. Alternatively, the p150/PtdIns 3-kinase complex may be localised to a novel, as yet uncharacterised compartment on the biosynthetic pathway, such as that which contains the AP-3 adaptor complex (Simpson *et al.*, 1997). It is interesting to note that whereas yeast has only one PI 3-kinase, Vps34p, higher eukaryotes have numerous PI 3-kinase isoforms. Consequently, it is possible that whilst the human Vps34p homologue, PtdIns 3-kinase may be implicated in vesicle trafficking events, its function may not be exactly comparable to that of yeast Vps34p. It is possible that human PtdIns 3-kinase has adopted a sub-specialisation in mammalian vesicle transport and that other PI 3-kinase isoforms may also function in membrane trafficking. In yeast, an allele of *VPS34* (*END12*), has been shown to be required for the proper targeting of endocytosed proteins in yeast (Munn and Riezman, 1994). However, in mammalian cells the class I_A p85α/p110α PI 3-kinase complex, is required for the delivery of endocytosed PDGF receptors to the lysosome (Joly *et al.*, 1994; Joly *et al.*, 1995). This suggests that distinct isoforms of PI 3-kinase, such as p85α/p110α can fulfil the role of the single enzyme present in lower eukaryotes such as yeast, although this does not totally preclude the possible involvement of human PtdIns 3-kinase in similar endocytic events.

Recent evidence has also shown that other PI 3-kinase enzymes may also play a role in membrane trafficking events (section 1.7.1.3.2). The 3'-phosphoinositides PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3, which are not considered to be products of PtdIns 3-kinase activity, have been shown to bind to proteins implicated in vesicular trafficking (section 1.7.1.2), (Roth and Sternweis, 1997). One example is the AP-2 adaptor complex which is present in clathrin coated pits at the plasma membrane. Generation of PtdIns(3,4)P_2 or PtdIns(3,4,5)P_3 increases the affinity of AP-2 for receptor signals tenfold and facilitates the formation of clathrin-associated AP-2 complex (Rapoport *et al*, 1997). In this way, these 3'-phosphoinositides can regulate the production of coated vesicles. PtdIns(3,4,5)P_3 has also been shown to bind cytohesin 1 (a guanine nucleotide excange factor for the small GTPase ARF 1; section 1.7.1.2) and localise cytohesin to a membrane site. PtdIns(3,4,5)P_3 which has been bound by cytohesin, in conjunction with ARF 1 and cytosolic coat proteins is thought to be required for maintaining the vesicular morphology of endosomes (Roth and Sternweis, 1997). PtdIns(3,4,5)P_3 has also been demonstrated to bind the Golgi coatamer protein α-COP I (Chaudhary *et al.*, 1998). It is possible that each 3'-phosphoinositide such as PtdIns3P, generated by a PtdIns 3-kinase or PtdIns(3,4)P_2/PtdIns(3,4,5)P_3 produced by a class I or II PI 3-kinase, could have a potentially specific role at particular vesicle transport steps and may explain the molecular heterogeneity observed amongst mammalian PI 3-kinase proteins.

To gain further insight into the role of p150 in intracellular signalling events, the functional relevance of the domains identified within p150 were investigated. Results presented in this thesis showed that systematic deletion of the p150 myristoylation site, protein kinase, HEAT and WD-40 domains did not affect the subcellular localisation of p150, or its ability to bind PtdIns 3-kinase (chapter 7). In addition, experiments examining the role of cytoskeletal proteins in p150 localisation, showed that p150 co-localised with the intermediate filament protein vimentin. This interaction was dependent on the iHt-WD domain, a 308 amino acid region between the HEAT repeats and WD-40 domain of p150. However, what involvement vimentin might have in p150/PtdIns 3-kinase function is presently unclear. Although the sequential deletion of the p150 domains did not effect the subcellular localisation of this protein or its ability to bind PtdIns 3-kinase, the role of these domains in events such as fluid phase endocytosis or transferrin receptor internalisation could be investigated with fluorophore conjugates of dextran and transferrin respectively. Lack of time however, did not permit such studies to be undertaken. Similarly, identification of the PtdIns 3-kinase binding site on p150 by further sytematic mutagenesis, would facilitate the production of a p150 dominant negative, which may prove to be a useful reagent for examining PtdIns 3-kinase mediated events.

The production of p150, PtdIns 3-kinase or p150/PtdIns 3-kinase 'knock out' mice (possibly in conjunction with the 'knock outs' of other PI 3-kinase family members) and cell lines derived from these genetically altered animals, would provide important information regarding the functional role of p150/PtdIns 3-kinase, especially with respect to cell morphology, growth and membrane trafficking. Mapping of the human chromosomal location of p150 may also generate more information, particularly if it is identified to a chromosomal locus associated with a disease process. Several human diseases have been attributed to defects in intracellular protein trafficking. These include Chediak-Higashi syndrome in which a defective 400 kDa HEAT repeat and WD-40 motif containing protein causes protein sorting defects resulting in hypopigmentation and immunodeficiency (chapter 3) (Nagle *et al.*, 1996). Another disease resulting in a protein sorting defect is I-cell disease, which is characterised by a deficiency of lysosomal enzymes. This is caused by a defect in the phosphotransferase enzyme responsible for the mannose-6-phosphate modification of lysosmal enzymes required for their correct targeting to lysosomal compartments (Amara *et al.*, 1992).

Membrane trafficking events represent a pivotal response to alterations in cellular environment. Consequently, a close relationship must exist between intracellular signalling pathways which lie downstream of cell surface receptors and the machinery for controlling membrane traffic. The characterisation of these interactions is an important

task for the future, as reciprocal relationships between signal transduction and membrane trafficking events begin to emerge. Toward this goal, further analysis of p150/PtdIns 3-kinase function will not only provide an improved understanding of vesicle trafficking processes and cellular regulation, but it will also facilitate in delineating the role of 3'-phosphoinositides in membrane transport events.

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