THE INTERACTION OF THE p85 SUBUNIT OF PI3K WITH RAB PROTEINS

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By

M. Dean Chamberlain

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Dr. D. Anderson

Saskatchewan Cancer Agency

Cancer Research Unit

20 Campus Dr.

University of Saskatchewan

Saskatoon, Saskatchewan

S7N 4H4

ABSTRACT

The p85 subunit of phosphatidylinositol 3'-kinase (PI3K) has long been thought of as a regulatory subunit that has no other function than the regulation of the p110 catalytic subunit. Our laboratory is studying other roles of the p85 subunit, in particular determining the role of the p85 BH domain. The BH domain has homology to GTPase activating protein (GAP) domains that are involved in the stimulation of monomeric G proteins to hydrolyze their bound GTP to GDP. This converts the G protein from its active conformation to its inactive conformation. We have determined that p85 interacts with Rab proteins, monomeric G proteins that regulate vesicle fusion during the endocytosis of receptors. We have shown that p85 binds to Rab5 regardless of nucleotide-bound state of Rab5. The p85 subunit of PI3K has *in vitro* GAP activity towards Rab5. It was determined that p85 also has *in vitro* GAP activity towards Rab4, Rab7, Rab6 as well as the Rho-family G proteins, Rac1 and Cdc42. This GAP activity was localized to the BH domain of p85 and mutation of Arg 274 to Ala abolishes the GAP activity of p85. When this p85R274A mutant was expressed in cells, PDGFR degradation was severely inhibited and there was a corresponding increase in the duration of MAPK and Akt signalling. This increase in cell signalling caused a transformed phenotype in cells expressing the p85 protein with the Arg 274 mutation. These cells have lost contact inhibition for growth, are able to grow independent of attachment as well as in the presence of limited growth factors. They also form tumours in nude mice. These cellular effects seem to be due to an increase in receptor recycling

because of the loss of the GAP activity of p85. This increase in receptor recycling may interfere with receptor targeting to the late endosome, which would cause the decrease in receptor degradation that is seen in the p85R274A cells.

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This work is dedicated to my wife

Leanne

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LIST OF ABBREVIATIONS

BH	breakpoint cluster region homology
BSA	bovine serum albumin
DMEM	Dulbecco's Modified Eagle Medium
DTT	dithiothreitol
CDKs	cyclin-dependent kinases
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
EEA1	early endosomal antigen 1
EGF	epidermal growth factor
EGFR/ErbB	epidermal growth factor receptor
EGTA	ethylene glycol-bis(2-aminoethylether) N,N,N',N'-tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
ERK	extracellular regulated kinase
ESCRT	Endosomal Sorting Complex Required for Transport
FACS	fluorescence activated cell sorting
FGFR	fibroblast growth factor receptor
FBS	fetal bovine serum

G418	Geneticin
GAP	GTPase activating protein
GDI	guanine dissociation inhibitor
GDF	GDI-dissociation factor
GEF	guanine nucleotide exchange factor
GLUE	GRAM-like ubiquitin binding in EAP45
GPCR	G protein-coupled receptor
Grb2	growth factor receptor-bound protein 2
GSK3	glycogen synthase kinase 3
GST	glutathione S-transferase
HA	hemagglutinin
Hepes	N-2-hydroxyethylpiperazine-H'-2-ethanesulfonic acid
HGFR	hepatocyte growth factor receptor
hr	hour
IGFR	insulin-like growth factor receptor
IPTG	isopropyl β–D-thiogalactopyranoside
IR	insulin receptor
LAMP-1	lysosomal associated membrane protein 1
LB	Luria-Bertani Broth
LBA	Luria-Bertani Broth plus ampicillin
MAPK	mitogen activated protein kinase
Mdm2	murine double minute 2
MEK	MAPK/Erk kinase
MVB	multivesicular body

NaPP	sodium pyrophosphate
Na_3VO_4	sodium orthovanadate
NF-κB	nuclear factor-kappa B
NGFR	nerve growth factor receptor
NSF	N-ethylmaleimide sensitive factor
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
PDGFR	platelet-derived growth factor receptor
PCR	polymerase chain reaction
PDK1	phosphoinositide (3)-dependent kinase 1
PDK2	phosphoinositide (3)-dependent kinase 2
PH	pleckstrin homology
PI3K	phosphatidylinositol 3'-kinase
PI	phosphatidylinositol
PI3P	phosphatidylinositol 3-phosphate
PI4P	phosphatidylinositol 4-phosphate
$PI3,4P_2$	phosphatidylinositol 3,4-bisphosphate
$PI3,4,5P_3$	phosphatidylinositol 3,4,5-trisphosphate
$PI4,5P_2$	phosphatidylinositol 4,5-bisphosphate
PLC-γ	phospholipase C γ
PMSF	phenylmethylsulfonyl fluoride
PS	phosphatidylserine
P/S	penicillin G/ streptomycin

PTEN	phosphatase and tensin homolog (mutated in multiple advanced cancers 1)
RT	room temperature
RTK	receptor tyrosine kinase
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SH2	Src homology region 2
SH3	Src homology region 3
Shc	Src homologous and collagen-like protein
SHP-2	SH2 domain-containing phosphatase 2
SNARE	soluble NSF attachment receptor
SOS	Son of Sevenless
STAT	signal transducer and activator of transcription
TEMED	N,N,N',N'-Tetra-methylethylenediamine
TLC	thin-layer chromatography
TMB	3,3'5,5'-tetramethylbenzidine
Tween 20	polyoxyethylenesorbitan monolaurate
VEGFR	vascular endothelial growth factor receptor

1.0 INTRODUCTION

1.1 Overview of Signal Transduction Pathways

Signal transduction is the process that allows a cell to interact with its environment. Cells are the basic building blocks of life and in higher organisms they are highly differentiated into specialized tissues. These specialized cells must communicate with cells in the same tissue and cells in other tissues for the organism to function properly. If this communication breaks down it can cause cell death or disease, which could lead to the death of the entire organism. Signal transduction allows the cell to regulate itself in the context of the larger organism, including when to grow and divide in response to the levels of nutrients available for these and other functions.

Signal transduction starts at the exterior of the cell with the binding of a ligand to a receptor. There are many types of receptors, which respond to varied stimuli such as temperature, light, sound, pressure, vitamins, hormones, neurotransmitters, growth factors, immunoglobulins, oxygen and other chemicals or environmental signals (Schuller, 1991). The receptor transduces the information supplied from the ligand at the exterior of the cell to the interior of the cell. The signalling cascade that is initiated by a few receptors is amplified by the activation of signalling proteins. The challenge for a cell is to convey all the information required, but to use as few unique signalling molecules and proteins as possible (Csiszar, 2006). Efficiency is required in order to allow for cellular survival. If the cell was obliged to have a large set of unique proteins and signalling molecules for each possible receptor, the energy demands required to

produce the molecules would be too large for the viability of the cell (Csiszar, 2006). By having a single protein function in multiple pathways within the cell, the total number of required proteins can be greatly reduced. In addition, if the cell had to design new proteins for every pathway, the time it would take to evolve new signalling pathways would be horrendously long (Csiszar, 2006). These signalling cascades are often illustrated as linear pathways but in reality the signalling cascades are complicated networks of intersecting pathways (Citri and Yarden, 2006). The activation of a receptor can induce many responses within the cell for both short-term and long-term cellular changes. For example, the short-term quick responses can be the activation of neurons or the mobilization of the cytoskeleton, while the long-term responses can be the differentiation or division of the cell. Activation of a single receptor can cause many different effects in the cell due to either the duration and/or strength of the signal, or the activation of multiple signalling cascades. Also, cells use the same downstream signalling proteins in multiple different signalling cascades that have various effects on the cell. Such intricacies create the problem of how cells use the same receptors and downstream proteins to control and activate multiple different pathways at one time. This is accomplished through modification of the proteins and sequestering proteins in space and/or time.

The most common way in which a signal is transmitted through the cell is by the serial phosphorylation or dephosphorylation of proteins to change their activation states. Proteins are typically phosphorylated on one or more tyrosine, serine and threonine residues. The level of protein activation or its binding specificity can be altered by these phosphorylation events. Other common modifications including methylation, acetylation, ubiquitylation, glycosylation, myristoylation and prenylation also alter the

activity or function of a protein. All of these modifications can have several different effects. They can increase or decrease the activity of enzymes, or alter their subcellular location. They can also change the conformation of the protein to form or unmask binding sites for lipids, small molecules and other proteins. For example, a phosphorylated tyrosine plus three to six adjacent amino acids form a binding motif for a specific Src homology region 2 (SH2) domain (Bradshaw and Waksman, 2002; Grucza *et al*., 1999). This SH2 binding motif is created by the phosphorylation of the tyrosine contained in the context of a specific sequence.

Apart from the physical modification of individual proteins, sequestering proteins in time and space can alter signalling cascades. The proteins can have varied expression profiles where the proteins can be expressed concurrently or individually and this can change the activities of the proteins. Proteins can also be degraded so that they cannot interact until they are resynthesized at the proper time. Protein function may be influenced through interactions with other proteins such as in cases where activation of a protein is dependent on a secondary protein. An example of this is the cell cycle proteins cyclin-dependent kinases (CDKs) and cyclins. The progression of cells through the cell cycle is regulated by CDKs, which in turn are activated by cyclins (Deshpande *et al*., 2005; Murray, 2004). The levels of CDKs remain constant in the cell while the level of cyclins are low in the cell until the appropriate stage of the cell cycle when the cyclin levels increase (Deshpande *et al*., 2005; Murray, 2004). The increase in the amount of cyclin causes the formation of the cyclin-CDK complexes that activate the CDK and allow the progression of the cell through the cell cycle (Deshpande *et al*., 2005; Murray, 2004).

Proteins are very commonly sequestered in space, either from each other, a co-activator, an inhibitor or a substrate. A classical example of this is the co-activator Ca^{++}. Calcium is a second messenger that is sequestered in the endoplasmic reticulum (ER) (Chin and Means, 2000; Thomas *et al*., 1996; Vetter and Leclerc, 2003). Upon release from the ER the calcium binds to calmodulin to bind and activate various proteins resulting in signal propagation (Chin and Means, 2000; Thomas *et al*., 1996; Vetter and Leclerc, 2003). In signalling cascades, all of the modifications and methods mentioned above can be used in combination to control and fine tune the signalling information sent to the cell. This allows the same signalling proteins to generate different signals for the cell. These signals are very diverse and regulate several signalling pathways including cell proliferation and survival, differentiation and chemotaxis of cells (Aaronson, 2005). At the whole organism level these cellular functions permit developmental processes, tissue differentiation, immune responses, wound healing and tissue repair (Aaronson, 2005). A deregulation of cell signalling can cause the cell to proliferate in an uncontrolled manner resulting in cancer. Elucidation of the various components of the signalling pathways and how the components interact and are deregulated will give a better understanding of cell function and how cancer develops.

1.2 Receptor Tyrosine Kinases

The receptor tyrosine kinase (RTK) family of proteins are integral membrane proteins that are composed of an extracellular ligand-binding domain, a transmembrane domain that passes once through the plasma membrane and an intracellular kinase domain. There are several RTK subfamilies identified according to the structural characteristics of the receptors and the ligands that bind to the receptors. The main

structural classifications are according to the organization of the extracellular domain and the presence of an insert in the kinase domain (Fig. 1.1)(Reith and Panayotou, 1997). The major RTK subfamilies include the epidermal growth factor receptors (EGFR/ErbB), the insulin and insulin-like growth factor receptors (IR and IGFR), the platelet-derived growth factor receptors (PDGFR), the vascular endothelial growth factor receptors (VEGFR), the fibroblast growth factor receptors (FGFR), the hepatocyte growth factor receptors (HGFR) and the nerve growth factor receptors (NGFR) (Reith and Panayotou, 1997). Each of these RTK subfamilies drives different but overlapping processes of cell survival and cell division with some specific functions. This can be illustrated with the insulin receptor. Although insulin binding to the receptor promotes cell survival similar to other RTKs, its main and unique function is energy regulation through glucose metabolism and glucose uptake via the glucose transporter, GLUT4 (Kanzaki, 2006; Saltiel and Kahn, 2001).

Another example of a specific but overlapping function is that activation of the PDGFR and VEGFR both have the ability to stimulate angiogenesis (Conway *et al*., 2001; Reigstad *et al*., 2005). The reason why growth factor receptors have overlapping function is redundancy. Redundancy is important because it allows the cell to function normally with a small amount of deregulation due to loss or mutation of proteins. Although most cell types can and do express several different receptors they may not be expressed at the same time or the ligand may not be available; therefore, the redundancy in signalling allows cells to function in changing environments and to integrate cellular responses to several external stimuli.

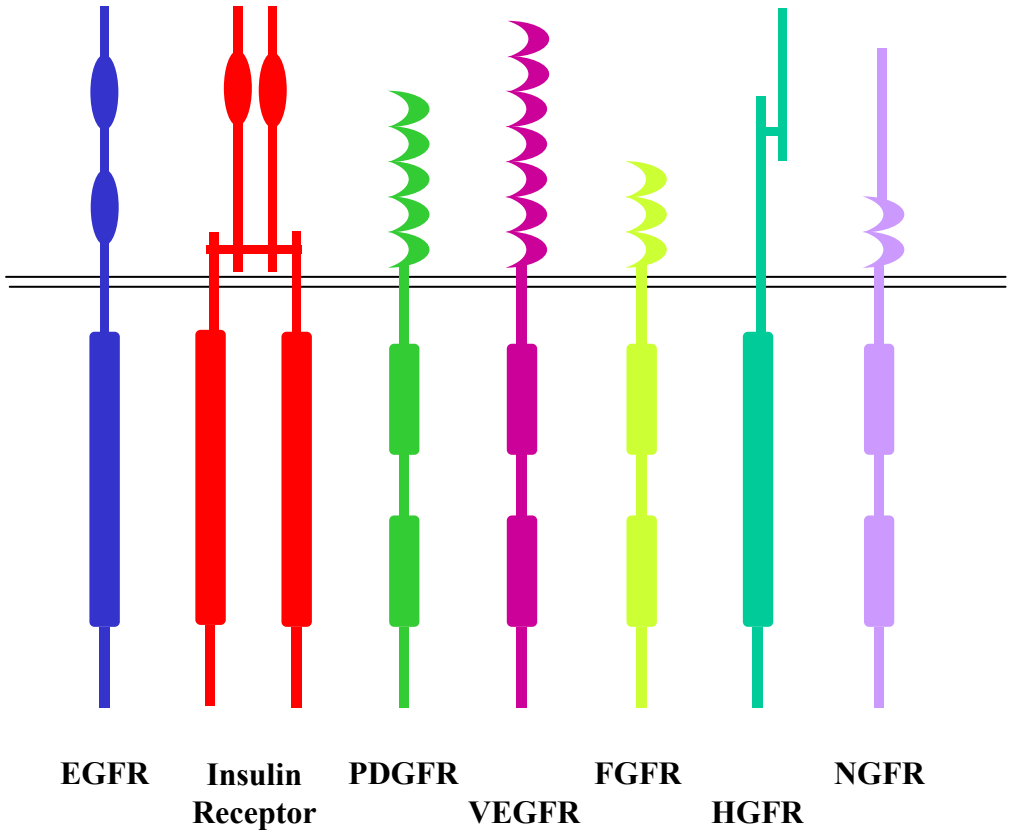


Figure 1.1 Receptor Tyrosine Kinase Subfamilies. The receptors are classified by three elements, the growth factors they bind, if they contain an insert in their kinase domain and the conformation of their extracellular domains.

The ligands that bind to RTK are proteins that are usually expressed as pro-ligands, which are proteolytically cleaved into the active ligand (Fredriksson *et al*., 2004; Harris *et al*., 2003; Heldin and Westermark, 1999; Reith and Panayotou, 1997). Most pro-ligands are bound within the membrane or extracellular matrix (Fredriksson *et al*., 2004; Harris *et al*., 2003; Heldin and Westermark, 1999; Reith and Panayotou, 1997) and are activated by proteolytic cleavage by proteinases (Fredriksson *et al*., 2004; Harris *et al*., 2003; Heldin and Westermark, 1999; Reith and Panayotou, 1997). The activation of the pro-ligand also allows for cross-talk between the G protein-coupled receptor (GPCR) signalling pathway and the RTK signalling pathway because GPCRs activate metalloproteinases which in turn, activate the pro-ligands of the RTK (Gschwind *et al*., 2001). The activated ligands are frequently growth factors that can act in an autocrine, paracrine or endocrine fashion. Most growth factors tend to work in an autocrine or paracrine function except for insulin that works in an endocrine fashion.

The activation of RTKs is due to autophosphorylation of the receptor upon binding of the ligand (Heldin and Ostman, 1996; Heldin and Westermark, 1999). The exact method of activation may vary between the different RTKs but some basic information is known about RTK activation mainly through the study of EGFR and insulin receptors. Most RTKs are initially monomeric and ligand binding causes receptor dimerization, activation and autophosphorylation (Heldin and Ostman, 1996). This raises the question of the actual mechanism of receptor activation upon ligand binding. The ligand could physically bring together the two receptor monomers, allowing the dimer to activate itself or it could also cause a conformational change in the dimerized receptor to activate the receptor (Heldin and Ostman, 1996). For the EGFR there is a shift from monomers to dimers with the addition of EGF; however, there is also a small amount of EGFR

dimers even in the absence of EGF (Boni-Schnetzler and Pilch, 1987). This research did not look at the downstream signalling events of the EGFR dimers without EGF to determine if they were active, but our knowledge of RTKs to date suggests that they are inactive (Boni-Schnetzler and Pilch, 1987). These dimerized but inactive receptors show an increased affinity for EGF, which would increase the sensitivity of the cell to the growth factor (Boni-Schnetzler and Pilch, 1987). It also leads to the question does the ligand stabilize receptor dimers or cause the dimerization. It has been suggested that the extracellular domain of receptors could have an inhibitory effect that is not overcome until the ligand is bound (Heldin and Westermark, 1999; Warren and Landgraf, 2006). However, when the receptor dimerizes it leads to the activation of the receptor and the trans-phosphorylation of the dimerized receptors. This increases the catalytic activity of the receptors and creates binding sites for signalling proteins. The dimerization of the receptor also allows for signalling diversity because the receptors can form heterodimers with other receptors within the same subfamily as well as homodimers (Heldin and Ostman, 1996). The EGFR subfamily has four isoforms that are expressed in various cells and can be overexpressed in cancers.

Many types of cancers either have RTK mutations or overexpression (Arteaga, 2003; Hanahan and Weinberg, 2000; Holbro *et al*., 2003; Pelengaris and Khan, 2006; Teich, 1997). This is because RTKs are one of the key components of the signalling pathways; therefore, unregulated activation of the receptor leads to increased signalling from the receptor. There are three ways in which RTKs are deregulated in cancer: 1) the receptor is overexpressed, increasing the amount of a normal receptor protein 2) there is a mutation or deletion that constitutively activates the receptor 3) a chromosomal translocation generates a fusion protein that activates the receptor (Li and Hristova,

2006). Different types of cancers are associated with activation of different subfamilies of RTKs. For example, the EGFR family is deregulated in many types of cancers including colon, pancreas, breast, ovary, gliomas and lung (Arteaga, 2003; Sebastian *et al*., 2006; Teich, 1997). A truncated form of EGFR (v-erbB) was one of the first oncogenes identified (Sebastian *et al*., 2006). It was discovered as an oncogenic protein expressed by the avian erythroblastosis virus (Holbro *et al*., 2003). Another oncogene that was identified around the same time as v-erbB was v-sis from the simian sarcoma virus and was found to be homologous to PDGF-B, the ligand for the PDGFR (Teich, 1997). PDGF and its receptors are also involved in several types of cancer and are overexpressed in many glial tumours, sarcomas, germ cell tumours and gastrointestinal carcinomas (Heldin and Westermark, 1999; Shih and Holland, 2006).

1.2.1 Platelet-derived Growth Factor Receptor

There are two isoforms of PDGFR, PDGFRα and PDGFRβ, which bind four different PDGF ligand isoforms A, B, C and D (Fig. 1.2). The PDGF isoforms dimerize to form five known active dimers, PDGF-AA, PDGF-AB, PDGF-BB, PDGF-CC and PDGF-DD (Reigstad *et al*., 2005). These growth factors are of the cysteine knot superfamily which is characterized by a conserved pattern of six cysteine residues making disulfide bridges that main the structure of the growth factor (Reigstad *et al*., 2005). PDGF A and B are secreted by the cell in an active form after undergoing dimerization in the ER and proteolytic cleavage in the trans-Golgi (Fredriksson *et al*., 2004). PDGF C and D are dimerized inside the cell but are activated by proteolytic cleavage outside the cell (Fredriksson *et al*., 2004).

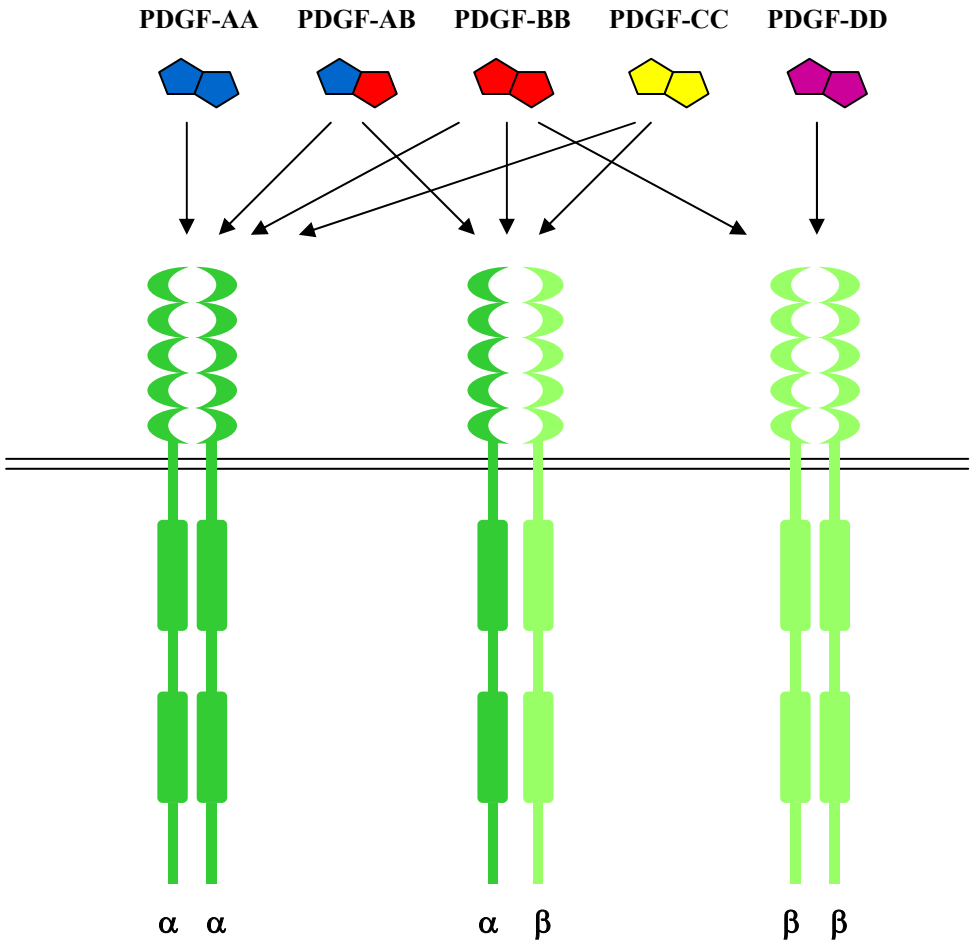


Figure 1.2 Binding of PDGF isoforms to the different PDGFR dimers. The PDGF dimers have different affinity for the three PDGFR dimers. The PDGFR-αα dimer interacts with PDGF-AA, -AB, -BB and -CC. The PDGFR-αβ dimer interacts with PDGF-AB, -BB and -CC. The PDGFR-ββ dimer interacts with PDGF-BB and -DD.

There are three different receptor dimers, PDGFRαα, PDGFRαβ and PDGFRββ, that bind to the PDGF isoforms with varying degrees of affinity (Bornfeldt *et al*., 1995; Claesson-Welsh, 1994; Reigstad *et al*., 2005). PDGF-AA binds to only PDGFRαα, PDGF-BB binds to all three receptors, PDGF-DD binds to PDGFRββ and PDGF-AB and PDGF-CC both interact with PDGFRαα and PDGFRαβ (Fig. 1.2). The PDGFR is activated by dimerization induced by the binding of the PDGF to the extracellular domain of the receptor monomers. The extracellular domain contains five immunoglobulin domains; the first three-immunoglobulin domains are thought to bind to the ligand. The fourth immunoglobulin domain of each allows the receptor monomers to interact with each other to stabilize the structure (Heldin *et al*., 1998). This dimerization allows the receptors to trans-phosphorylate each other to activate the dimer. The phosphorylated tyrosine residues on the receptors have two functions. The first is to control the activation state of the receptor and the second is to create binding sites for adaptor and signalling proteins. The binding of multiple growth factors to the different PDGFR dimers allows for specificity in the activated signalling pathways (Claesson-Welsh, 1994). This is because each of the PDGFR isoforms have different phosphorylation sites that activate several different downstream signalling proteins (Fig. 1.3)(Claesson-Welsh, 1994). The two major signalling pathways associated with the PDGFR are the phosphatidylinositol 3'-kinase (PI3K)-Akt and Ras-mitogen activated protein kinase (MAPK) signalling pathways (discussed in greater detail below).

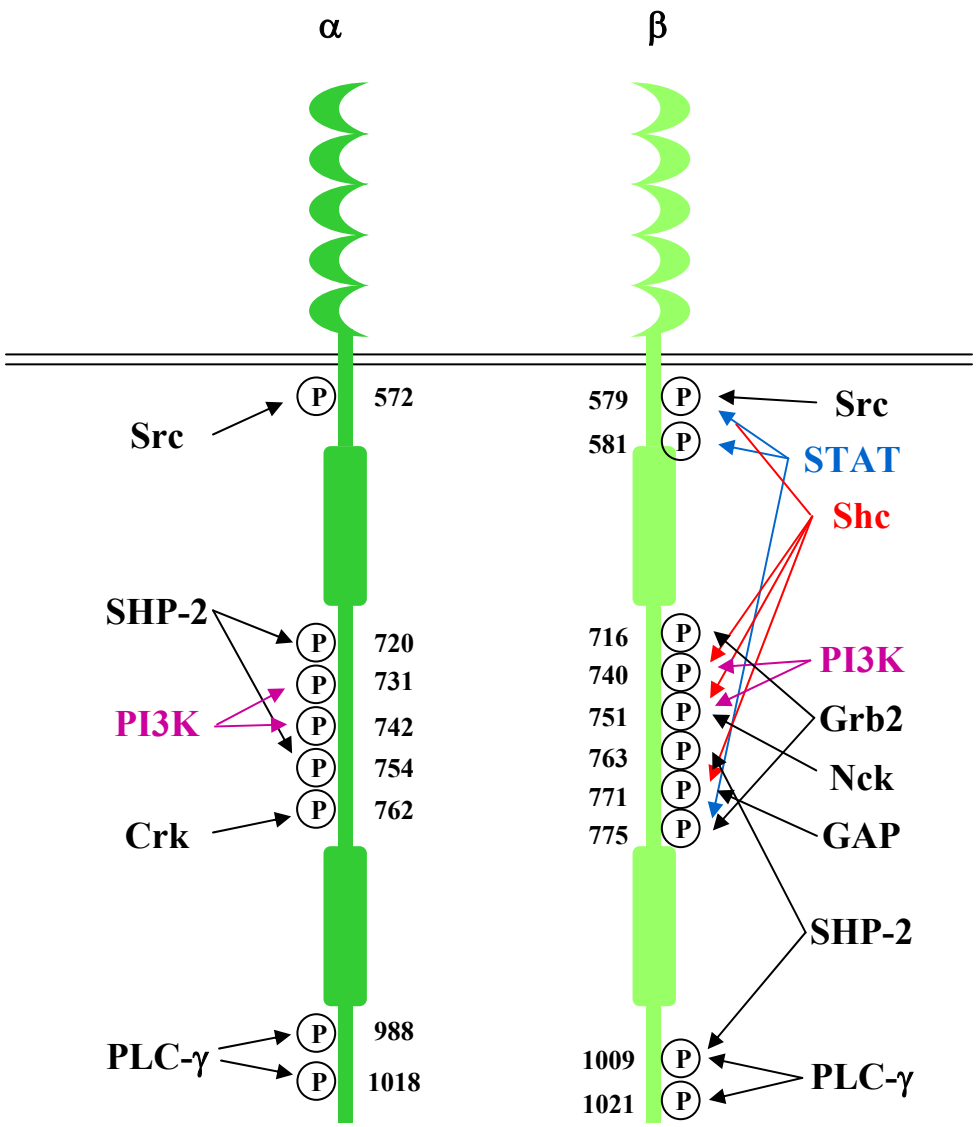


Figure 1.3 Binding sites for adaptor and signalling proteins created by tyrosine phosphorylation of PDGFR isoforms. The PDGFR-α is phosphorylated at 8 sites and is known to bind to 5 different protein families. The PDGFR-β is phosphorylated at 10 sites and is known to bind to 9 different protein families.

The two PDGFR isoforms have overlapping biological functions for the cell (Table 1.1)(Claesson-Welsh, 1994).

Table 1.1 The Physiological Effects of the PDGFR Isoforms.

PDGFRα	PDGFRβ
Proliferation	Proliferation
Migration*	Migration
Membrane Ruffles	Membrane Ruffles
Cytoskeletal Rearrgement	Cytoskeletal Rearrgement
Calcium Flux	Calcium Flux
Phosphatidylinositol Turnover*	Phosphatidylinositol Turnover
Timing of Differentiation	Angiogenesis

* cell type dependent

Not only do the two isoforms have some specialized biological functions, but they are also expressed at different levels in various cell types. For example, in smooth muscle cells, the PDGFRβ is expressed at levels 10 times higher than PDGFRα (Bornfeldt *et al*., 1995). Both PDGFR α and β are expressed in fibroblasts, kidney mesangial cells, Leydig cells, vascular smooth muscle cells, neurons, Schwann cells and retinal pigment epithelial cells (Heldin and Westermark, 1999). Only PDGFRα is expressed in liver sinusoidal endothelial cells, astrocytes, platelets and megakaryocytes, and PDGFRβ is the only isoform expressed in Itoh cells of the liver, myoblasts, capillary endothelial cells, pericytes, mammary epithelial cells, T cells, myeloid hematopoietic cells and macrophages (Heldin and Westermark, 1999). This means that the different cell types can have different responses to the same PDGF stimulation, which causes the tissues to act in different ways when stimulated in the same way.

After the PDGFR is activated it is endocytosed (discussed below). The internalized receptor continues to signal until it is downregulated. There are two

methods of downregulation. Immediate downregulation is accomplished via dephosphorylation while the definitive long-term downregulation occurs by receptor degradation (discussed below). Upon PDGF stimulation of the receptor only 30 to 40 % of the total amount of the receptor in the cell is degraded (Chiarugi *et al*., 2002). This allows the PDGFR to be stimulated several times before the receptor levels are depleted. The major mechanism for deactivation of the receptor is dephosphorylation by tyrosine phosphatases (Chiarugi *et al*., 2002).

1.3 Signalling Pathways from PDGFR

There are many signalling pathways that are activated by the PDGFRs (Fig. 1.3). Two major pathways that are activated by the PDGFR are the Ras/MAPK and PI3K/Akt signalling pathways (Sections 1.3.1 and 1.3.2). These pathways are involved in many cellular events that are important for cell survival and proliferation. There are also several other signalling pathways activated from the PDGFR.

Several signalling pathways are activated by both isoforms of the PDGFR, including the Src, phospholipase C γ (PLC-γ), SH2 domain-containing phosphatase 2 (SHP-2) and PI3K pathways. The Src protein is a cytoplasmic tyrosine kinase that binds to the receptor and activates pathways involved in cell division and cytoskeletal rearrangement (Erpel and Courtneidge, 1995). PLC-γ is a phospholipase C that converts phosphatidylinositol 4,5-bisphosphate into inositol 1,4,5-trisphosphate and diacylglycerol that mobilize cellular Ca^{++} and activate several members of the protein kinase C family (Heldin and Westermark, 1999). SHP-2 is a tyrosine phosphatase that dephosphorylates the PDGFR to turn off its signalling (Heldin and Westermark, 1999).

The alpha isoform of the PDGFR also binds Crk, an adaptor protein that binds to several proteins including the E3 ubiquitin ligase Cbl (Yokote *et al*., 1998). There are also several proteins that bind only the beta isoform of the PDGFR. These include growth factor receptor-bound protein 2 (Grb2), Nck, GTPase activating protein (GAP), signal transducer and activator of transcription (STAT) and Src homologous and collagen-like protein (Shc). Grb2 is an adaptor protein that is part of the Ras/MAPK signalling pathway. Nck and Shc are also adaptor proteins that activate downstream signalling pathways. Nck interacts with PAK1 and NIK to activate the JNK/SAPK signalling pathway (Heldin and Westermark, 1999). Shc binds to Grb2, activating the Ras/MAPK signalling pathway (Heldin and Westermark, 1999). GAP is a RasGAP that inactivates the Ras/MAPK signalling pathway (Heldin and Westermark, 1999). The STAT family of proteins are transcription factors with several family members that bind to PDGFR and are phosphorylated. This causes them to dimerize and translocate into the nucleus where they function (Heldin and Westermark, 1999).

1.3.1 The Ras/MAPK Signalling Pathway

The Ras/MAPK signalling pathway is important for cell proliferation, one signalling output of growth factor receptors including PDGFR. The importance of this signalling pathway in this thesis is that the phosphorylation of MAPK is used as a measurement of signalling output from the activated PDGFR. The Ras/MAPK signalling pathway starts with Shc binding to the activated PDGFR through the phosphotyrosines 579, 740 and 771 (Claesson-Welsh, 1994; Heldin *et al*., 1998). This allows the receptor to phosphorylate Shc on tyrosine 317, which forms a binding site for the Grb2-Sos complex (Heldin *et al*., 1998). Grb2 can also bind directly to the PDGFR

through interaction with phosphotyrosines 716 and 775 (Claesson-Welsh, 1994; Heldin *et al*., 1998). Sos (Son of Sevenless) is a guanine nucleotide exchange factor (GEF) for the monomeric G protein Ras; therefore, Sos activates Ras by causing it to release GDP and bind GTP (Anderson, 2006; Heldin *et al*., 1998; Heldin and Westermark, 1999). Activated Ras binds to and activates the Raf serine/threonine kinase (Avruch *et al*., 1994; Barnard *et al*., 1995). MAPK/Erk kinase (MEK), is activated by phosphorylation on serine residues 218 and 222 by Raf (Zheng and Guan, 1994). MEK is a duel threonine/tyrosine kinase which continues the signalling cascade by phosphorylating mitogen activated protein kinase (MAPK), also known as extracellular regulated kinase (ERK), on threonine 183 and tyrosine 185 (Payne *et al*., 1991). Activated MAPK translocates into the nucleus where it regulates various transcription factors including Myc, Fos and Jun by phosphorylation (Fig. 1.4) (Pelengaris and Khan, 2006). These transcription factors are involved in the expression of genes required for cell cycle progression and cell proliferation.

1.3.2 The PI3K/Akt Signalling Pathway

When the PDGFR is activated, the receptor is phosphorylated on multiple tyrosine residues including 740 and 751 (Fig. 1.3)(Claesson-Welsh, 1994; Heldin *et al*., 1998). This forms a binding site for the SH2 domains of p85 (Piccione *et al*., 1993; Shoelson *et al*., 1993). p85 is the regulatory subunit of phosphatidylinositol 3'-kinase (PI3K) (Carpenter and Cantley, 1996; Fruman *et al*., 1998). This regulatory subunit is associated with the catalytic subunit, p110; therefore, p85 brings p110 to the activated receptor and the plasma membrane where PI3K is activated. The main products of p85-p110 are phosphatidylinositol 3,4-bisphosphate ($PI3,4P_2$) and phosphatidylinositol 3,4,5-

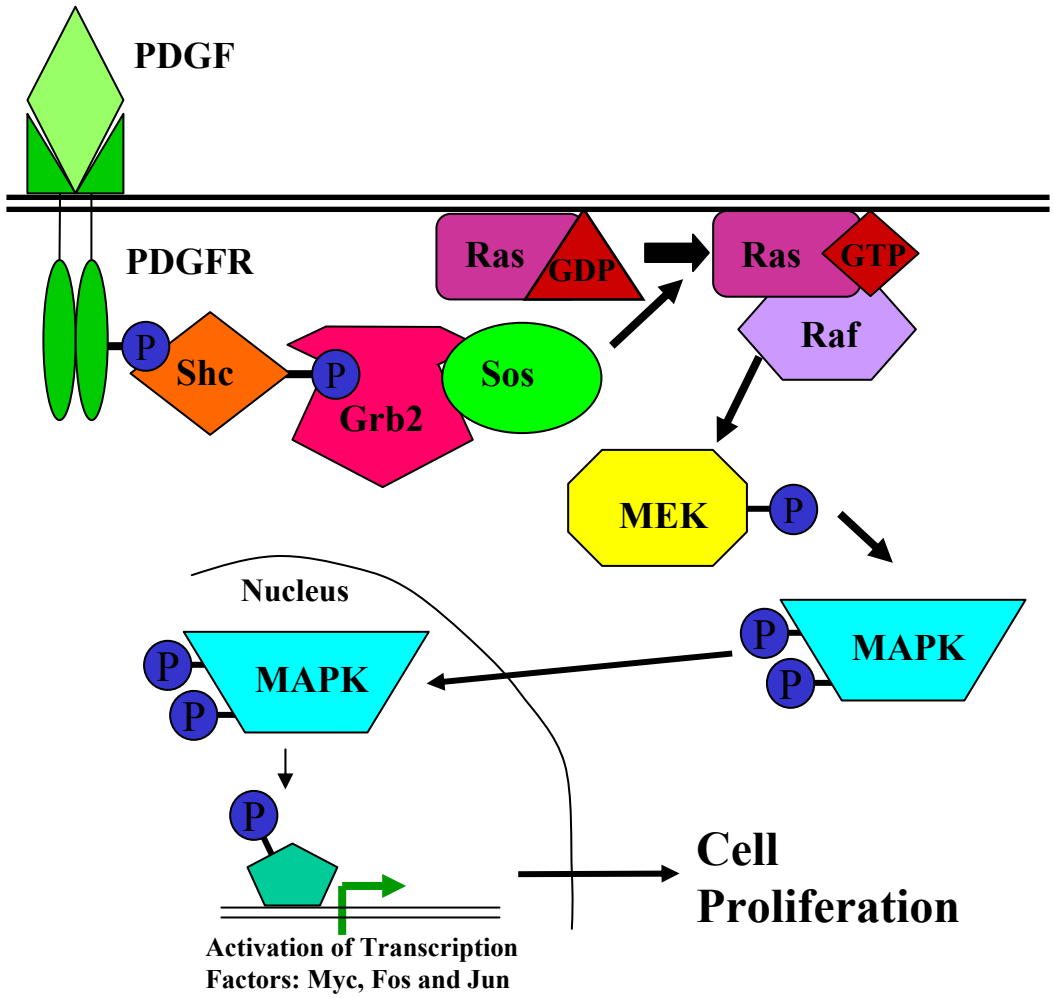


Figure 1.4 The Ras/MAPK signalling pathway. The binding of PDGF to PDGFR activates it to dimerize and autophosphorylate. This forms a binding site on the PDGFR for Shc. The receptor phosphorylates Shc, forming a binding site for Grb2, which is bound to Sos. The relocalized Sos activates Ras by exchanging GDP for GTP. Ras-GTP binds Raf, activating it to phosphorylate MEK, which phosphorylates MAPK. MAPK enters the nucleus and regulates various transcription factors including Myc, Fos and Jun.

trisphosphate (PI3,4,5P_3) (Carpenter and Cantley, 1996; Fruman *et al*., 1998). These lipids are dephosphorylated by the lipid phosphatase PTEN (phosphatase and tensin homolog (mutated in multiple advanced cancers 1)). PTEN removes the phosphate from the 3' position of the phosphatidylinositol, which was added by PI3K, downregulating this signalling pathway (Maehama and Dixon, 1998). PI3,4,5P_3 and PI3,4P_2 lipids act as second messengers in several important cellular pathways. They cause Akt to migrate to the plasma membrane where it is activated by phosphorylation of threonine residue 308 by phosphoinositide (3)-dependent kinase 1 (PDK1) (Song *et al*., 2005). Complete activation of Akt also requires phosphorylation of serine residue 473. The kinases responsible for phosphorylation of Ser473 are not well understood since several possible kinases may phosphorylate this site. Four possibilities are phosphoinositide (3)-dependent kinase 2 (PDK2), PDK1, integrin-linked kinase (ILK) or Akt itself (Song *et al*., 2005). More recently it has been suggested that Ser473 is phosphorylated by the TORC2 complex (Jacinto *et al*., 2006). Akt is a serine/threonine kinase that phosphorylates many downstream proteins including glycogen synthase kinase 3 (GSK3), Bad, S6 kinase, murine double minute 2 (Mdm2), FoxO Forkhead and nuclear factor-kappa B (NF-κB) (Fig. 1.5)(Song *et al*., 2005). The downstream effects of the PI3K/Akt signalling pathway include cell survival, protein synthesis, cell growth and gene expression.

1.3.2.1 Phosphatidylinositol 3'-kinase

The PI3K family of kinases is a large super-family with three classes of lipid kinases. Class I PI3K is a heterodimer that consists of a p110 catalytic subunit and a p85 regulatory subunit and are the only PI3Ks activated in response to RTK activation

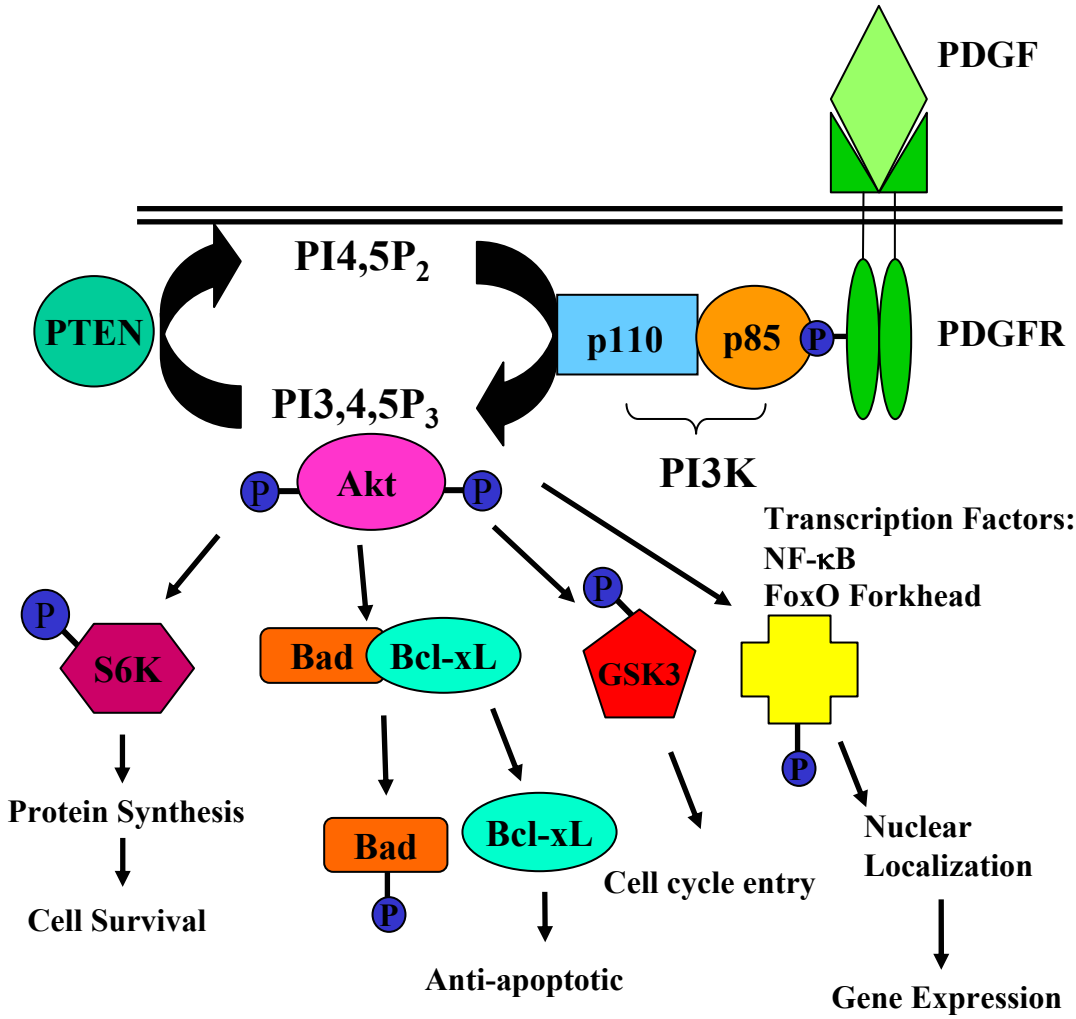


Figure 1.5 The PI3K/Akt signalling pathway. The binding of PDGF to the PDGFR activates it to dimerize and autophosphorylate. This forms a binding site on the PDGFR for the regulatory subunit of PI3K, p85, which recruits p110 to the membrane where it can phosphorylate $PI4,5P_2$ to form $PI3,4,5P_3$. This lipid product recruits Akt to the membrane which allows it to be activated. Akt phosphorylates several other downstream proteins that activate cell survival and proliferation pathways.

(Carpenter and Cantley, 1996; Fruman *et al*., 1998). PI3K can also be activated through direct interactions between the p110 catalytic subunit and Ras-GTP, although p85 can inhibit this activation (Jimenez *et al*., 2002; Kodaki *et al*., 1994; Kodaki *et al*., 1995). There are several isoforms of each subunit. For the catalytic subunit there are the p110α, β, γ and δ isoforms (Fig. 1.6)(Fruman *et al*., 1998).. For the regulatory subunit there are the p85α and β isoforms, as well as subunits of different sizes p55γ and p101 (Fig. 1.6)(Fruman *et al*., 1998).

The p85α subunit also has two splice variants, p55α and p50α, which lack the N-terminal portion of the protein (Fruman *et al*., 1998). The p110 subunit is both a protein serine kinase and a lipid kinase that can catalyze the phosphorylation of phosphatidylinositol (PI), PI4-phosphate (PI4P) and PI4,5-bisphosphate ($PI4,5P_2$) to produce the lipid products phosphatidylinositol 3-phosphate (PI3P), $PI3,4P_2$ and $PI3,4,5P_3$ respectively (Carpenter and Cantley, 1996). While p85-p110 can generate PI3P *in vitro*, the main physiological products of p85-p110 are the phospholipids $PI3,4P_2$ and $PI3,4,5P_3$. These phosphorylated lipids act as second messengers in several important cellular pathways. The p85 regulatory subunit controls p110 lipid phosphorylation activity by inhibiting the activity of p110 until p85 binds the receptor. This causes a conformational change that relieves the inhibitory effects of p85 (Yu *et al*., 1998). The p85 subunit also localizes the p110 catalytic subunit in the cytosol in quiescent cells and then relocates it to the plasma membrane through interactions with activated growth factor receptors, including the PDGFR (Engelman *et al*., 2006).

The p85 subunit has a domain structure that includes several protein-protein interaction domains (Fig. 1.7). These domains are an N-terminal Src homology 3 (SH3)

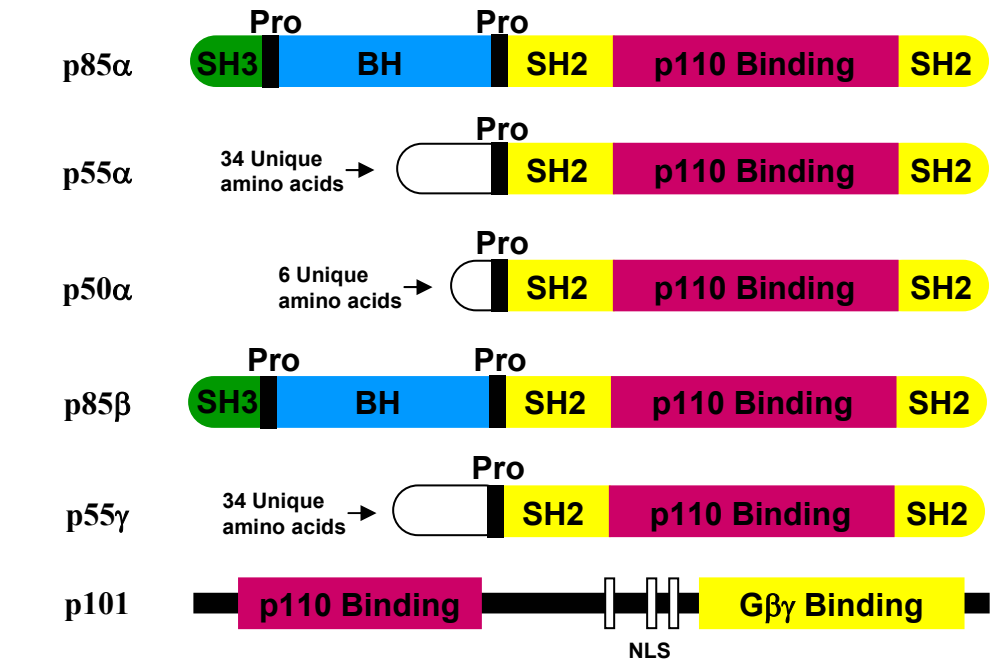


Figure 1.6 The isoforms of PI3K. There are six different regulatory subunits of p85. The first three subunits, p85α, p55α and p50α are formed from alternative splicing of a single gene, while the other three regulatory subunits are from different genes. The regulatory subunit p101 is the only one that binds to p110γ. The p101 protein also has a different domain structure from the other regulatory subunits and interacts with G protein coupled receptors. All of the other subunits have the same domain structures and bind to RTKs. The four p110 isoforms are all similar in domain structure containing a p85 binding domain, a Ras binding domain, two domains of homology (HD) and a kinase domain except for p110γ that has a lipid binding, pleckstrin homology (PH) domain instead of a p85 binding domain.

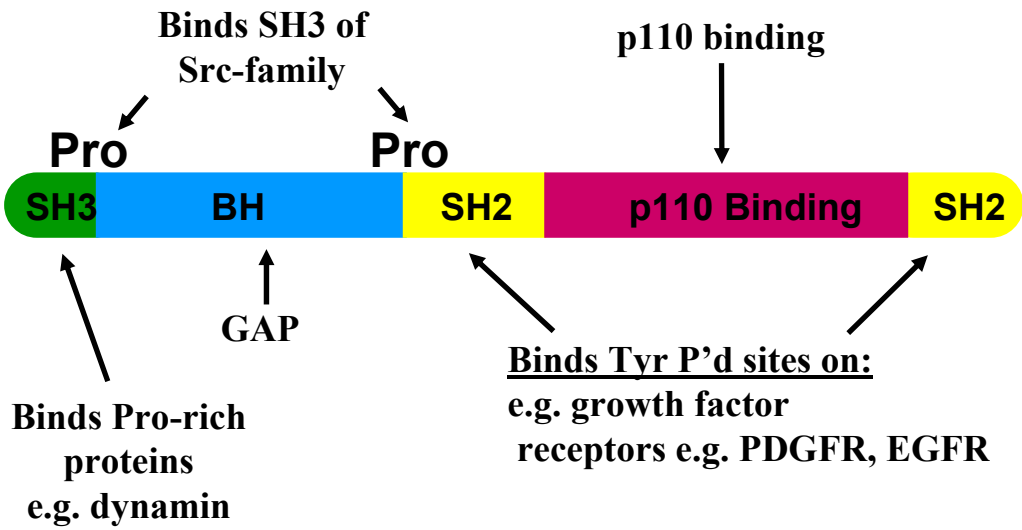


Figure 1.7 The domain structure of PI3K. The p85 regulatory subunit of PI3K contains several modular domains. An SH3 domain binds proline-rich regions. A BH domain has GAP homology and is being tested for GAP activity in this thesis. The BH domain is flanked by two proline-rich regions that bind to SH3 domains. The C-terminus of the protein has the p110 binding domain flanked by two SH2 domains that bind to phosphorylated tyrosines on activated growth factor receptors.

domain, a breakpoint cluster region homology (BH) domain flanked by two proline rich regions, an N-terminal SH2 domain, a p110 binding domain that binds the PI3K catalytic subunit and a C-terminal SH2 domain (Fruman *et al*., 1998). The SH3 domain of p85 binds the consensus motif φ-P-p-φ-P, where P is a proline, p is a weakly conserved proline and φ is an aliphatic amino acid. Although the SH3 domain is not well characterized in terms of function, it is known to interact with the GTPase dynamin, focal adhesion kinase, Cbl and the N-terminal proline rich region in p85 causing p85 to dimerize (Booker *et al*., 1993; Guinebault *et al*., 1995; Harpur *et al*., 1999; Hunter *et al*., 1997). The BH domain can also dimerize although the function of this interaction is unknown (Harpur *et al*., 1999). The proline rich regions of p85 are also not well characterized for function but they are known to bind the SH3 domains of Src family tyrosine kinases and the Abl tyrosine kinase (Kapeller *et al*., 1994; Pleiman *et al*., 1994). The BH domain contains sequence homology to GTPase activating proteins (GAPs) and will be discussed in greater detail later. The two SH2 domains bind to the phosphorylated tyrosine sequence pY-X-X-M (where pY is phosphotyrosine) in proteins such as activated receptors, to activate PI3K (Piccione *et al*., 1993; Shoelson *et al*., 1993).

The class II PI3K is a single catalytic protein with no regulatory protein. There are three proteins in this class C2α, C2β and C2γ (Carpenter and Cantley, 1996; Fruman *et al*., 1998). The main lipid product of the class II PI3K is PI3P. The class III PI3K is a heterodimer between the p150 regulatory subunit and the hVps34 catalytic subunit (Carpenter and Cantley, 1996; Fruman *et al*., 1998). The class III PI3K is involved in membrane trafficking (discussed in detail later) and its main lipid product is also PI3P.

1.4 Endocytosis

Endocytosis is the process by which a cell internalizes extracellular material. There are two types of endocytosis: phagocytosis and pinocytosis (Fig. 1.8). Phagocytosis means "to eat" and is the uptake of large material by the cell such as apoptotic bodies, bacteria and viruses. In the body, specialized cells like macrophages primarily perform phagocytosis but any cell type can be induced to phagocytose material under specific circumstances such as the uptake of apoptotic bodies (Mukherjee *et al*., 1997). Pinocytosis means "to drink" and is the uptake of smaller material such as proteins, nutrients and solutes. There are several types of pinocytosis but the three best characterized are: macropinocytosis, caveolae endocytosis and clathrin-mediated endocytosis (Fig. 1.8)(Mukherjee *et al*., 1997).

Macropinocytosis is the non-specific bulk uptake of extracellular fluid. The cell membrane invaginates and pinches off to form a vesicle. This type of endocytosis tends to occur at the leading edge of migrating cells and at cellular ruffles. Caveolae endocytosis is a specific process where the uptake happens at special structures called caveolae. Caveolae, which means little cave, are enriched in cholesterol and glycosphingolipids and are thought to be a specialized form of lipid raft (Anderson, 1998; Miaczynska and Zerial, 2002). They are invaginations of the plasma membrane containing the protein caveolin. Caveolin binds to the membrane in a spiral pattern that may force the membrane to curve in to form the caveolae (Anderson, 1998). Caveolae are involved in the uptake of receptors and lipids. Clathrin-mediated endocytosis is the best studied type of endocytosis. Clathrin is a protein lattice that forms a mesh around the membrane to form a clathrin-coated pit (Edeling *et al*., 2006). Clathrin-mediated

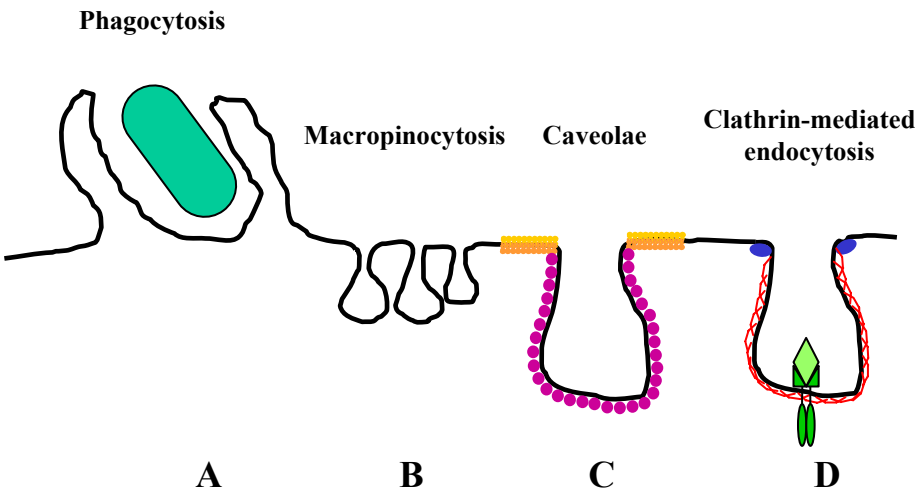


Figure 1.8 Types of Endocytosis. A) Phagocytosis is the uptake of large molecules such as bacteria or apoptotic bodies. B) Macropinocytosis is the bulk uptake of fluid and small molecules that happens at the leading edge of cells or membrane ruffles. Several invaginations of the cell membrane form in close proximity to each other. C) Caveolae form in lipid rafts (yellow) and endocytose several types of lipids and receptors. Caveolin (purple) binds to the membrane and forms a coat that causes the membrane to invaginate. D) Clathrin-mediated endocytosis is the main method by which receptors (green) are endocytosed. A clathrin cage (red) forms around the invaginating membrane containing the receptors. The invaginated membrane is pinched off the plasma membrane by dynamin (Blue).

endocytosis is the main route for the endocytosis of several receptors so it is also called receptor-mediated endocytosis (reviewed in greater detail below).

Regardless of the initial method of endocytosis, all material taken up by the cell is routed through the endosomal pathway. The endosomal pathway is made up of several dynamic non-permanent fluid membrane structures called vesicles and endosomes. Vesicles are small membrane structures that transport proteins between the endosomal structures. Endosomes are larger membrane structures, semi-permanent in nature. The main types of endosomes are early/sorting endosomes, recycling endosomes and late endosomes (Miaczynska and Zerial, 2002). Vesicles that originate at the plasma membrane from the various forms of endocytosis fuse together and form an early/sorting endosome. Material in the early endosome can be sorted either for degradation in the lysosome through the late endosome, or recycled back to the plasma membrane directly or via the recycling endosome (Fig. 1.9). The flow of material through the endosomal pathway will be explained in more detail below using receptors as an example. The endosomal organelles are distinct structures that perform specific functions but at the same time are fluidly and dynamically connected to each other. So the question is how do they maintain their structure, specificity and directionality of protein trafficking. Some models suggest that they may be composed of distinct protein and lipid domains that self-assemble, which allows these endosomal domains to maintain their organization as they exchange material (Miaczynska and Zerial, 2002).

1.4.1 Rab Proteins

A family of monomeric G proteins called Rab proteins regulate the composition of these endosomal domains as well as the fusion and fission of endosomes. There are

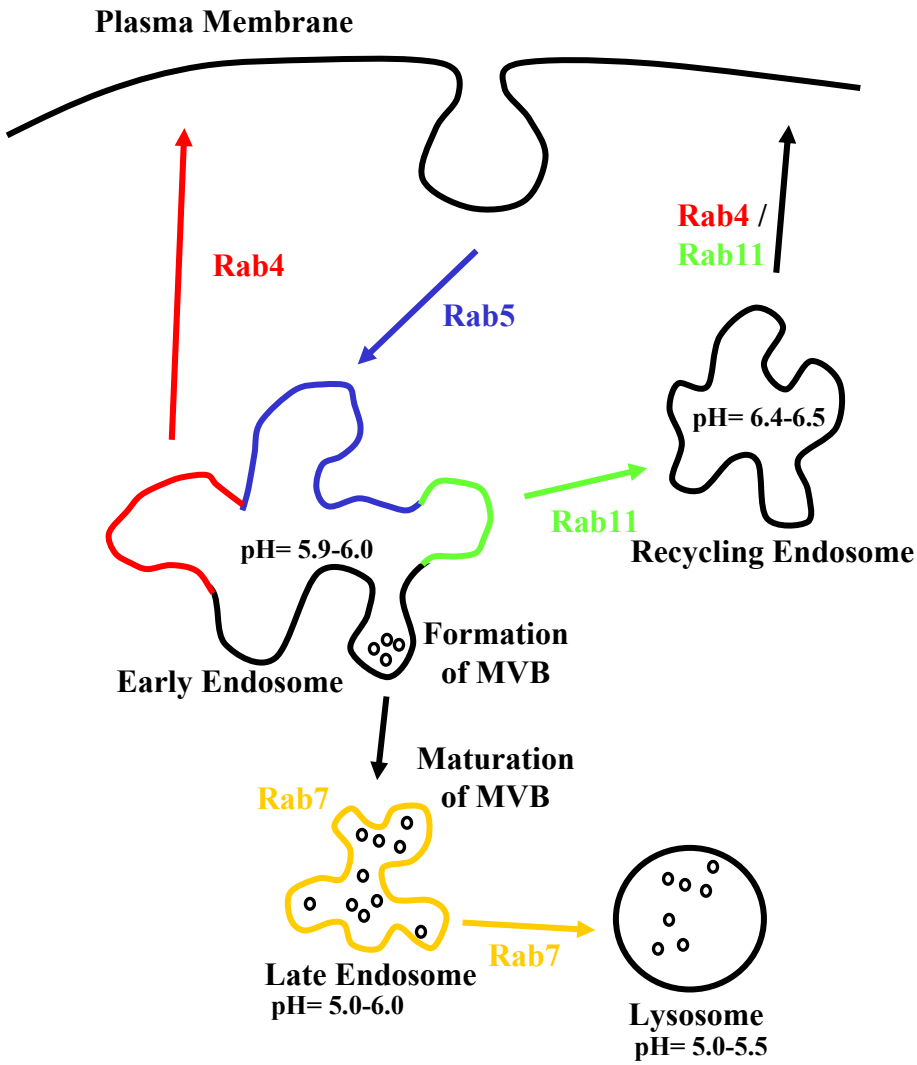


Figure 1.9 Rab proteins involved in the Endosomal Pathway. Rab5 (blue) allows the fusion events between the plasma membrane and the early endosome to take place. Rab4 (red) is involved with recycling membranes and receptors back to the plasma membrane from the early endosome and recycling endosome. Rab11 (green) is involved in transport through the recycling endosome. The multivesicular body (MVB) form in the early endosome and mature to form the late endosome. Rab7 (orange) is on the late endosome and allows the fusion of the late endosome to the mature MVB and the lysosome. The early endosome contains at least four domains that are involved in these different functions.

more than 60 Rab proteins that have been reported in the literature (Seabra and Wasmeier, 2004; Stenmark and Olkkonen, 2001). Each Rab protein governs a specific function of vesicle trafficking or endosomal domain structure (Fig. 1.9). Some of the Rab proteins, such as Rab5, are ubiquitously expressed in all tissues. While other Rab proteins have very specific functions and are expressed in only a few tissues such as Rab27 involved in the movement of melanosomes to the periphery of melanocytes and the lytic granules in cytotoxic T cells (Stenmark and Olkkonen, 2001).

Rab proteins have two different states and they act as molecular switches by alternating between these states. The active state is the GTP-bound conformation and the inactive state is the GDP-bound conformation (Seabra and Wasmeier, 2004; Stenmark and Olkkonen, 2001). Binding of the GTP molecule to the Rab protein causes a conformational change in two switch regions of the Rab protein allowing effector proteins to bind (Pereira-Leal and Seabra, 2000). This can be seen in the crystal structure of Sec4p, a yeast Rab, bound to both GDP and a GTP analog (Fig. 1.10)(Deneka *et al*., 2003).

The inactive, GDP-bound form of Rab proteins are found in the cytosol bound to a guanine dissociation inhibitor (GDI) protein (Fig. 1.11). The GDI binds the Rab protein and forms a pocket around the prenyl group used by the Rab protein to associate with the membrane. The prenyl group of the Rab protein is inserted into the membrane with the help of the GDI-displacement factors (GDFs). The active, GTP-bound form of Rab proteins can be associated with endosomes, vesicles or other cellular structures. Two classes of proteins, GEFs and GAPs, facilitate the conversions between these conformations (Fig. 1.10)(Seabra and Wasmeier, 2004; Stenmark and Olkkonen, 2001). GEFs facilitate the exchange of bound GDP to GTP whereas GAPs stimulate the

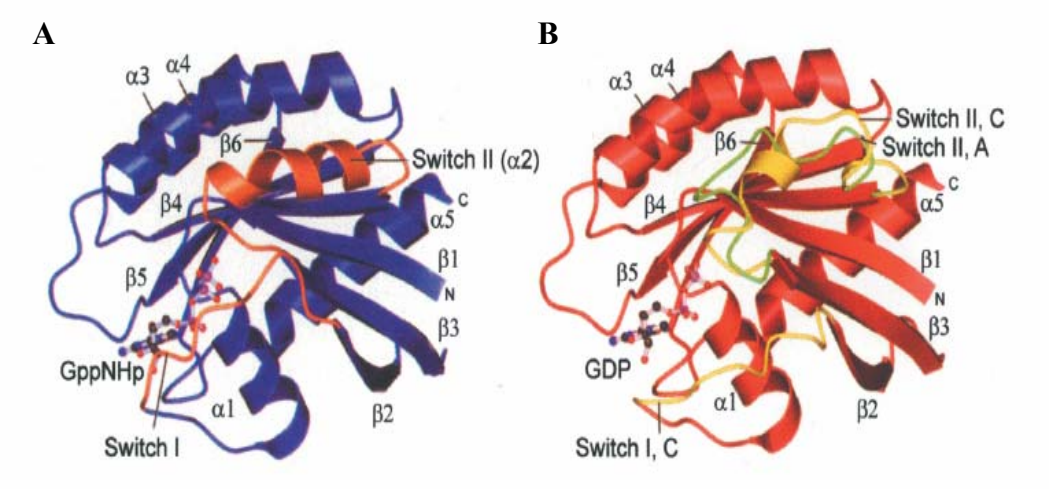


Figure 1.10 Conformational change of the Switch Regions. The crystal structures of Sec4p, a yeast Rab protein, show the conformations when binding GTP and GDP. A) The conformation of active Sec4p bound to GTP. B) The conformation of inactive Sec4p bound to GDP. Modified from Deneka, *et al.*, 2003.

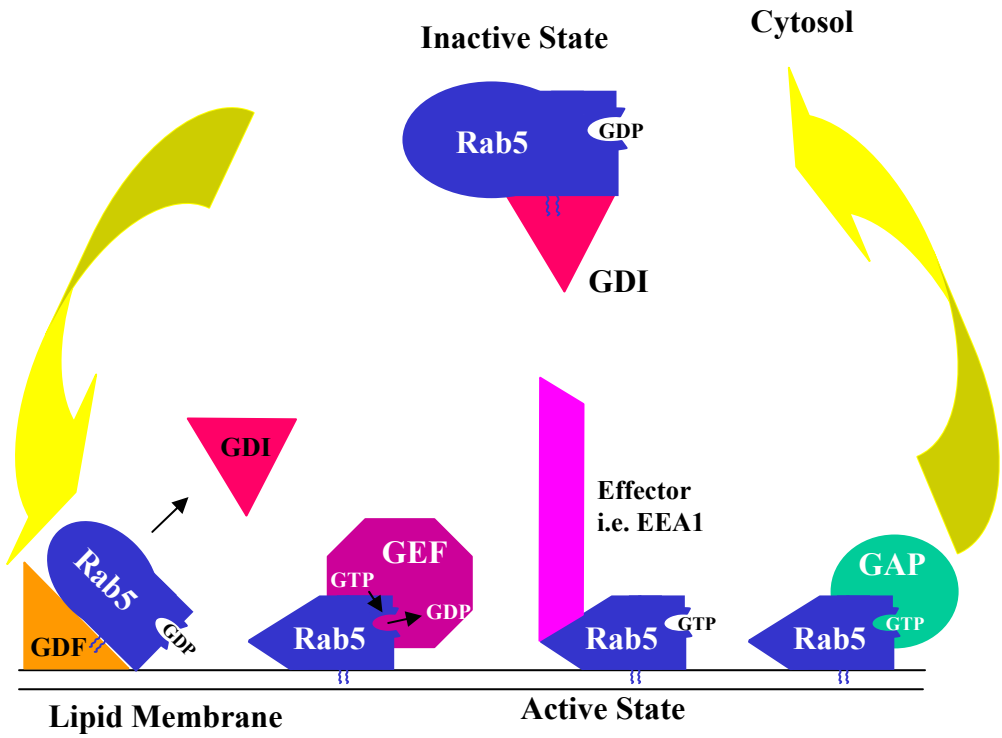


Figure 1.11 Regulatory proteins of Rab function and model of Rab cycle. The inactive Rab5-GDP is bound to GDI in the cytosol. The Rab5-GDP is then recruited to the membrane, dissociates from GDI and is inserted into the membrane by the GDF complex. A GEF protein then causes Rab5 to release the GDP and bind to GTP. The active Rab5-GTP binds to effector proteins such as EEA1 to carry out its function. GAP proteins stimulate Rab5 to hydrolyse bound GTP to GDP to inactivate Rab5 and GDI removes it from the membrane.

intrinsic GTPase activity of the Rab protein to hydrolyse bound GTP to GDP. The inactive Rab protein is removed from the membrane and binds GDI.

There are still many questions surrounding the method by which Rab proteins are inserted into membranes, but some of the basics are known. There are only two known GDI proteins in humans that act as carriers of all the Rab proteins in the cytosol (Stenmark and Olkkonen, 2001). There is also a protein with high homology to the GDI proteins called Rab escort protein. It has exactly the same function as the GDI but it only binds to newly synthesized Rab proteins and allows them to be prenylated by geranylgeranyl transferase so that they can be inserted into the membrane (Stenmark and Olkkonen, 2001). This lipid modification is added to the C-terminal end of the Rab protein in a cysteine motif (CXXX, CC, CXC, CCXX or CCXXX where X is any amino acid) (Stenmark and Olkkonen, 2001). With only two GDIs and 60 Rab proteins in humans, the membrane targeting of the Rab proteins cannot come from the GDI.

The mechanism of this targeting to a specific endosomal domain is currently unknown, but it is thought that the C-terminal hypervariable region of the Rab protein interacts with unidentified targeting factors on the membrane (Pfeffer and Aivazian, 2004). After the Rab-GDI complex associates with the membrane, the Rab protein must be associated with the membrane by insertion of the lipid group into the membrane. The disassociation of the Rab protein from GDI and the insertion of the lipid group into the membrane are carried out by a complex of proteins called GDI-displacement factors (GDFs). The GDFs in humans have not been well characterized but in yeast the Yip proteins function as GDFs (Pfeffer and Aivazian, 2004). Once the Rab protein is associated with the membrane, it will interact with a GEF and become activated by the

exchange of its GDP with GTP. The activated Rab protein recruits effector proteins to the endosome/vesicle to promote membrane fusion or fission.

The best described example is fusion of vesicles from the plasma membrane with the early endosome, which is governed by the Rab5 protein. The active form of Rab5 is associated with the early endosome or clathrin-coated vesicles (Armstrong, 2000; Mills *et al*., 1999; Mohrmann and van der Sluijs, 1999; Somsel Rodman and Wandinger-Ness, 2000; Ullrich *et al*., 1994). The active Rab5-GTP, along with PI3P, a lipid product of class III PI3K, recruits the effector protein early endosomal antigen 1 (EEA1) to the endosome. The presence of EEA1 is an absolute requirement for the fusion event between endosomes (Christoforidis *et al*., 1999a; Mills *et al*., 1998; Rubino *et al*., 2000; Simonsen *et al*., 1998). EEA1 tethers the two membranes together, allowing the formation of a soluble NSF (N-ethylmaleimide sensitive factor) attachment receptor (SNARE) complex to form that fuses the membranes together (Fig. 1.12) (McBride *et al*., 1999). Membrane fusion by SNARE complexes is the method by which most endosomal compartments are formed and is how receptors are transported through the endosomal system.

1.4.2 Receptor-mediated Endocytosis

Receptor-mediated endocytosis is the main method of permanent downregulation of signalling receptors and the major pathway of nutrient uptake by receptors such as iron by the transferrin receptor (Conner and Schmid, 2003). When a growth factor receptor is activated on the plasma membrane, it migrates to a clathrin-coated pit (Fig. 1.12). In the clathrin-coated pit, the receptor associates with several adaptor proteins that link the receptor to clathrin and lock it into the invaginating membrane. There are at

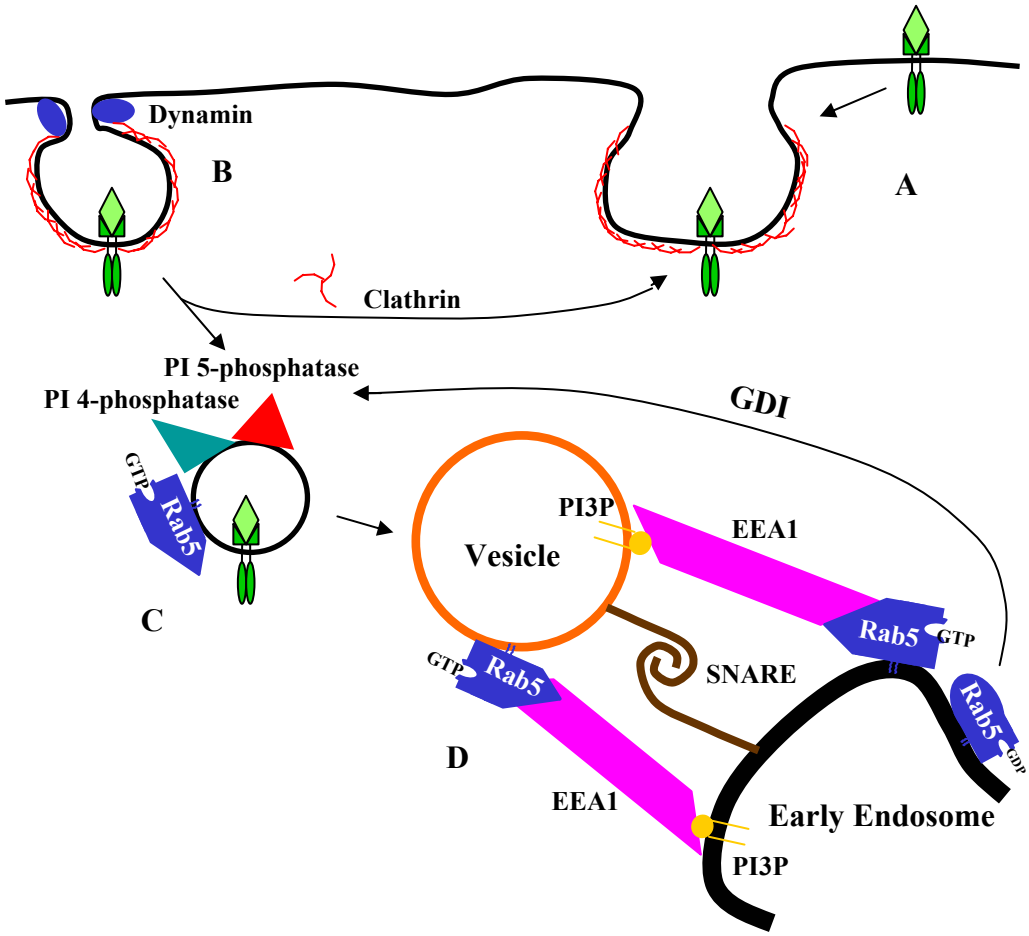


Figure 1.12 Regulation of receptor transport and endosomal fusion. A) The activated receptor travels into the clathrin-coated pit during the signalling process. B) The clathrin-coated pit is pinched off from the plasma membrane by dynamin. C) The vesicle is uncoated and the clathrin is recycled to a newly forming pit. Also Rab5-GTP binds to vesicles and binds effector proteins including PI 4-phosphatase and PI 5-phosphatase that convert PI3,4,5P_3 to PI3P. D) Rab5-GTP and PI3P bind to EEA1 to form a tether between vesicles and the early endosome. This brings the vesicle into close proximity to the endosome allowing the SNARE complex to form and fuse the two membranes together. After the fusion of the membranes, some of the Rab5-GTP is downregulated to Rab5-GDP and recycled by GDI back to the newly forming vesicles to continue the fusion process.

least 20 adaptor proteins that are involved in binding of receptors, clathrin and the lipid membrane. Adaptor proteins such as AP2 can interact with both the membrane, via a PI4,5P_2 binding domain and to the cargo (i.e. the receptor) via several protein binding motifs that bind YXXΦ (where X is any amino acid and Φ is a hydrophobic amino acid), NPXY or acidic dileucine motifs (eg. [DE]XXXL[LI]) (Bonifacino and Traub, 2003; Owen *et al*., 2004). Other adaptor proteins can also bind cargo via a ubiquitin-binding site (Bonifacino and Traub, 2003; Owen *et al*., 2004). This allows complexes of adaptor proteins and other proteins to bind together, locking cargo into the clathrin-coated pit. These adaptor complexes vary depending on the cargo being endocytosed. The invaginated membrane is pinched off from the plasma membrane by the large GTPase dynamin and the clathrin coat is dissociated from the nascent vesicle (Hinshaw, 2000).

Rab5-GTP is recruited to the forming vesicle to target the new vesicles to the early endosome (Section 1.4.1). The Rab5 GAP, RN-tre, regulates the formation of the new vesicles that bud from the plasma membrane. RN-tre interacts with Grb2 and Eps8 at the plasma membrane to decrease the rate of EGFR endocytosis (Lanzetti *et al*., 2000; Martinu *et al*., 2002). This allows the proper amount of EGFR signalling to take place and overexpression of RN-tre has been linked to cancer (Matoskova *et al*., 1996). The active Rab5 recruits several effector proteins including EEA1. Rab5 also recruits two lipid phosphatases, PI 4-phosphatase and PI 5-phosphatase, which convert PI3,4,5P_3 to PI3P on the early endosome (Shin *et al*., 2005). This suggests that althought the class III PI3K is thought to be responsible for PI3P generation on the early endosome some of the PI3P can come from class I PI3Ks. Multiple vesicles fuse together and then fuse with the early endosome. This traffics the activated receptor complex into the early endosome. Throughout this process and in the early endosome, the activated receptor

complex continues to signal (Burke *et al*., 2001; Cavalli *et al*., 2001a; Wang *et al*., 2004; Wiley and Burke, 2001). At some point in the endosomal pathway the ligand dissociates from its receptor due to the change in pH. The pH in the endosomal compartments decreases as the endosomes mature towards the lysosome (Fig. 1.9)(Maxfield and McGraw, 2004). The receptor is also dephosphorylated by phosphatases to deactivate it that are recruited to the activated receptor either directly or indirectly by phosphorylated tyrosines on the receptor (Ostman and Bohmer, 2001). This downregulated receptor is then diverted to either the recycling or degradation pathway. There are two possible fates for the receptor in the early endosome; it is either diverted back to the plasma membrane or targeted for degradation in the lysosome. The exact methods of receptor sorting are not completely elucidated but a basic understanding of each pathway is known.

The early endosome contains three different and distinct Rab domains that function in different roles in the flow of material through the early endosome (Fig. 1.9). These domains are characterized by the content of Rab proteins. There is a Rab5 domain where receptors enter the early endosome (Miaczynska and Zerial, 2002). Rab4 and Rab11 domains from which receptors are recycled back to the plasma membrane (Miaczynska and Zerial, 2002). There is also another domain in the early endosome, which is not known to have a Rab protein associated with it, where receptors are targeted to the late endosome and then to the lysosome for degradation (Cavalli *et al*., 2001a; Deneka *et al*., 2003). It is believed that the receptor is diverted to the recycling pathway by default and that there needs to be a specific molecular mechanism for the receptor to be targeted for degradation (Mayor *et al*., 1993). There are two known methods of receptor recycling that seem to work concurrently (Sheff *et al*., 1999). The

first method, mediated by Rab4, involves direct recycling of the receptor from the early endosome to the plasma membrane. The second method, mediated by both Rab4 and Rab11, involves a less direct route, traveling from the early endosome through the recycling endosome and then to the plasma membrane (Sheff *et al*., 1999).

The process of receptor degradation is complicated but much better characterized. Receptors targeted for degradation travel to a domain of the early endosome that forms a multivesicular body (MVB) that matures into the late endosome (Cavalli *et al*., 2001a; Deneka *et al*., 2003). The late endosome can then fuse with the lysosome to degrade the receptors. Targeting of the receptor for degradation is achieved by the mono-ubiquitylation of the receptor by the protein Cbl (Haglund *et al*., 2003; Holler and Dikic, 2004; Levkowitz *et al*., 1998). Cbl is an E3 ubiquitin ligase that associates with several activated RTKs and mono-ubiquitylates them on several sites.

Mono-ubiquitylated receptors are targeted into the MVB by interactions with ubiquitin binding domains within proteins in the Hrs-STAM complex and the Endosomal Sorting Complex Required for Transport (ESCRT) protein complexes (Raiborg and Stenmark, 2002). There are three ESCRT complexes plus the Hrs-STAM complex that help mono-ubiquitylated receptors enter into the interior vesicles of the MVB (Fig. 1.13)(Hurley and Emr, 2006; Katzmann *et al*., 2002; Row *et al*., 2005). The mono-ubiquitylated receptor first interacts with the Hrs-STAM complex, targeting the receptor to the ESCRT-I complex. Three ESCRT complexes in co-operation target the receptor into the MVB vesicle in the lumen of the endosome and remove the ubiquitin from the receptor. By being sequestered in the interior vesicles of the MVB the receptors cannot interact with any signalling components in the cytosol of the cell. This ensures that the receptors are permanently downregulated and cannot be reactivated by

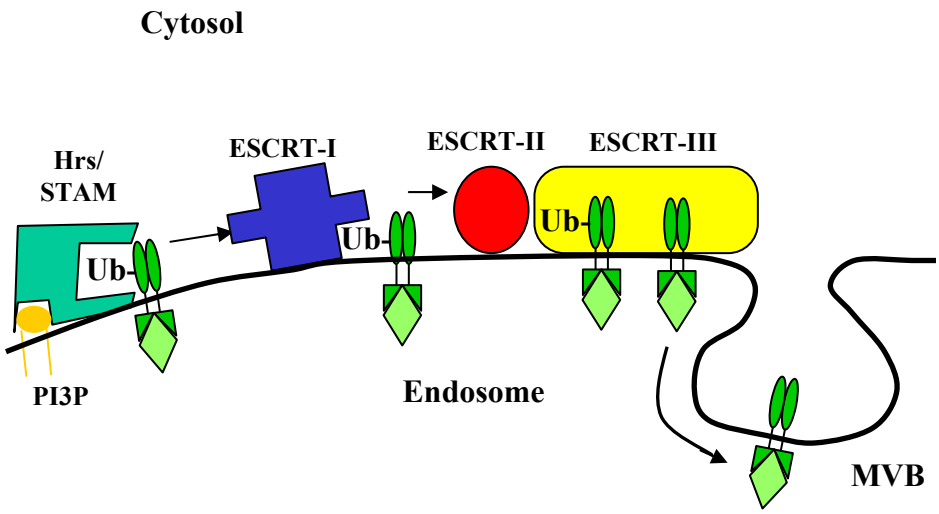


Figure 1.13 Targeting of the mono-ubiquitylated receptors into the interior vesicles of the MVB. The Hrs-STAM complex binds to the mono-ubiquitylated receptor. The receptor is past from the ESCRT-I, ESCRT-II and ESCRT-III complexes and into an invaginating pit in the endosome that forms the MVB. As the receptor enters the forming MVB the ubiquitin tag is removed. Modified from Katzmann, *et al.*, 2002.

mistake. The nascent MVB that is forming from the early endosome matures into a tubular structure that may bud off the early endosome. This MVB then matures into and/or fuses with a late endosome in a Rab7 dependent manner (Bucci *et al*., 2000; Ceresa and Bahr, 2006). This also allows the enzymes of the lysosome access to the receptors to degrade them. Cells must synthesize new receptor proteins to regain their responsiveness to the ligand.

The PDGFR was first shown to be ubiquitylated in 1992 (Mori *et al*., 1992). It was believed at the time to be poly-ubiquitylated and it was shown that the receptor needed to be functional for ubiquitylation to occur and was not ubiquitylated if it was missing the last 98 amino acids of the receptor (Mori *et al*., 1992). It was later shown that PDGFR is mono-ubiquitylation on multiple sites (Haglund *et al*., 2003).

1.5 The Interplay between Signalling and Endocytosis

As more information about signalling and endocytosis pathways has become available, it has become obvious that the two pathways are intimately intertwined. When examining how these two pathways are regulated, there are many places where cross-talk is possible between the two processes. There have been many studies that show that several different receptors are activated and continue to signal not only from the plasma membrane but also from intracellular structures like the early endosome (Burke *et al*., 2001; Miaczynska *et al*., 2004b; Pennock and Wang, 2003; Sorkin and Von Zastrow, 2002; Wang *et al*., 2002a; Wang *et al*., 2002b; Wang *et al*., 2004; Wiley and Burke, 2001). It has also been shown that an activated receptor can change its signalling output as it is endocytosed (Burke *et al*., 2001; Miaczynska *et al*., 2004b; Pennock and Wang, 2003; Sorkin and Von Zastrow, 2002; Wang *et al*., 2002a; Wang *et*

al., 2002b; Wang *et al*., 2004; Wiley and Burke, 2001). Most of these studies investigating signalling from endosomal compartments examined only the Ras/MAPK signalling pathway and found it to be active in endosomal compartments (Burke *et al*., 2001; Miaczynska *et al*., 2004b; Pennock and Wang, 2003; Sorkin and Von Zastrow, 2002; Wang *et al*., 2002b; Wiley and Burke, 2001). There are scaffolding proteins of the Ras/MAPK pathway that bind to Raf, MEK and ERK specifically at the plasma membrane, early endosome and late endosome (Anderson, 2006; Kolch, 2005).

Several groups have also shown that PI3K/Akt signalling can be propagated from the early endosome (Ceresa *et al*., 1998; Kelly and Ruderman, 1993; Wang *et al*., 2002a; Wang *et al*., 2002b; Wang *et al*., 2004). However, there is some debate about PI3K/Akt signalling from the early endosome since some studies have shown that the lipid substrate ($PI4,5P_2$) and product ($PI3,4,5P_3$) of class I PI3K (p85-p110) are not found in the early endosome (Haugh and Meyer, 2002; Sorkin and Von Zastrow, 2002). Some laboratories have even suggested there is a specific sub-population of early endosomes, coined "signalling endosomes", involved in compartmentalized signalling (Miaczynska *et al*., 2004b). A signalling endosome is thought to a sub-population of endosomes that may contain specialized signalling complexes from which activated receptors signal. These signalling endosomes also may have different fates for their cargo and different paths of travel when compared to endosomes that contain transferrin.

There may be several different types of signalling endosomes that carry out specific signalling roles with different subsets of activated receptors. An example of this is the APPL protein, which interacts with a subset of activated EGFR within a distinct sub-population of early endosomes (Miaczynska *et al*., 2004a). This interaction activates APPL, allowing it to translocate into the nucleus where it interacts with the

histone deacetylase complex, NuRD/MeCP1, thereby altering transcriptional regulation (Miaczynska *et al*., 2004a). These results suggest that the location of the activated receptor can change the level and type of signalling pathway activated. Therefore, the process of endocytosis may directly alter signalling of activated receptors.

Another major area of cross-talk between signalling and endocytosis is the lipid PI3P, produced primarily by the class III PI3K (Carpenter and Cantley, 1996; Fruman *et al*., 1998). Over the last decade it has come to light that PI3P is involved in endocytosis as well as signalling. The early endosome is enriched with PI3P (Di Paolo and De Camilli, 2006; Miaczynska and Zerial, 2002). It has been suggested that some of the PI3P in the early endosome may come from the dephosphorylation of the $PI3,4,5P_3$ lipids generated by class I PI3K (p85-p110) and not from class III PI3K (Behnia and Munro, 2005; Ivetac *et al*., 2005; Munro, 2002). The Corvera laboratory found that the vesicle tethering protein, EEA1, binds to PI3P via its FYVE domain and suggested that this lipid helps in the recruitment of EEA1 to the membrane (Patki *et al*., 1998; Patki *et al*., 1997). Wortmannin, an inhibitor of PI3Ks, causes cells to produce enlarged endosomes and the PDGFRs are not targeted to the lysosome for degradation (Lawe *et al*., 2000; Shpetner *et al*., 1996). A constitutively activated Rab5 mutant (Rab5Q79L; locked in its GTP-bound state due to lost GTPase activity) can reverse the inhibition of endocytosis caused by wortmannin (Lawe *et al*., 2000). This suggests that although the PI3P lipid products of PI3K play a role in the endocytosis process, they are not absolutely mandatory for receptor endocytosis to take place.

It has also been discovered that Hrs, part of the Hrs-STAM complex, has a FYVE domain that binds PI3P and is recruited to the membrane by PI3P (Komada and Soriano, 1999). Interestingly, when PI3P production is blocked by wortmannin or a FYVE

domain is overexpressed to block protein binding, the transport of EGFR to the late endosome, but not bulk solvent transport, is blocked (Petiot *et al*., 2003). This blockage of receptor degradation occurs because Hrs is important for receptor transport into the late endosome but not for the transport of bulk solvent. Several other ESCRT complex proteins contain GLUE (GRAM-like ubiquitin binding in EAP45) domains that bind several phosphatidylinositides including PI3P, PI4P, PI3,4P_2, PI3,5P_2 and PI3,4,5P_3 (Teo *et al*., 2006). The interaction between EEA1, Hrs and ESCRT proteins with 3'-phosphatidylinositides can explain the effects of the wortmannin treatment on endocytosis. The interaction of several proteins involved in endocytosis with phosphatidylinositides suggests that several classes of PI3K and other lipid kinases and phosphatases may also have a role to play in regulating lipid structures important for endocytosis.

There is other evidence to suggest that the class I PI3Ks are involved in receptor endocytosis. Mutations that block the binding of the p85 subunit of PI3K to the PDGFR disrupt the trafficking so that the receptor is internalized but is then exclusively recycled back to the cell membrane instead of being targeted to the lysosome for degradation (Hiles *et al*., 1992; Kapeller *et al*., 1993; Schu *et al*., 1993). It has recently been suggested that this is because the binding site on PDGFR has a dual function of binding p85 and acting as a tyrosine internalization motif (Wu *et al*., 2003). This study replaced the p85 binding motifs (G**Y**^{740}MDMSKAESVD**Y**^{751}MPM) with two copies of the lysosomal associated membrane protein 1 (LAMP-1) sorting motif (GY^{740}**QTI**SKDESV**G**Y^{751}**QTI**), causing the receptor to be degraded "normally" even though it does not bind p85. LAMP-1 is normally associated with the lysosome and the targeting of it to the lysosome is by the GYQTI motif that was added to the PDGFR.

The problem with this experiment is that it does not prove that PI3K has no role in targeting for degradation. It only determines that another method of receptor targeting can be used. It has also been shown that the catalytic activity of p85-p110 is not needed for the degradation of the EGFR (Chen and Wang, 2001).

The Ras/MAPK signalling pathway also regulates the endocytic pathway. During endocytosis of the μ opioid receptor, which is a GPCR, p38 MAPK phosphorylates two Rab5 effector proteins EEA1 and Rabenosyn-5 (Mace *et al*., 2005). These two effector proteins are involved in early endosome fusion and contain FYVE domains that bind to PI3P. EEA1 is phosphorylated on threonine 1392 and Rabenosyn-5 is phosphorylated on serine 215. These phosphorylation sites are located in the FYVE domains of the proteins and increase the membrane recruitment of the two proteins. This increases the rate of receptor endocytosis.

MAPK can also phosphorylate GDI, which also increases the rate of endocytosis. When GDI is phosphorylated on serine 121, GDI is activated to remove Rab5 from the membrane (Cavalli *et al*., 2001b). This allows GDI to deliver Rab5 to the membranes of vesicles that are waiting to fuse with the early endosome, thus increasing the rate of endocytosis because the amount of Rab5 is the rate limiting factor (Cavalli *et al*., 2001b). The authors also reported the same interaction between Rab7 and GDI although they did not show the results (Cavalli *et al*., 2001b).

Several proteins in the Ras/MAPK pathway also interact directly with Rab proteins. ERK1 phosphorylates Rab4, causing it to be redistributed to the cytosol from GLUT4 containing endosomes after stimulation with insulin (Cormont *et al*., 1994). The catalytic subunit, p110β, of PI3K also binds to Rab5-GTP although the function of this interaction is not known (Christoforidis *et al*., 1999b).

To summarize, there are several levels of cross-talk between signalling and endocytosis pathways and it is likely that more will be discovered in the future. Both of the main signalling pathways of RTKs are involved in endocytosis. Several PI3K lipid products recruit endocytic machinery to the membrane; while, Ras/MAPK is involved in phosphorylating several endocytic proteins to regulate their activity and change the rate of endocytosis. We wanted to determine if there was cross-talk between the PI3K/Akt signalling pathway and the endocytosis pathway in addition to the known role the PI3K lipid products play in the recruitment of the endocytosis machinery.

2.0 RATIONALE AND OBJECTIVES

Our laboratory studies the interaction of the class I PI3K, p85-p110, with other proteins and the role of these protein complexes in signalling pathways. We have studied the interaction of PI3K with A-Raf (Anderson, 2006; Fang *et al*., 2002; Johnson *et al*., 2005; King *et al*., 2000; Mahon *et al*., 2005), ankyrin3 (Ignatiuk *et al*., 2006) and PTEN (Chagpar *et al*., 2007). We are particularly interested in the role of p85 subunit interactions with other proteins. To determine the different roles of p85 we decided to look at the possible function(s) of its BH domain. The BH domain of p85 has a high degree of sequence homology with known GAP domains. GAP domains have been shown to increase the catalytic rate of G proteins. G proteins possess intrinsic GTPase activity to catalyze the hydrolysis of GTP to GDP. They do this at a very slow rate but GAP proteins increase the rate of catalysis of G proteins (Fidyk and Cerione, 2002; Scheffzek *et al*., 1998).

The GAP protein increases the catalytic rate in two ways. First, it binds to the switch regions of the G protein stabilizing it in such a way that the GAP protein changes the conformation of the active site, which increases the rate of hydrolysis (Fidyk and Cerione, 2002; Scheffzek *et al*., 1998). Second, GAP protein participates directly in the hydrolysis of the GTP by inserting a catalytic arginine into the reaction which is often referred to as an arginine finger (Fidyk and Cerione, 2002; Scheffzek *et al*., 1998). This arginine stabilizes the transition state of the hydrolyzing GTP, allowing the reaction to proceed (Fidyk and Cerione, 2002; Scheffzek *et al*., 1998).

The BH domain of p85 has not previously been shown to have GAP activity towards any G proteins that it has so far been tested against. Although it has been shown to bind to the GTP-bound forms of the G proteins, Rac1 and Cdc42, it did not have GAP activity towards them at p85 concentrations of up to 1 μM (Tolias *et al*., 1995). Cdc42 and Rac1 are G proteins that are involved in many cellular processes including maintaining cell shape, cell movement, cell adhesion and vesicle movement, by regulating the actin cytoskeleton structure (Wittmann and Waterman-Storer, 2001).

In the literature there is evidence for the possibility of p85 interacting with the Rab family of monomeric G proteins. There are two sets of experimental data that suggested p85 interacts with Rab proteins, specifically Rab5, and could regulate receptor degradation. First, Rab5 has been shown to bind to the catalytic subunit of PI3K, p110β (Christoforidis *et al*., 1999b; Kurosu and Katada, 2001). The p85α protein also associates with the p110β:Rab5-GTP complex. This suggested that p85 could be a GAP towards Rab proteins, specifically Rab5 because p85 and Rab5 are localized on the same membranes in the early endosome and they both bind to p110β. The second set of experiments that supported the role of p85 in receptor endocytosis was that mutations, which block the binding of the p85 subunit of PI3K to the PDGFR disrupt trafficking. Trafficking was disrupted such that the receptor is internalized but is then exclusively recycled back to the plasma membrane instead of some fraction of the receptor being targeted to the lysosome for degradation. The lipid kinase activity of PI3K is not required for the degradation of the receptor, suggesting this role for the p85-p110 (PI3K) is not dependent on the generation of its lipid products (Chen and Wang, 2001; Hiles *et al*., 1992; Kapeller *et al*., 1993; Schu *et al*., 1993). This data leads to the following:

2.1 Hypothesis

That the p85 regulatory subunit of PI3K is a GAP for Rab proteins of the early endosome (eg. Rab5) that regulate the endocytosis of the PDGFR.

2.2 Objectives

The goal of this thesis is to characterize the role of the p85 subunit of phosphatidylinositol 3-kinase in the regulation of endocytosis and downregulation of the PDGFR through the interaction with and regulation of Rab proteins.

2.3 Specific Objectives of Thesis Work

1) To determine if p85 can bind directly to Rab5 and if this interaction is dependent upon the nucleotide bound state of the Rab protein (i.e. GTP, GDP and GDP+AlF_4).
2) To determine if p85 has GAP activity towards Rab5.
3) To determine if p85 has GAP activity towards other Rab proteins that are involved in endocytosis.
4) To determine if p85 mutants have effects on Rab regulation and on receptor endocytosis, recycling and degradation in cells.
5) To characterize cell lines containing p85 and the p85 mutants, p85ΔBH and p85R274A, for transformed properties.

The information obtained from these experiments will give us a better understanding of the role of p85 in growth factor receptor endocytosis. From this data we will be able to construct a model of receptor endocytosis that includes p85 and Rab

proteins. This model can then be used to test the role of p85 in endocytosis and give us ideas for future experiments to better understand the mechanism of receptor endocytosis and downregulation.

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Reagents and Supplies

All of the chemicals were of analytical grade or higher and were purchased from VWR, Sigma or BDH unless otherwise stated. Glutathione Sepharose beads (Amersham Pharmacia Biotech) were prepared according to manufacturer's instructions and were used for purification of GST fusion proteins and pull-down experiments. Glutathione Sepharose beads were stored at 4°C and were stable for several months. The radioisotopes, [α-^{32}P]GTP (Cat #: BLU506H) and [γ-^{32}P]ATP (Cat #: BLU502A) were purchased from PerkinElmer and were stored at 4°C. All experiments using radioisotopes met with regulations as mandated by the University of Saskatchewan. The Swiss nude mice were purchased from Charles River Laboratories and all animal handling was done according to the University of Saskatchewan guidelines.

The following primary antibodies were used for the experiments: anti-Akt (New England Biolaboratory, 9272), anti-FLAG M2 (Sigma, F3165), anti-MAPK (Transduction Laboratory, M12320), anti-p85 (Upstate Biotechnology Institute, 05-217), several anti-p85 (specific for amino acids: 78-332 or 312-722, (Chamberlain *et al*., 2004; Fang *et al*., 2002; King *et al*., 2000)), anti-pAkt (New England Biolaboratory, 9271, pS473), anti-pMAPK (Cell Signalling, 9101, phospho-44/42 MAPK T202/Y204) and

anti-Rabaptin5 (Transduction Laboratory, R78620). Several of the primary antibodies and the secondary antibodies used were from Santa Cruz Biotechnology. The primary antibodies are anti-GST (sc-138), anti-Myc (sc-789), anti-PDGFR (sc-432), anti-pTyr (sc-508) and anti-Rab5 (sc-309). The secondary antibodies used were horseradish peroxidase-coupled anti-rabbit IgG (sc-2004), horseradish peroxidase-coupled anti-mouse IgG (sc-2005) and horseradish peroxidase-coupled anti-goat IgG (sc-2020).

3.2 Methods

If a specific method for basic molecular biology and protein science, such as DNA digests, ligations, competent cell production, transformation of competent cells and transfection of cells is not giving in specific detail, then the method comes from either Current Protocols in Molecular Biology or Current Protocols in Protein Science (Ausubel *et al*., 2007; Coligan *et al*., 2007).

3.2.1 Plasmids and Vectors

GST-fusion Proteins

The pGEX2T, pGEX3X and pGEX6P1 vectors (Amersham Pharmacia) are designed for inducible protein production in bacteria. Transformation with these vectors produces high levels of proteins fused with glutathione S-transferase (GST) after induction with isopropyl β–D-thiogalactopyranoside (IPTG). They all contain ampicillin resistance genes and have a similar overall architecture. The major difference between the three vectors is that they have different protease cleavage sites between the fusion protein and GST. The pGEX2T has a thrombin cleavage site (LVPR*GS, the asterisk indicates the cleavage site) whereas pGEX3X has a factor Xa cleavage site

(I(E/N)GR*) and pGEX6P1 has a PreScission cleavage site (LEVLFQ*GP). Several plasmids encoding different proteins have been used (Table 3.1). Dr. G. Li (Liu and Li, 1998) generously provided the pGEX3X plasmids encoding human Rab5 (wild type and the mutants Q79L and S34N) as well as human Rab4 and human Rab6. The human Rab7, Rab11, Rac1 and Cdc42 cDNA were obtained by reverse transcription (Promega kit) from total RNA obtained from HeLa cells, followed by polymerase chain reaction (PCR) with Pfu (Stratagene). Primers were designed to incorporate 5' *Bam*HI and 3' *Eco*RI sites immediately upstream of the start codon and downstream of the stop codon, respectively. The amplified products were cloned into pGEX6P1 and sequenced to verify the accuracy of the sequence (Chamberlain *et al*., 2004).

GAPette is the catalytic domain of the p120 Ras GAP and is used as a positive control in the GAP assays (Liu and Li, 1998). A plasmid containing human p120 RasGAP previously obtained from F. McCormick (University of California, San Francisco) was used to generate the GAPette. The fragment encoding the catalytic domain of p120 RasGAP (GAPette) was obtained by digesting the pUC101a plasmid encoding p120 RasGAP with *Sca*I and *Eco*RI and the resulting insert was gel purified on a 0.8% low melt agarose gel (VWR). The insert was ligated into the pGEX2T vector digested with *Sma*I and *Eco*RI. This plasmid encoded a GST-GAPette protein containing amino acids 450-1047 of human p120 RasGAP.

Table 3.1 Plasmids encoding the Proteins used in this Thesis.

Protein	Species	Amino Acids	Vector Backbone	Expression Vector	Tag	Selection
Cdc42	Human	1-192	pGEX6P1	Bacterial	GST	Ampicillin
Rab4	Human	1-218	pGEX3X	Bacterial	GST	Ampicillin
Rab5	Human	1-215	pGEX3X	Bacterial	GST	Ampicillin
Rab5	Human	1-215	pHA_3	Mammalian	NA	Geneticin
Rab5Q79L	Human	1-215	pGEX3X	Bacterial	GST	Ampicillin
Rab5S34N	Human	1-215	pGEX3X	Bacterial	GST	Ampicillin
Rab6	Human	1-208	pGEX3X	Bacterial	GST	Ampicillin
Rab7	Human	1-207	pGEX6P1	Bacterial	GST	Ampicillin
Rab11	Human	2-216	pGEX6P1	Bacterial	GST	Ampicillin
Rac1	Human	1-192	pGEX6P1	Bacterial	GST	Ampicillin
p110α	Human	1-1068	$pMyc_3$	Mammalian	Myc	Geneticin
p85	Bovine	1-724	pGEX2T	Bacterial	GST	Ampicillin
	Bovine	1-724	pGEX6P1	Bacterial	GST	Ampicillin
	Bovine	1-724	$pFLAG_3$	Mammalian	FLAG	Geneticin
	Bovine	1-724	pGEX6P-His	Bacterial	GST/His	Ampicillin
p85SH3	Bovine	1-83	pGEX2T	Bacterial	GST	Ampicillin
p85BH	Bovine	77-322	pGEX2T	Bacterial	GST	Ampicillin
p85(N+C)SH2	Bovine	314-724	pGEX2T	Bacterial	GST	Ampicillin
p85ΔBH	Bovine	1-83,314-724	pGEX2T	Bacterial	GST	Ampicillin
	Bovine	1-83,314-724	$pFLAG_3$	Mammalian	FLAG	Geneticin
p85R151A	Bovine	1-724	pGEX6P1	Bacterial	GST	Ampicillin
p85R274A	Bovine	1-724	pGEX6P1	Bacterial	GST	Ampicillin
	Bovine	1-724	$pFLAG_3$	Mammalian	FLAG	Geneticin

The plasmids pGEX2T and pGEX6P1 were both used to express the bovine p85 wild type and p85 mutants/domains (p85SH3, p85BH, p85(N+C)SH2, p85ΔBH, p85R151A and p85R274A) (Chamberlain *et al*., 2004; King *et al*., 2000). The p85ΔBH mutant was generated by fusing the p85 sequences encoding the p85SH3 domain (amino acids 1-83) and the p85(N+C)SH2 (amino acids 314-724) as described (Chamberlain *et al*., 2004; King *et al*., 2000). The point mutations (p85R151A and p85R274A) were generated using the Quikchange mutagenesis method (Stratagene)(Chamberlain *et al*.,

2004). Wild type p85 was also expressed with a C-terminal His_6 tag from an altered pGEX6P1 plasmid, pGEX6P-His, for use in the enzyme-linked immunosorbent assay (ELISA) binding assay (Chamberlain *et al*., 2004). The altered pGEX6P1 plasmid, pGEX6P-His, was generated by inserting a pair of oligonucleotides encoding six histidine residues and a stop codon, between the *Eco*RI and *Sal*I sites such that the sequence of this region is now (5'GAA TTC CAT CAT CAC CAT CAC CAT TGA GTC GAC-3'). The p85 encoding insert (not including the stop codon), amplified by polymerase chain reaction, was subcloned into the *Bam*HI and *Eco*RI sites of pGEX6P-His.

The vectors $pFLAG_3$, $pMyc_3$ and pHA_3 are all mammalian expression plasmids. They were used for expression of proteins in eukaryotic cell lines. All three vectors contain both ampicillin and geneticin selection genes.

HA-Rab Proteins

The pHA_3 vector was previously generated by Yun Fang and is based on the pRc/CMV2 vector (Invitrogen) originally modified and obtained from the Tremblay laboratory (Charest *et al*., 1995). Inserts expressed in this vector encode three copies of the hemagglutinin (HA)-epitope (YPYDVPDYA) fused to the N-terminus of the protein (King *et al*., 2000). The Rab5 insert was excised from the pGEX3X vector using *Bam*HI and *Eco*RI and subcloned into *Bgl*II and *Eco*RI digested pHA_3. The *Bam*HI and *Bgl*II restriction cleavage sequences have compatible overhanging cleavage sites that can join together by ligation but this destroys both the *Bam*HI and *Bgl*II sites.

FLAG-p85 Proteins

The $pFLAG_3$ vector was derived from the pHA_3 vector (Chamberlain *et al*., 2004). The small *Hind*III-*Bgl*II fragment upstream of the multiple cloning site in the pHA_3 vector was replaced by the following sequence to make the new $pFLAG_3$ vector: 5'-AA GCT TCC ACC ATG GAC TAC AAG GAC GAC GAT GAC AAG GCT AGT GAC TAC AAG GAC GAC GAC GAT AAA GCG GCC GCT GAT TAC AAG GAC GAC GAC GAT AAA GCT AGC AGA TCT-3' (Chamberlain *et al*., 2004). The newly generated plasmid was verified by DNA sequencing. The $pFLAG_3$ vector encodes three copies of the FLAG-epitope (DYKDDDDK) in place of the three HA sequences fused to the N-terminus of the protein. Inserts encoding full-length wild type p85, p85ΔBH, p85R151A and p85R274A were excised from the pGEX vectors described above, using *Bam*HI and *Eco*RI and subcloned into *Bgl*II and *Eco*RI digested $pFLAG_3$ (Chamberlain *et al*., 2004). The *Bam*HI and *Bgl*II restriction cleavage sequences have compatible overhanging cleavage sites that can join together by ligation but this destroys both the *Bam*HI and *Bgl*II sites.

Myc-p110 Proteins

The $pMyc_3$ vector was modified from the $pFLAG_3$ vector and encoded three copies of the Myc-epitope (EQKLISEEDL) upstream of the multiple cloning site. The small *Hind*III-*Nhe*I fragment from the $pFLAG_3$ vector was replaced by the following sequence in the new $pMyc_3$ vector: 5'-AA GCT TCC ACC ATG GAA CAG AAA CTG ATC AGC GAA GAG GAT CTG CTG AGC GAG CAG AAA CTG ATC AGC GAG GAA GAA CTG GCC GCG GAA CAG AAA CTG ATC AGC GAA GAG GAT CTG

GCT AGC-3' (Chamberlain *et al*., 2004). A pair of oligonucleotides (5'-AAT TCG CGG CCG CGG GCC-3' and 5'-CGC GGC CGC G-3') were ligated into the *Eco*RI-*Apa*I-digested FLAG-modified vector, as described above, to alter the multiple cloning site so that it contained a new unique *Not*I site between these two restriction sites (Chamberlain *et al*., 2004). This entire region of the new $pMyc_3$ vector was verified by DNA sequencing. An insert encoding full-length wild type mouse p110α was amplified by PCR from the pCMV-p110myc plasmid (generously provided Dr. L.T. Williams (University of California San Francisco, CA)). The *Bam*HI-*Not*I-digested insert DNA (p110α) was subcloned into the *Bgl*II-*Not*I-digested $pMyc_3$ vector. The integrity of all inserts containing PCR-generated DNA inserts were verified by sequencing to ensure that no mutations had been introduced.

3.2.2 Bacterial Strains

For protein purification, the pGEX2T, pGEX3X, pGEX6P1 and pGEX6P-His plasmids containing inserts were expressed in *Escherichia coli* (*E. coli*) BL21 cells [F^- *ompT hsdS*$_B$ ($r_B^- m_B^-$) *gal dcm*] (Novagen). These cells are protease deficient to minimize protein degradation during purification. The *E. coli* BL21 cells were grown in Luria-Bertani Broth (LB, Sigma) containing 1%(w/v) bacto-tryptone, 0.5%(w/v) bacto-yeast extract and 1%(w/v) sodium chloride (NaCl) pH 7.0, per litre. All of the plasmids contained an ampicillin resistance gene, therefore the *E. coli* BL21 cells were grown in LB containing 100 μg/mL ampicillin (LBA, Sigma). For the production of plasmid DNA, *E. coli* TOP10 cells [F^- *mcrA (mrr*$^-$*hsdRMS*$^-$*mcrBC) 80lacZM15 lacX74 recA1*

ara139 (ara-leu)7697 galU galK rpsL (StrR) endA1 nupG] (Invitrogen) were used and they were grown in the same conditions as the *E. coli* BL21 cells.

3.2.3 Mammalian Cell Lines

Mouse fibroblast cells, NIH 3T3 and African green monkey kidney fibroblast-like cells, COS-1, were obtained from American Type Culture Collection (CRL-1658) and grown in Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Hyclone), 100 units/mL penicillin G and 100 μg/mL streptomycin (P/S, Gibco) at 37°C with 5% CO_2. NIH 3T3 cells transfected with plasmids derived from $pFLAG_3$ were grown in selection media (DMEM + 10% FBS + P/S) containing 400 μg/mL Geneticin (G418, Gibco). COS-1 cells were transiently transfected with $pFLAG_3$-p85 and/or $pMyc_3$-p110 and/or pHA_3-Rab5 and grown in media (DMEM + 10% FBS + P/S) at 37°C with 5% CO_2.

3.2.4 Protein Analysis

3.2.4.1 Induction and Purification

LBA (100 mL) was inoculated with *E. coli* BL21 cells transformed with the plasmid encoding the protein of interest fused to GST and grown overnight at 37˚C. The next day 1 L of LBA was inoculated with the entire 100 mL overnight culture. The culture was grown at 37ºC until an OD_{600} of 0.5-0.7 was reached (~ 1 hr). IPTG (Sigma) was added to a concentration of 0.1 mM to induce protein expression. After the addition of IPTG, the culture was grown at 37ºC for 4 hr and the cells were harvested by centrifugation at 4200 x g for 15 min at 4ºC. The cell pellet was frozen at –20ºC or lysed immediately.

For lysis, the cell pellet was resuspended in 10 mL phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM sodium phosphate, 1.4 mM potassium phosphate, pH 7.3) containing 10 μg/mL aprotinin (Sigma), 10 μg/mL leupeptin (Sigma) and 1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma), which were added fresh. The cells were lysed by 3 bursts of sonification for a duration of 30 sec. at the setting of 2.5 using a Model 250/450 Sonifier (Branson Ultrasonics) with 2 min of chilling on ice between bursts. Triton X-100 (Sigma) was added to a concentration of 1% (to minimize protein-protein interactions and reduce contamination of the sample) and the sample was centrifuged at 12,000 x g for 30 min at 4ºC to remove cell debris. The supernatant was filtered through a 0.45 μM cellulose acetate membrane (Nalgene) and mixed with 1 mL of a 50% slurry of Glutathione Sepharose beads (Amersham Biosciences). The lysate and beads were incubated together for 1 hr at room temperature (RT) with rocking. The beads were recovered by centrifugation at 800 x g for 5 min and washed 3 times with 50 mL of ice-cold PBS.

For pull-down experiments, the GST fusion proteins were left bound to the glutathione Sepharose beads, resuspended in PBS to make a 50% slurry and were stored at 4°C. A sample of each protein suspension was resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie blue to determine protein purity, integrity and recovery. The protein concentrations were also determined by visually comparing GST-fusion protein amounts to bovine serum albumin (BSA) standards on the SDS-PAGE gel.

To cleave the protein of interest from the GST tag, the immobilized GST-fusion protein was treated with a protease. The protease added depended on the cleavage site

present in the vector and was either 100 NIH units of Thrombin (Sigma), 50 units of Factor Xa (Amersham Biosciences) or 35 units of PreScission protease (Amersham Biosciences). The Thrombin and Factor Xa cleavage reactions were carried out in PBS. For PreScission cleavage, the beads were washed 3 times in 20 mL PreScission buffer (50 mM Tris-HCl, pH 7.0, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT)) and the cleavage reaction was performed in PreScission buffer. The cleavage reactions were carried out for 4 hr or overnight at RT (4˚C for PreScission) depending on the protein. A four hour cleavage time was used for Rab5 as longer cleavage times sometimes resulted in proteolysis of the Rab5 protein. To recover the cleaved protein, the sample was centrifuged at 2000 x g and the supernatant containing the purified protein was collected. The beads were washed 4 times with 1 mL of PBS or PreScission buffer to recover any residual protein trapped between the beads.

After PreScission cleavage from GST, the C-terminal His_6-tag was used to further purify p85-His_6. The protein that had been previously buffer-exchanged into Talon extraction/wash buffer (50 mM NaH_2PO_4, 300 mM NaCl) using an Amicon Ultra Centrifugal Filter (MW 30,000, Millipore) was bound to 1 mL of a 50% slurry of Talon beads (Clontech). Samples were mixed end-over-end for 20 min at RT. Beads were recovered by centrifugation at 800 x g for 5 min and washed in 20 mL Talon extraction/wash buffer 3 times, recovering the washed beads by centrifugation as described above. The Talon beads were transferred into a small column using additional Talon extraction/wash buffer. The p85-His_6 protein was eluted from the resin using 3 successive 1 mL additions of Talon elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 150 mM imidazole) and collecting the elution buffer containing the p85-His_6 protein.

For storage the p85-His_6 protein was concentrated and exchanged into Talon extraction/wash buffer (not protein storage buffer) as above. Glycerol was added to 20% and aliquots were stored at –80ºC.

For the ELISA-based binding assay, the Rab5 protein was further purified after factor Xa cleavage from GST using gel filtration chromatography on a Sephacryl HR-200 column in phosphate buffer (50 mM NaH_2PO_4, pH 7.0, 150 mM NaCl) before exchange into protein storage buffer. Also for the ELISA assay, purified GST protein was eluted from glutathione Sepharose beads using 15 mM reduced glutathione. To concentrate all the proteins, the samples of protein were pooled and concentrated using an Amicon Ultra Centrifugal Filter (Millipore) with a molecular weight cut-off 2 to 3 times smaller than the molecular weight of the protein being purified. The protein was buffer exchanged into protein storage buffer (50 mM N-2-hydroxyethylpiperazine-H'-2-ethanesulfonic acid (Hepes), pH 7.5, 50 mM NaCl, 1 mM EDTA) and glycerol was added to a concentration of 10% to 40%. All purified proteins were flash-frozen in liquid nitrogen and stored at –80ºC. A sample of all proteins was resolved by SDS-PAGE and stained with Coomassie blue to determine purity. The protein concentrations were determined by Bradford assay (Bio-Rad).

The PreScission protease (Amersham Biosciences), 3C, was expressed as a GST-3C fusion protein, purified as described above except that it was eluted from the Glutathione Sepharose beads using 15 mM reduced glutathione (Sigma) in 50 mM Tris-HCl pH 8.0 and stored at –20°C.

3.2.4.2 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were resolved by SDS-PAGE for protein staining with Coomassie blue or for Western blot analysis (Laemmli, 1970). SDS-PAGE was performed using the Mini-Protean II apparatus (Bio-Rad). Gels were cast between two glass plates with one mm spacers. The resolving gels contained between 7.5% to 15% acrylamide solution (29.2:0.8 acrylamide: bisacrylamide), 37 mM Tris-HCl pH 8.8 and 0.1%(w/v) SDS. Polymerization was initiated by the addition of 0.06%(w/v) ammonium persulfate and 0.1%(v/v) N,N,N',N'-Tetra-methylethylenediamine (TEMED). After the resolving gel was set, a 2 mL stacking gel containing 4.5% acrylamide solution, 125 mM Tris-HCl pH 7.0 and 0.1%(w/v) SDS was added to the top of the gel and a comb was inserted to form wells. The stacking gel was polymerized with the same procedure as the resolving gel. After the stacking gel was set, the gel was assembled into the electrophoresis apparatus. The protein samples were prepared in SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 5%(v/v) β–mercaptoethanol, 2.3%(w/v) SDS, 0.1%(w/v) bromophenol blue, 10%(v/v) glycerol) and heated to 100°C for 5 min. The samples and prestained molecular weight markers (Sigma, SDS-7B or Fermentas, SM0671) were loaded on the gel and resolved. The electrophoresis was performed at a constant voltage of 180V in running buffer (25 mM Tris, 250 mM glycine and 0.1%(w/v) SDS) until the bromophenol blue dye had run off the bottom of the gel as described (Laemmli, 1970). For protein staining, the gel was incubated in Coomassie blue stain (0.14% w/v Coomassie blue R-250 (Bio-Rad), 41.4% v/v methanol and 5.4% acetic acid) at RT for 30 min and destained in destaining solution (41.4% v/v methanol and 5.4% acetic acid) until bands were visible. The gel was dried onto 3MM paper and scanned using an Epson Perfection 1260 scanner.

3.2.4.3 Western blot analysis

Following protein separation by electrophoresis, the SDS-PAGE gel was soaked in transfer buffer (48 mM Tris, 39 mM glycine, 0.0375% SDS, 20% methanol) for 15 min. At the same time, 6 pieces of 3MM filter paper (Whatman) were soaked in transfer buffer and one piece of nitrocellulose membrane (Schleicher and Schuell) was rehydrated in distilled water. The proteins were transferred to the nitrocellulose membrane using a Panther semi-dry Electroblotter Owl Separation System (VWR) at constant current of 400 mA for 15 min per gel being transferred. After transfer, the nitrocellulose was blocked in blocking solution [5% Carnation skim milk powder in TBST (100 mM Tris-HCl, pH 8, 150 mM NaCl, 0.05% polyoxyethylenesorbitan monolaurate (Tween-20))] for 1 hour at RT or overnight at 4˚C. The membrane was incubated with primary antibody in blocking solution for one hour at RT or overnight at 4˚C and washed 3 times in TBST for 5 min each. The membrane was probed with horseradish peroxidase conjugated secondary antibody for one hour at RT or overnight at 4˚C and washed as described above. The nitrocellulose membrane was incubated for 1 min in Western Lightning enhanced chemiluminescence reagent (PerkinElmer) and exposed to X-Omat Blue XB-1 film (Kodak) for up to 5 min in a darkroom. The film was developed in a Kodak M35A X-Omat processor.

To reprobe the membrane with subsequent antibodies, the membrane was washed as described above and incubated with stripping buffer (62.5 mM Tris-HCl pH 6.8, 2%(w/v) SDS, 100 mM β-mercaptoethanol) for 30 min at 60°C. The membrane was washed again and incubated in blocking solution before reprobing using the method described above.

3.2.4.4 GAP assay

This three-step assay measures the hydrolysis of GTP by the Rab5 protein by determining the amount of GDP produced. The Rab5 protein was loaded with [α-^{32}P]GTP, a hydrolysis reaction converts the bound [α-^{32}P]GTP to [α-^{32}P]GDP and the [α-^{32}P]GDP was separated from the [α-^{32}P]GTP by thin-layer chromatography (TLC). Two different variations of the GAP assay were used that were adapted from the method used by the Li laboratory (Liu and Li, 1998). The first was the Steady State GAP Assay, which was simple to perform but could not measure the catalytic rate. The second was the Single Turnover GAP Assay used to determine the catalytic rate.

It should be noted that high concentrations of glycerol adversely affected both GAP assays. Thus, if the concentrations of the p85 or Rab5 proteins were low, it was necessary to add less glycerol to the proteins prior to freezing. We found with 40% glycerol in the Rab5 protein, there was no experimental effect from the glycerol if the Rab5 was diluted 1:100 with loading buffer before using it for nucleotide loading. For p85, we found that 10% glycerol could be added without a significant effect on the GAP assay even though the p85 was not always diluted before addition into the assay reaction mixture. We also noted that different preparations of the proteins had slightly different specific activities; therefore, the same batches of Rab5 and p85 proteins were used for particular sets of experiments.

3.2.4.4.1 Steady State GAP Assay

This assay measured the amount of GDP generated via hydrolysis by Rab5 for a fixed time with increasing concentrations of the GAP protein, p85. This assay allowed

the possibility of new [α-^{32}P]GTP loading onto the Rab5 protein and therefore could not be used to determine the catalytic rate.

Preparation for the GAP Assay

The TLC chamber was pre-equilibrated with the developing solvent (0.75 M KH_2PO_4) for 1 - 24 hr. The PEI Cellulose F plates (VWR, Cat #: CAM05725-01) were labelled using a pencil. Lanes were marked with a small "x" 2 cm from the bottom edge and at 1.5 cm intervals starting 2 cm from the outside edge of the plate. Labelled TLC plates were dried for 1 to 24 hr at ~75˚C.

GAP Assay

1) Nucleotide loading of the Rab protein

The Rab5 protein was diluted in loading buffer (20 mM Tris-HCl, pH 8.0, 2 mM EDTA, 1 mM DTT) to a final concentration of 200 nM. [α-^{32}P]GTP (Perkin Elmer; 3000 Ci/mmol; 0.51 μL) was added to the Rab5 protein to a final concentration of 85 nM. The total reaction volume of the Rab5 protein plus the [α-^{32}P]GTP was 20 μL per sample. The reaction mix was incubated at RT for 30 min, which allowed the formation of the Rab5-[α-^{32}P]GTP complex. A negative control of nucleotide ([α-^{32}P]GTP) without any Rab5 protein was prepared as well.

2) Hydrolysis Reaction

As the [α-^{32}P]GTP/Rab5 mix incubated, tubes containing the various concentrations of GAP protein (p85 or GAPette) were prepared. The hydrolysis reaction

was carried out in a total volume of 30 μL: 9 μL p85 protein (diluted in loading buffer; sufficiently concentrated to give a final concentration of 0 – 35 μM in the 30 μL assay volume), 1 μL $MgCl_2$ (300 mM made fresh; final concentration of 10 mM), and 20 μL of the [α-^{32}P]GTP/Rab5 (prepared above in section 1). Therefore, a set of tubes containing various concentrations of p85 and the $MgCl_2$ in a total volume of 10 μL were prepared, that gave the appropriate final concentrations of p85 and $MgCl_2$ after the 20 μL of [α-^{32}P]GTP/Rab5 were added. For the positive control of GAPette, 9 μL of GAPette plus 1 μL of 300 mM $MgCl_2$ was used with a final concentration of 0.8 μM of GAPette in the 30 μL assay volume and for the negative control 9 μL loading buffer and 1 μL of 300 mM $MgCl_2$ was used.

After the Rab5 protein and the nucleotide mixture, from step 1, was finished incubating, 20 μL of this mix was added to the p85 protein plus $MgCl_2$, as well as to each of the control tubes. This was mixed by inversion and incubated for 10 min at RT. The $MgCl_2$ locked the [α-^{32}P]GTP into the Rab5-[α-^{32}P]GTP complex. After 10 min the reaction was stopped by the addition of 6 μL of elution buffer (1% SDS, 25 mM EDTA, 25 mM GDP, 25 mM GTP) and heated for 2 min at 65˚C.

3) Separation of [α-^{32}P]GTP/[α-^{32}P]GDP by TLC

The samples were spotted onto the TLC plates 5 μL at a time until the entire sample was applied, allowing the spots to dry between additions. After the entire sample was added, the plate was allowed to dry at RT. When the plate was inserted into the TLC chamber, the developing solvent was several mm below the line marking the lanes so that the developing solvent did not smear the spots. The samples were allowed

to migrate in the TLC chamber until the solvent front was ~1 cm from the top of the plate (~2 hr). After the plate was removed and dried at RT, the separated [α-^{32}P]GTP and [α-^{32}P]GDP were visualized using a phosphorimager (Molecular Imager FX Pro Plus; Bio-Rad) and quantified using Quantity One software (Bio-Rad). The amount of GTP hydrolyzed was calculated as femtomoles of [α-^{32}P]GDP produced or as a relative GAP activity using the ratio of [α-^{32}P]GDP to [α-^{32}P]GTP on the TLC plate. The value from the nucleotide alone (negative control) sample was subtracted from the experimental values since it was the background of [α-^{32}P]GDP present in the absence of Rab5. The relative GAP activity was determined by normalizing the data to the amount of [α-^{32}P]GDP produced by the Rab5 protein in the absence of a GAP protein. The results were graphed and statistical analysis was performed using Prism software (GraphPad Software, Inc.).

3.2.4.4.2 Single Turnover GAP Assay

This assay measured the rate of Rab5 GTP hydrolysis over time with a fixed concentration of p85 protein. There was no possibility of [α-^{32}P]GTP reloading due to the presence of a large excess of unlabeled GTP. Using this assay it was possible to measure the catalytic rate of GTP hydrolysis by the Rab5 protein and how that rate was changed by the addition of the p85 protein.

The single turnover GAP assay was performed the same way as the steady state GAP assay with the following changes. Unlabeled GTP (1.7 mM) was added to the tube containing the p85 protein and the $MgCl_2$. Also the concentration of the p85 protein was held constant and the time of incubation was varied from 0 to 25 min in 5 min intervals.

When choosing a concentration to use for the p85 protein, it was ideal to choose a concentration that was sub maximal in the steady state GAP assay. This allowed for either increases or decreases in rates to be observed easily. For the purposes of our experiments we used 10 μM p85.

3.2.4.5 ELISA

The ELISA binding assay was used to determine if there was an interaction between the Rab5 and p85 proteins. The first step was to load the Rab5 protein with nucleotides and then bind the complex to the ELISA plate. The second step was to incubate the p85 protein with the Rab5 and the last step was to develop the assay and read the absorbance.

Rab5 was diluted to 40 μg/mL in PBS + 2 mM EDTA and incubated for 20 min at RT to remove any bound nucleotide. To prepare Rab5 with no bound nucleotide, an equal volume of 20 mM $MgCl_2$ in PBS was added. To prepare Rab5 with bound GDP, an equal volume of 20 mM $MgCl_2$ + 200 μM GDP (Sigma) in PBS was added. To prepare Rab5 with bound GTPγS (a non-hydrolyzable form of GTP), an equal volume of 20 mM $MgCl_2$ + 200 μM GTPγS (Sigma) in PBS was added. Each was incubated for a further 20 min at RT to load each of the nucleotides onto the Rab5 protein.

Immulon 4 flat-bottomed 96-well ELISA plates (VWR) were used for the assay. All incubations were carried out at RT, unless otherwise indicated, in a humidified plastic container (i.e. damp paper towels in the bottom of a plastic container with a lid). Determinations were carried out at least in duplicate. The wells around the perimeter of the plate were avoided since they showed larger well-to-well differences in absorbance

(at 450 nm) even when empty (0.036-0.042), as compared to the interior wells (0.036-0.039).

The Rab5 protein that had been preloaded with nucleotide was bound to 12 wells for each test protein (20 μg/mL in PBS, 50 μL per well; 1 μg total) overnight. For controls, 4 wells were prepared with PBS. The next day, wells were emptied and excess liquid was removed by inverting the plates onto paper towels. The wells were blocked by filling each well completely with blocking buffer (5% Carnation skim milk, 0.2% Tween-20 in PBS) and incubated for 2 hours. Blocking was removed, excess liquid was removed by percussion and the wells were washed 4 times with distilled water. To wash the ELISA plate, a plastic basin was filled 1/2 to 3/4 full of distilled water and with the ELISA plate at a 45˚ angle, it was submerged to fill the wells with water. This was repeated, tipping a different edge of the plate down each time until all 4 edges had been done. Excess water was removed onto a paper towel. In duplicate wells, 50 μL of a serial dilution of the p85-His_6 or GST (control for background non-specific binding) in blocking buffer was added. For the dilutions 2-fold serial dilutions of protein in blocking buffer (e.g. 250 nM, 125 nM, 62.5 nM, 31.25 nM, 15.6 nM), as well as 0 nM wells were used. As an additional control, the highest concentration of p85-His_6 or GST test protein (e.g. 250 nM) was added to wells, which received PBS instead of Rab5 protein initially. Samples were incubated for 2 hours at RT. After the incubation, 16 washes were carried out in distilled water: 4 times for one leading edge, then the water was changed and repeated for each of the 3 remaining edges of the plate. Excess water was removed as before. To the p85-His_6 wells (including wells that did not receive any test protein) 100 μL of anti-p85 (affinity purified rabbit antibody directed against p85

amino acids 314-724) at a concentration of 0.25 μg/mL in blocking buffer was added. To the wells where GST had been added (including 0 nM wells), 100 μL of anti-GST at a concentration of 0.0625 μg/mL in blocking buffer was added. Primary antibody was incubated for 1 hour at RT. ELISA plates were washed 16 times as before. The anti-p85 wells had 100 μL of horseradish peroxidase-coupled anti-rabbit IgG (0.125 μg/mL) in blocking buffer added to them. The anti-GST wells had 100 μL of horseradish peroxidase-coupled anti-mouse IgG (0.25 μg/mL) in blocking buffer added to them. The secondary antibodies were incubated for 1 hour at RT. The ELISA plates were washed 16 times as before. To each well a 100 μL 3,3'5,5'-tetramethylbenzidine (TMB) solution (Kirkegaard & Perry Labs) was added and incubated for 30 min at RT. Reactions were stopped by adding 100 μL of 1 M H_3PO_4 per well and the absorbance was read at 450 nm.

In the absence of Rab5 bound to the plate, wells containing 250 nM p85-His_6 typically had an absorbance of 0.06-0.09, while those containing 250 nM GST had an absorbance of 0.05. When Rab5 was bound to the plate, the "background absorbance" in the absence of p85-His_6 protein (i.e. 0 nM test protein in step 5) was relatively small (for Rab5 it was 0.06-0.07; for Rab5-GDP it was 0.09-0.11; for Rab5-GTPγS it was 0.11). The corresponding background absorbance in the absence of GST protein was 0.06-0.08. Each experiment was carried out at least twice, with duplicate determinations each time. After subtracting the "background absorbance" from each experimental value, the results were averaged and plotted using Prism software (GraphPad Software, Inc.) ± standard error from the mean.

Before starting the ELISA assay, titrations of the primary (anti-p85, generated in our laboratory; 1, 0.5, 0.25, 0.125, 0.0625 μg/mL) and secondary (anti-rabbit horseradish peroxidase; 0.25, 0.125, 0.0625 μg/mL) antibodies were carried out using p85-His_6 protein bound to the wells. The lowest concentrations of antibody that still gave maximal sensitivity were selected and these were the ones used in the above method. A similar antibody titration has been carried out for the anti-GST antibody (Fang *et al*., 2002).

3.2.4.6 Purified protein pull-down experiments

An aliquot of bead suspension for each different immobilized GST-fusion protein (GST, GST-Rab5, GST-Rab5S34N and GST-Rab5Q79L prepared in section 3.2.4.1) corresponding to 5 μg was transferred to a 1.5 mL microcentrifuge tube. An additional 20 μL of 50% glutathione Sepharose beads was added to aid in visibility of the bead pellet during the wash steps. Beads were centrifuged at 2000 x g for 1 min and supernate was aspirated. The GST-Rab5 proteins were loaded with nucleotide. To each sample, 500 μL of Buffer A (20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM DTT, 5% glycerol, 0.1% Triton X-100, 10 μg/ml aprotinin, 10 μg/mL leupeptin) + 10 mM EDTA was added to remove nucleotide. The beads were incubated for 20 min at RT and centrifuged at 2000 x g for 1 min and the buffer removed. One of the following was added:

i. For no nucleotide: 400 μL Buffer A + 10 mM EDTA

ii. For Mg/GTPγS: 400 μL Buffer A + 10 mM $MgCl_2$ + 200 μM GTPγS

iii. For Mg/GDP: 400 μL Buffer A + 10 mM $MgCl_2$ + 200 μM GDP

iv. For Mg/GDP-AlF_4 (the transition state analogue): 400 μL Buffer A + 10 mM $MgCl_2$ + 1 mM $AlCl_3$ + 10 mM NaF + 1 mM GDP

Samples were allowed to bind nucleotide for 30 min at RT. The beads were centrifuged at 2000 x g for 1 min and supernate was aspirated. The p85 test protein (10 μg) was added in a volume of 50 μL of the corresponding buffer used to load the nucleotide plus 5% Carnation skim milk powder. Samples were incubated for 1 – 2 hours at 4˚C. The beads were centrifuged at 2000 x g for 1 min and washed 3 times with 500 μL wash buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% nonidet P-40). After the final wash the beads were resuspended in SDS sample buffer and resolved on SDS-PAGE gel followed by anti-p85 Western analysis.

3.2.5 Cell Culture Techniques

3.2.5.1 Growth Factor Stimulation

NIH 3T3 cells were grown as described in section 3.2.3 until they were approximately 80% confluent. The cells were deprived of serum for 24 hrs in DMEM containing 0.5% FBS. The cells were stimulated for the time indicated in the experiments with 50 ng/mL of PDGF-BB (Calbiochem) in the DMEM that the cells were starved in that contained 0.5% FBS at 37°C. The cells were placed on ice, the media removed and they were washed with cold PBS. The cells were lysed in either SDS sample buffer or PLC lysis buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM ethylene glycol-bis(2-aminoethylether) N,N,N',N'-tetraacetic acid (EGTA), 10 mM sodium pyrophosphate

(NaPP) and 100 mM NaF) containing 10 μg/ml aprotinin, 10 μg/mL leupeptin, 1 mM PMSF and 1 mM sodium orthovanadate (Na_3VO_4).

The SDS-solubilized cell lysates were used as whole cell lysates for Western blot analysis. The cells lysed in SDS sample buffer were passed through a 27 gauge needle several times to reduce the viscosity of the cell lysates. The cell lysates were heated to 100°C for 5 min to completely denature the proteins. The lysates were used immediately or stored at –80°C. The protein concentration of the lysates was determined by Lowry protein assay (Sigma) according to the manufacturer's directions.

The cells lysed in PLC buffer were used for immunoprecipitation. The PLC lysates were clarified by centrifugation (14,000 x g) for 10 min at 4°C. The supernatants were used immediately or stored at –80°C. The protein concentration of the samples were determined using the Lowry protein assay (Sigma) according to the manufacturer's directions.

3.2.5.2 Immunoprecipitation

To standardize immunoprecipitation experiments, a given amount (either volume or concentration) of lysate was used. For all experiments where a standard concentration was used, 200 μg total protein was used. The lysates were pre-cleared with 2 μg of anti-rabbit/mouse IgG agarose conjugate beads (depending on the species of the primary antibody) and 20 μL of the 50% suspension of Protein A or G beads (depending on the species of the IgG; Sigma). The lysates were pre-cleared with nutation for 1 hr at 4°C and the supernatant was collected after pelleting the beads at 14000 x g for 10 min. The proteins of interest were immunoprecipitated from the

precleared supernatant with 10 μg of anti-FLAG (mouse) or 5 μg of anti-PDGFR (rabbit). 20 μL of Protein G (for mouse) or A (for rabbit) beads (depending on the species of the primary antibody; Sigma) were also added with the antibodies and incubated for 90 min at 4°C with nutation. The beads were pelleted at 2000 x g for 1 min, the supernatant discarded and the beads were washed 3 times with 500 μL HNTG (20 mM Hepes, pH 7.5, 150 mM NaCl, 0.1%(v/v) Triton X-100 and 10% glycerol). The samples were either assayed for PI3K activity (section 3.2.5.3) or used in a Western blot analysis (section 3.2.4.3).

3.2.5.3 PI3K assay

Samples were immunoprecipitated using either anti-PDGFR antibodies (5 μg, Santa Cruz Biotechnology) or anti-FLAG M2 (10 μg, Sigma) as described in section 3.2.5.2. Immunoprecipitates were washed twice with each of the following: wash 1 (PBS), wash 2 (100 mM Tris-HCl pH 7.4, 50 mM LiCl), and wash 3 (10 mM Tris-HCl pH 7.4, 100 mM NaCl, 1 mM EDTA). Excess liquid was removed from the immunoprecipitates. Lipid micelles were generated by sonicating phosphatidylserine (PS) and phosphatidylinositol (PI) in PI3K assay buffer (25 mM Hepes pH 7.4, 10 mM $MgCl_2$) in a sonicating water bath for 20 min. Each sample was incubated with lipid micelles (5 μg PS + 2.5 μg PI) in PI3K assay buffer and 10 μCi [γ-^{32}P]ATP in a total volume of 50 μl for 15 min at 20˚C. The reaction was stopped by the addition of HCl (to 1.7 M). Lipids were extracted by chloroform: methanol (1:1 v/v) and washed with methanol: 1 N HCl (1:1 v/v). The reaction products were dried down, resuspended in chloroform: methanol (1:1 v/v) and spotted onto a Silica Gel 60 TLC plate (VWR). The

plate was developed in 1-propanol: water: acetic acid (17.4: 7.9: 1) in a chromatography chamber for 4 hours, dried and exposed to a phosphorimager screen. Results were visualized using a phosphorimager (Molecular Imager FX Pro Plus; Bio-Rad) and quantified using Quantity One software (Bio-Rad), and the statistical analysis was performed using Prism software (GraphPad Software, Inc.).

3.2.5.4 Proliferation assay

Cell proliferation was assayed using the Cell Titer 96 nonradioactive proliferation kit (Promega) according to the manufacturer's instructions. The cells were serum-starved in 0.5% fetal bovine serum-containing media for 24 hrs before being plated at a density of 5000 cells per well in a 96-well plate. The cells were tested for growth over 5 days in media containing either 2% fetal bovine serum or 50 ng/ml PDGF BB. Each experiment was done in duplicate in at least three independent experiments. The results were plotted using Prism software (GraphPad Software Inc.) and the standard error of the mean was indicated with error bars.

3.2.5.5 Apoptosis assay

Apoptotic cells were assayed using Vybrant Apoptosis Assay Kit 2 (Invitrogen) according to the manufacturer's instructions. Two 10 cm plates of proliferating cells were treated for 4 hrs with 1 mM hydrogen peroxide, washed with PBS and treated with trypsin-EDTA to remove them from the plate. The cells were washed once with media followed by a wash with PBS. The cells were counted and 10^6 cells were used for the assay according to the manufacturer's instructions. The number of viable cells were determined by lack of annexin V and propidium iodide staining by fluorescence

activated cell sorting (FACS) analysis. For a control, cells not treated with hydrogen peroxide were also assayed. The results from three independent experiments were plotted using Prism software and the standard error of the mean was indicated with error bars.

3.2.5.6 Contact inhibition assay

For the contact inhibition assay, cells (100 000) were seeded on a 10 cm plate and allowed to grow for 12 days. The media was changed every two to three days. Foci were counted and photographs were taken under phase contrast using a Coolpix 990 digital camera (Nikon) attached to an Eclipse TE300 microscope (Nikon). The total magnification was 100x. The plates of cells were fixed with methanol and stained with Giemsa stain (Sigma) according to manufacturers instructions. Photographs of each entire plate were taken with a Gel Doc 2000 (Bio Rad). The results shown are typical for three independent experiments.

3.2.5.7 Colony formation assay

To measure anchorage independent growth, a colony formation assay was performed in which the cells were grown in soft agar. An underlay of 0.6% agar containing media (DMEM, 10% FBS, 5x MEM vitamin solution (Gibco, 11120-052), 0.6% agar (Sigma, A-7002)) was poured into each 6 cm dish (approximately 5 mL) and allowed to set. Growing cells were trypsinized and counted. Three different amounts of cells (50 000, 100 000 and 250 000) were suspended in 2 mL soft agar (DMEM, 10% FBS, 3.75x MEM vitamin solution, 0.36% agar) at 40°C and added on top of the agar underlay. The cells were grown for 15 days at 37°C and 5% CO_2 and fed by adding

either 1 mL of soft agar or 2 mL of DMEM plus 10% FBS and P/S to each plate every three days. The feeding was alternated between the soft agar or DMEM every feeding starting with the soft agar feeding. Photographs were taken as described in section 3.2.5.6. The colonies formed were reported as the number of colonies per 10 000 cells seeded. Colonies were determined by their ability to increase in size over time. Colonies often had uneven edges with smaller satellite colonies growing around them. Three independent experiments were done.

3.2.5.8 Tumour formation assay

Male Swiss nude mice were injected subcutaneously with 2.5 x 10^6 cells (either NIH 3T3 or p85R274A) in the right flank as described (Cairns *et al*., 2001). The cells for injection were grown in 10 cm plates until they were approximately 80% confluent and harvested by trypsinization. The cells were resuspended in PBS and washed 3 times with 25 mL of PBS before injection into the mice. The cells were resuspended in PBS so that the total volume of the PBS and cells was 100 μL for injection. Tumour development was monitored every two to three days by measuring the length and width of the tumour using callipers. The tumour volume was determined by the equation $V=(4/3)\pi*length*width^2$. The average tumour volume was plotted using Prism software and the standard error of the mean was indicated with error bars. After the tumour reached approximately 1 cm by 1 cm the animal was sacrificed and the tumour excised. The tumour was cut into three pieces; one was frozen at –80˚C, one was fixed with formalin and one used for tissue culture. The paraffin-embedding and hematoxylin-eosin staining was performed as described (Cairns *et al*., 2001).

For generation of tumour cell lines, half of the tumour was washed aseptically with sterile PBS and incubated with 0.1% Trypsin-EDTA for 4 hr at 4˚C to breakdown the extracellular matrix of the tumour. The tumour was passed through a sterile fine steel mesh to break the tumour into single cells. These cells were incubated on 6 cm plates in DMEM media until the cells attached and started dividing. The media was not changed for at least 48 hr and more media was added if it took longer for the cells to attach. Once the cells attached, the media was changed normally. When the cells were 80% confluent, the cells were transferred to two 10 cm plates. One plate was treated with selection media containing 400 μg/mL G418 and the other was continued to be grown in DMEM media without selection. Once the cells were expanded, cell lysates were tested for FLAG expression by Western blot analysis and a sample of cells where frozen for long-term storage.

3.2.5.9 Endocytosis assay

Cells were grown on 10 cm plates to approximately 80% confluence and starved for 24 hr with DMEM plus 0.5% FBS and P/S. The cells were placed on ice and washed three times with 5 mL of ice-cold PBS^{++} (PBS plus 1 mM $MgCl_2$ and 2.5 mM $CaCl_2$). The cells were incubated with 2 mL of 1.5 μg/mL biotin (sulfo-NHS-SS-biotin; Pierce, 21331) in PBS^{++} for 30 min at 4°C. The cells were washed three times with 5 mL of 50 mM glycine (EM science) in PBS^{++} at 4°C to remove and inactivate the excess biotin. The cells were stimulated for the time indicated with 50 ng/mL of PDGF-BB (Calbiochem) in DMEM containing 0.5% FBS and P/S at 37°C and 5% CO_2. The cells were washed once with 5 mL of ice-cold PBS^{++} and incubated with 5 mL of glutathione cleavage buffer (50 mM glutathione in 75 mM NaCl, 10 mM EDTA, 1% BSA and 0.075

N NaOH) for 20 min at 4°C three times to remove the surface-bound biotin. The cells were washed three times with 5 mL of 5 mg/mL iodoacetamide (Sigma) at 4°C to inactivate the glutathione. Cells were trypsinized and resupended in 10 mL of ice-cold PBS. The cells were pelleted (2000 x g) and fixed with 2% paraformaldehyde (Electron Microscope Sciences) overnight at 4°C. They were washed with 10 mL of PBS and pelleted at 2000 x g. The cells were permeabilized with 0.05% saponin (Sigma) for 30 min at RT with gently mixing. They were washed with PBS as above and incubated with Streptavidin (SA)-FITC (eBioscience, 11-4317-87). The amount of endocytosed biotin was measured by FACS analysis.

3.2.6 Statistical Analysis

All of the statistical analysis, plotting of graphs and lines were done using Prism software (GraphPad Software, Inc.). Unless otherwise noted, ANOVA statistical analysis followed by a Tukey's test was used to determine the significance of the differences between experimental groups.

4.0 RESULTS

4.1 Interaction of p85 with Rab5

It has been shown in two studies that Rab5-GTP binds to the p110β subunit of PI3K; however, one of the studies did not test p85 and the other could not detect a direct interaction between p85 and Rab5 using *in vitro* translated p85 (Christoforidis *et al*., 1999b; Kurosu and Katada, 2001). Because p85 was shown to bind other monomeric G proteins (Rac1 and Cdc42; (Fidyk and Cerione, 2002; Zheng *et al*., 1994)) and there was some evidence that p85 is involved in endocytosis, we set out to determine if p85 could bind to Rab5. We looked for interaction between p85 and Rab5 by three different methods including: immunoprecipitation, enzyme-linked immunosorbent assay (ELISA) and a pull-down assay using purified proteins.

The first experiment consisted of immunoprecipitating FLAG-tagged p85 with HA-tagged Rab5 or vice versa from COS-1 lysates that were either unstimulated or stimulated with EGF for 5 min. These experiments did not show any interaction between the two proteins (data not shown) but in the interim we learned that the nucleotide bound states of G proteins are fragile because nucleotide dissociation can occur if the lysates are not used quickly. The lysates were routinely frozen at –80°C so we may have lost the interaction between p85 and Rab5 due to the nucleotide dissociating from Rab5. This may cause the complex between p85 and Rab5 to fall apart, especially if there is a multiprotein complex formed around Rab5-GTP. Another possible reason for the inability to see the Rab5-p85 complex was that it may be very

transient and relatively small amounts of p85 and Rab5 were likely interacting at any one time. The other experiments to test the direct interaction between p85 and Rab5 used purified proteins.

For the ELISA experiments, Rab5 was preloaded with nucleotide (either no nucleotide, GDP or GTPγS (a non-hydrolyzable form of GTP)) and was immobilized on an ELISA plate. After blocking, samples were incubated with increasing concentrations of purified p85 protein, or a control protein (glutathione S-transferase (GST)). Bound p85 and GST proteins were detected using anti-p85 and anti-GST antibodies, respectively (Fig. 4.1A). The control GST protein was not able to bind to Rab5. The p85 protein bound directly to Rab5 in a concentration-dependent manner, and this interaction was similar in the absence of nucleotide, or in the presence of GDP or GTPγS.

As a complementary approach, we also used a pull-down assay with immobilized GST-Rab5 fusion proteins preloaded with different nucleotides (Fig. 4.1B). Purified p85 protein bound best to Rab5 (no nucleotide), followed by Rab5-GTPγS. Significant amounts of p85 also bound to the transition state analogue conformation of Rab5 (GDP-AlF_4), and to Rab5-GDP. As a control we performed the same experiment with Rabaptin5 instead of p85. Rabaptin5 is reported to bind to only Rab5-GTP and not other nucleotide-bound states of Rab5 (Stenmark *et al*., 1995). It was shown to bind to only Rab5-GTP as expected, confirming that the nucleotide loading of Rab5 was successful (Fig. 4.1C). We found similar results using a pull-down assay with two Rab5 mutants (Fig. 4.1D). The Rab5S34N mutant preferentially binds to GDP, while the Rab5Q79L mutant is unable to hydrolyze bound GTP since it lacks GTPase activity. Purified p85

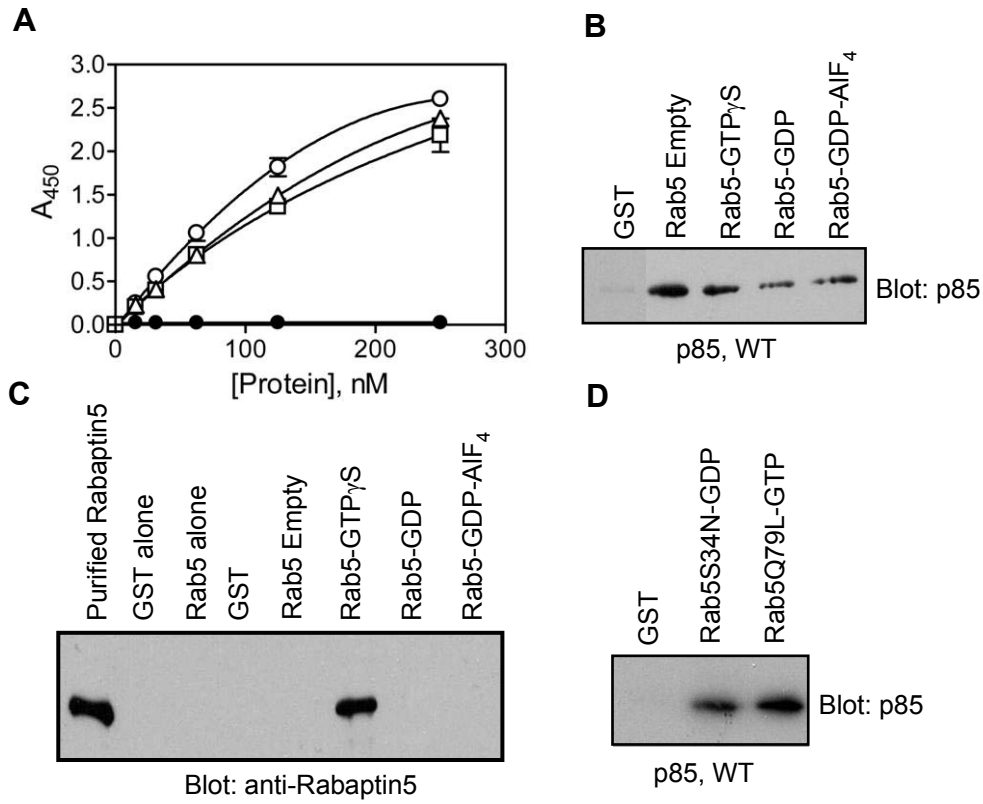


Figure 4.1 The p85 protein binds directly to Rab5. A) Wells containing Rab5 (1 μg) in the absence of nucleotide (circles), Rab5-GDP (triangles), or Rab5-GTPγS (squares) were blocked and incubated with increasing concentrations of purified p85 protein (open symbols) or control GST protein (closed symbols). Bound p85 (or GST) protein was detected using anti-p85 (or anti-GST) antibodies, followed by a secondary antibody conjugated to horseradish peroxidase and quantified by measuring the absorbance of the acidified product at 450 nm. The mean absorbance was plotted for three replicate experiments. B) GST and GST-Rab5 were immobilized on glutathione Sepharose beads and loaded with the indicated nucleotide. Purified p85 (10 μg) was added in blocking solution to the samples and incubated for 1 to 2 hr at 4˚C. The unbound p85 was washed off. The samples were resolved by SDS-PAGE and transferred to nitrocellulose. Using Western blot analysis, p85 was detected with anti-p85 antibodies and corresponding secondary antibodies conjugated to horseradish peroxidase. The blot was visualize using chemiluminescence and film. C) GST and GST-Rab5 were immobilized on glutathione Sepharose beads and loaded with the indicated nucleotide. The beads were incubated with purified Rabaptin5 and after washing, bound Rabaptin5 was detected by Western blot analysis with anti-Rabaptin5 antibodies. D) GST and GST-Rab5 mutants known to selectively bind GDP (S34N) or GTP (Q79L) were used in a pull-down assay with wild type p85 protein, as described for panel B. The results shown are typical for at least three independent experiments.

bound to both GST-Rab5S34N and GST-RabQ79L, immobilized on glutathione Sepharose beads, preloaded with GDP and GTP, respectively (Fig. 4.1D). These results indicate there was a direct interaction between p85 and Rab5, which was not significantly altered by the nucleotide-bound state of Rab5.

4.2 The GAP activity of p85

Having shown p85 and Rab5 interact, we determined whether p85 had GAP activity towards Rab5. A putative GAP domain had been reported within p85 but it was not clear whether this domain was functional. To determine if p85 had GAP activity towards Rab5, a steady state GAP assay and a single turnover GAP assay were performed. The steady state GAP assay measured the hydrolysis of GTP to GDP and allowed for the rebinding of GTP to the Rab protein, whereas the single turnover GAP assay did not allow rebinding of radiolabelled GTP to the Rab protein. Thus, it measured the catalytic rate (Anderson and Chamberlain, 2005).

4.2.1 p85 has GAP activity towards Rab5

The GAP activity of p85 towards Rab5 was determined using a GAP assay in which Rab5 was preloaded with [α-^{32}P]GTP and assayed alone, or with increasing concentrations of p85 protein. The reaction was initiated by the addition of $MgCl_2$, and the resulting [α-^{32}P]GTP and [α-^{32}P]GDP were separated by thin layer chromatography, prior to detection using a phosphorimager (Fig. 4.2A). The mean fmoles of radiolabelled GDP generated by Rab5 GTPase activity from three independent experiments were plotted as a function of p85 concentration (Fig. 4.2B).

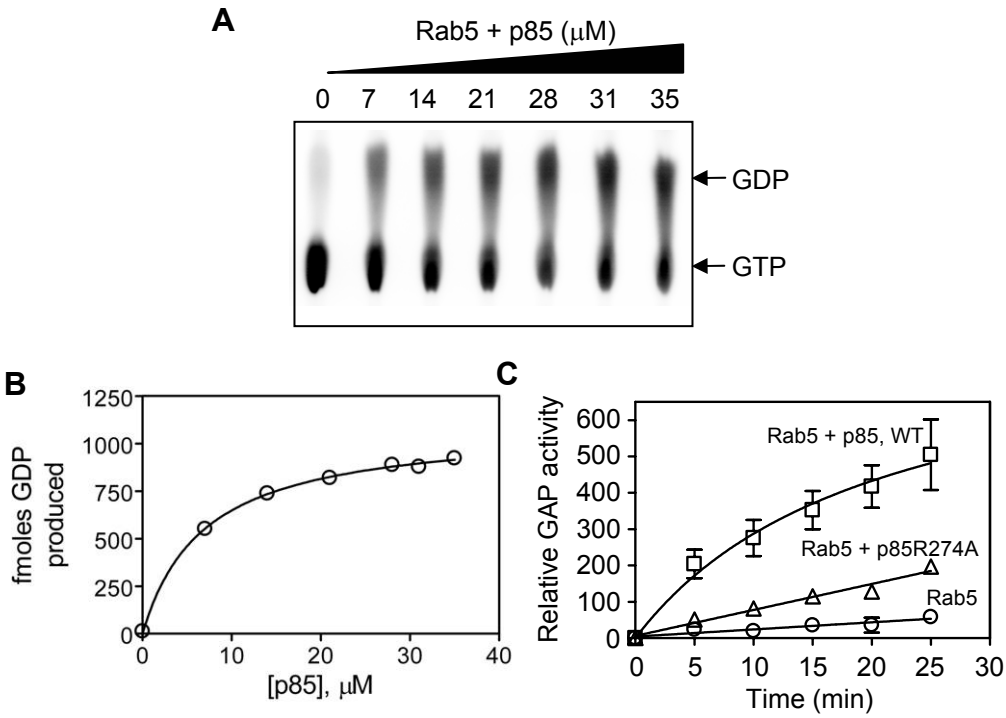


Figure 4.2 The p85 protein stimulates Rab5 GTP hydrolysis. A) Rab5 (200 nM) was loaded with [α-^{32}P]GTP, and hydrolysis to [α-^{32}P]GDP was assayed for 10 minutes in the absence and presence of increasing concentrations of p85 as indicated. Nucleotides were resolved by thin layer chromatography and visualized using a Phosphorimager. B) The results in panel A were quantified with Quantity One software and the fmoles of GDP calculated. The results of three independent determinations were used to calculate the mean and the SEM error bars, which are smaller in size than the circles of the data points. C) Rab5 was loaded with [α-^{32}P]GTP and hydrolysis to [α-^{32}P]GDP was assayed for different times in the presence of a large excess of unlabelled GTP (1.7 mM), either alone (circles), or in the presence of wild type p85 (10 μM, squares), or the mutant p85R274A (10 μM; triangles). The results were quantified with Quantity One software and the relative GAP activity was calculated. The mean of three independent experiments are shown and the error bars are the SEM.

The GAP activity of two different p85 preparations was tested. The p85 prepared using the PreScission cleavage method (max. 900 fmol GDP produced in 10 min) was found to be higher in activity than that of the p85 liberated by thrombin cleavage (max. 300 fmol GDP produced in 10 min). The thrombin cleaved p85 was used in all subsequent figures. The p85 protein stimulated the hydrolysis of GTP to GDP by Rab5 in a concentration-dependent manner with a maximum stimulation of approximately 570 to 1700-fold, depending upon the preparation of p85 protein used.

To ensure p85 could accelerate the catalytic rate of Rab5 GTP hydrolysis and not act by stimulating nucleotide exchange, we used a single turnover GAP assay. Rab5 was preloaded with [α-^{32}P]GTP and the reaction was carried out in the presence of excess unlabelled GTP, in the presence or absence of added p85. Aliquots were taken at various time points and the amount of hydrolyzed [α-^{32}P]GDP generated was visualized after separation by TLC and quantified using a phosphorimager (Fig. 4.2C). The addition of p85 to the assay substantially accelerated the Rab5-mediated hydrolysis of the bound [α-^{32}P]GTP to [α-^{32}P]GDP, suggesting that p85 stimulated GTP hydrolysis by Rab5 and not by simply promoting nucleotide exchange. A point mutation in p85, changing Arg274 to Ala, is described in more detail in section 4.2.5 and was found to compromise the GAP activity of p85 towards Rab5 (Fig. 4.2C)

4.2.2 p85 has GAP activity towards other Rab proteins

To test whether the GAP activity of p85 was specific for the Rab5 GTPase, or would also stimulate the GTPase activities of other Rab proteins, p85 was added to GAP assays containing several different Rab proteins: Rab4, Rab6, Rab7 and Rab11 (Fig.

4.3). Rab proteins play important roles in membrane fusion events of various trafficking steps in the endosomal pathways. Each Rab protein regulates a specific part of the endosomal pathway. Rab4 and Rab11 are involved in transport of receptors from early endosomes to recycling endosomes and the plasma membrane, whereas Rab7 is involved in receptor transport from late endosomes to lysosomes for receptor degradation. Rab6 is involved in membrane fusion events important during retrograde transport from the Golgi back to the endoplasmic reticulum. The p85 protein displayed similar GAP activity towards Rab4, Rab5 and Rab7 (Fig. 4.3). The GTPase activity of Rab6 was also stimulated by p85, but to a lesser degree, while Rab11 GTPase activity was not significantly altered by p85 addition. These results suggest that p85 is a GAP protein for several different Rab-family GTPases; in particular, it can stimulate the downregulation of Rab4, Rab5 and Rab7.

4.2.3 p85 has GAP activity towards Rho-family proteins

Previous studies have tested the ability of p85 to bind to and act as a GAP protein towards Rac1 and Cdc42, two Rho GTPases involved in the regulation of actin structures (Bokoch *et al*., 1996; Zheng *et al*., 1994). While p85 was shown to bind Rac1 and Cdc42 at concentrations of 0.1-1 μM, at these concentrations p85 was not found to possess GAP activity towards these two G proteins. There have, however, been reports of GAP proteins (e.g. ArfGAP) that require μM concentrations to stimulate GTP hydrolysis towards their cognate monomeric G protein (Goldberg, 1999). Since the concentration of p85 used in our Rab GAP assays was typically in the 10-20 μM range, and given the precedent for some GAP proteins requiring higher concentrations, we

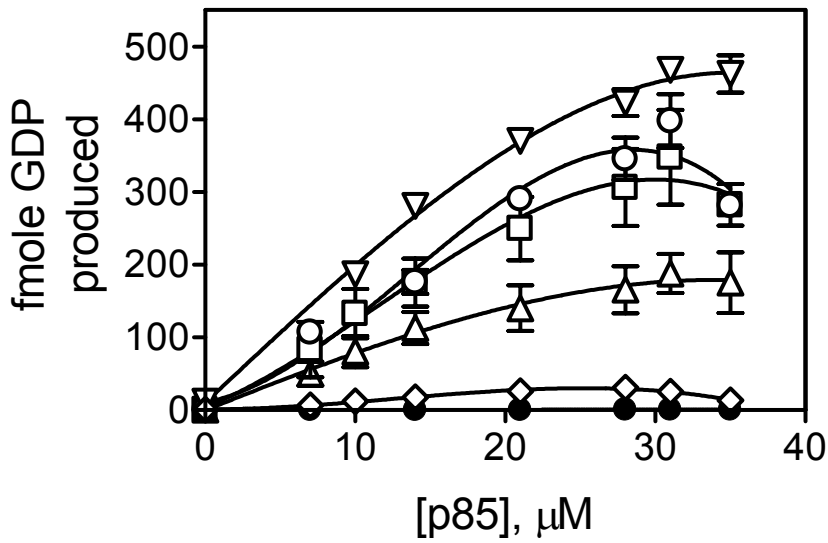


Figure 4.3 The GAP activity of p85 towards GTPases of the Rab family. Increasing concentrations of p85 were added to each Rab (200 nM) preloaded with [α-^{32}P]GTP and assayed as for GAP activity in Figure 4.2A-B. Rab5 (circles), Rab4 (squares), Rab6 (triangles), Rab7 (inverted triangles), Rab11 (diamonds), no Rab (closed circles). The mean of at least three independent experiments are shown and the error bars are the SEM.

retested p85 for GAP activity towards Rac1 and Cdc42 (Fig. 4.4). At these higher concentrations of p85, we observed p85 did have GAP activity towards both Rac1 and Cdc42. The GAP activity was similar in magnitude to the activity towards Rab5. Thus, while p85 does not act as a general GAP towards all Rab proteins (i.e. not Rab11, and to lesser extent towards Rab6), it is able to regulate the GTP hydrolysis of some Rab proteins and also some Rho proteins.

4.2.4 The BH domain of p85 contains the GAP activity

Given the sequence similarity between the BH domain of p85 and several other GAP domains (Musacchio *et al*., 1996; Zheng *et al*., 1994), we suspected it was the BH domain of p85 which encoded the observed Rab5 GAP activity. We generated polypeptides encompassing different p85 domains (Fig. 4.5A), and tested each for Rab5 GAP activity (Fig. 4.5B). As expected, the BH domain of p85 contained substantial Rab5 GAP activity. The p85 SH3 domain, the (N+C)SH2 domains (which included the p110-binding domain of p85), and a mutant of p85 lacking the BH domain (ΔBH; missing amino acids 84-313), showed little or no GAP activity towards Rab5. Although the isolated BH domain was not as active as the full-length p85 protein, these results suggest it is the BH domain of p85 that possesses Rab5 GAP activity and it can adopt a suitable conformation to demonstrate partial activity when expressed as an isolated domain.

4.2.5 p85 has an arginine finger

GAP proteins typically act in two ways to accelerate GTP hydrolysis of the GTPase (Scheffzek *et al*., 1998). First, many GAP proteins contain an arginine finger

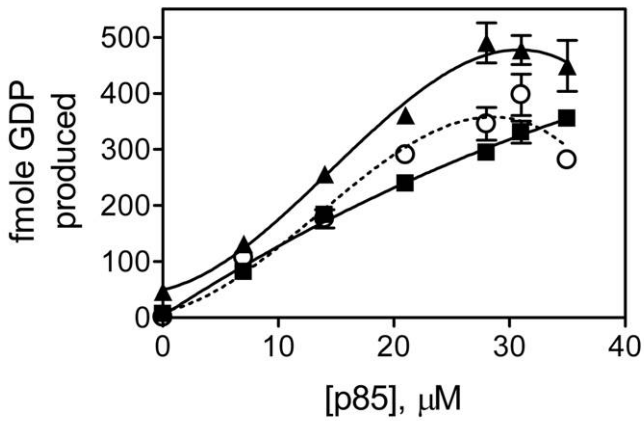


Figure 4.4 The GAP activity of p85 towards GTPases of the Rho family. The ability of p85 to stimulate Rac1 (triangles) and Cdc42 (squares) GTP hydrolysis was tested as in figure 4.2A-B. For comparison, the dashed line indicates p85 GAP activity towards Rab5 (open circles). The mean of at least three independent experiments are shown and the error bars are the SEM.

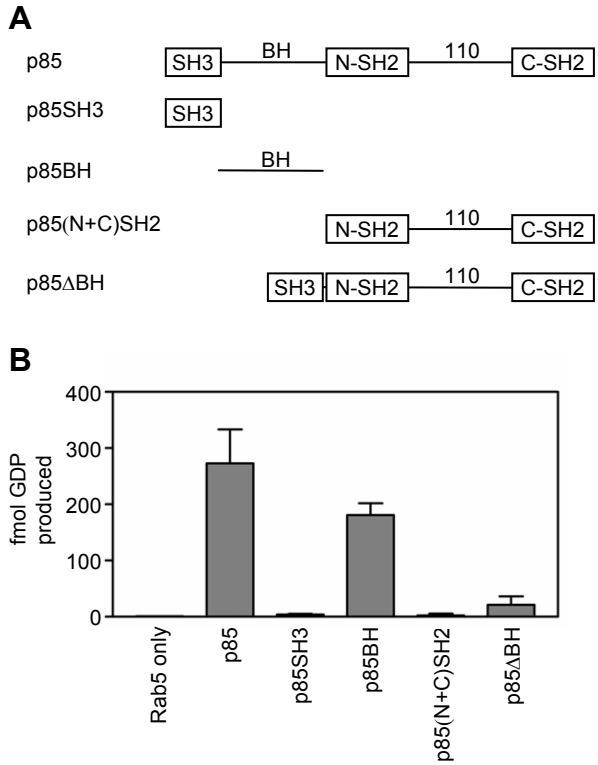


Figure 4.5 The BH domain of p85α encodes Rab5 GAP activity. A) The regions of p85 that were bacterial-expressed and purified. B) GAP assay testing the ability of different domains of p85 (10 μM each) to stimulate the GTPase activity of Rab5, as described in figure 4.2A-B. The mean of at least three independent experiments are shown and the error bars are the SEM.

positioned in the active site of the GTPase, which stabilizes the transition state during the hydrolysis reaction. Second, GAPs bind to the switch II region of the GTPase, which stabilizes and orients the critical glutamine residue within the active site of the GTPase. The literature on GAP proteins and their folded structures suggests it is often difficult to predict from a sequence alignment which arginine residue corresponds to the arginine finger. The BH domain of human p85 has been crystallized and a pocket of the domain containing a cluster of conserved residues has been proposed as a monomeric G protein binding site (Musacchio *et al*., 1996). There are two arginine residues (R151 and R274) within this proposed monomeric G protein binding site of the p85 BH domain. Each of these residues were mutated to alanine in the context of the full length p85 protein, and each mutant was tested for Rab5 GAP activity (Fig. 4.6). The p85R151A protein had about 65% of the GAP activity of wild type p85, whereas the p85R274A mutant had less than 5% GAP activity remaining. The GAP activity of the p85R274A mutant protein was also assayed using the single turnover GAP assay and was found to have little GAP activity, as compared to the wild type p85 protein (Fig. 4.2C). Thus, while both arginine residues are required for full p85 Rab5 GAP activity, R274 appears to play a more critical role than R151.

The ability of the p85 mutants deficient in GAP activity (p85ΔBH, p85R151A and p85R274A) to bind to Rab5S34N-GDP and Rab5Q79L-GTP was explored to determine if the loss of GAP activity was because the p85 mutants could no longer bind to Rab5 (Fig. 4.7). All three mutants, including the p85 protein lacking the entire BH domain, still bound some form of Rab5 suggesting additional domains other than the BH domain of p85 participate in Rab5 binding. The p85R151A protein, which retains 65%

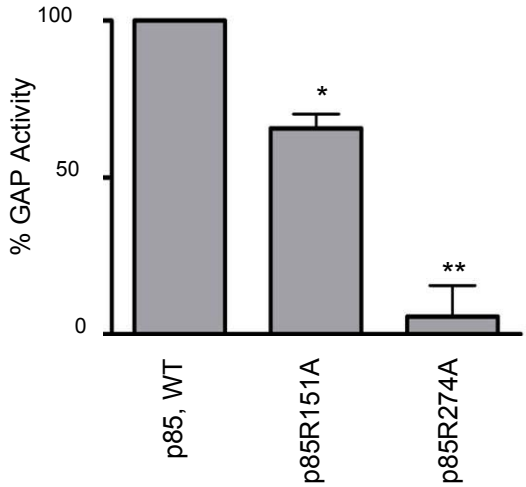


Figure 4.6 The p85 protein has an arginine finger. GAP assay comparing the ability of wild type (p85, WT) and mutant p85 proteins (p85R151A, p85R274A) to accelerate the GTPase activity of Rab5. The assay was performed as described in figure 4.2A-B and the GAP activity was compared to wild type p85 as a percentage. The mean of at least three independent experiments are shown and the error bars are the SEM. An ANOVA was done to determine the significance of the differences between wild type p85 and the p85 mutants. * = $p < 0.05$; ** = $p < 0.001$.

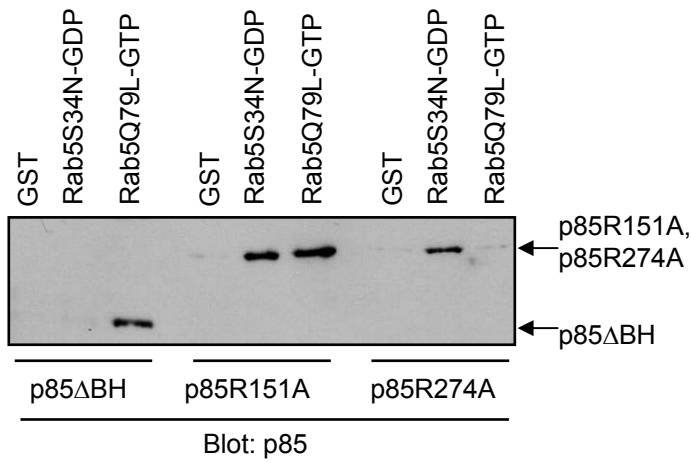


Figure 4.7 Binding of p85 mutants to Rab5. The indicated p85 mutants were tested for their ability to bind to GST (control), GST-Rab5S34N-GDP and GST-Rab5Q79L-GTP. GST and GST-Rab5 mutants were immobilized on glutathione Sepharose beads and loaded with the indicated nucleotide. Purified p85 mutants were added and bound p85 mutants were detected after washing, using an Western blot analysis with anti-p85 antibodies as described in figure 4.1B and D. This is a typical results for at least three independent experiments.

of its GAP activity, bound to the GDP- and GTP-bound forms of Rab5, much as the wild type p85 protein. In contrast, the two BH mutations (p85ΔBH and p85R274A) that dramatically decreased the GAP activity of p85 had large and opposite effects in their abilities to bind Rab5. The p85ΔBH mutant only bound Rab5Q79L-GTP, whereas the p85R274A mutant only bound Rab5S34N-GDP. This suggests that other domains in addition to the BH domain bind to Rab5 but the BH domain may have control over the nucleotide form of Rab5 that p85 binds to. This showed that although the p85 mutants do not have GAP activity, they can all bind to Rab5 to some degree.

4.3 Cellular effects of expression of p85 mutants defective for GAP activity

To determine the effects of mutations within the BH domain of p85 on cellular functions, we first needed to establish whether these mutations also compromised other known functions of p85 such as binding to the catalytic subunit of PI3K, p110, and growth factor dependent receptor binding. COS-1 cells were co-transfected with a Myc-epitope tagged p110α (Myc-p110α), and either a wild type or mutant version of a FLAG-epitope tagged p85 (FLAG-p85). Lysates from these transfected cells were confirmed to express both Myc-p110α and FLAG-p85 fusion proteins, as determined using Western blot analysis with anti-Myc and anti-FLAG antibodies (Fig. 4.8A). Anti-FLAG immunoprecipitates of these cell lysates were divided in half and either used for Western blot analysis to test for the presence of Myc-p110 protein, followed by FLAG-p85 protein (Fig. 4.8B), or used in a PI3K assay (Fig. 4.8C). As a control, anti-p85 immunoprecipitates from untransfected cells were also assayed for PI3K activity. These

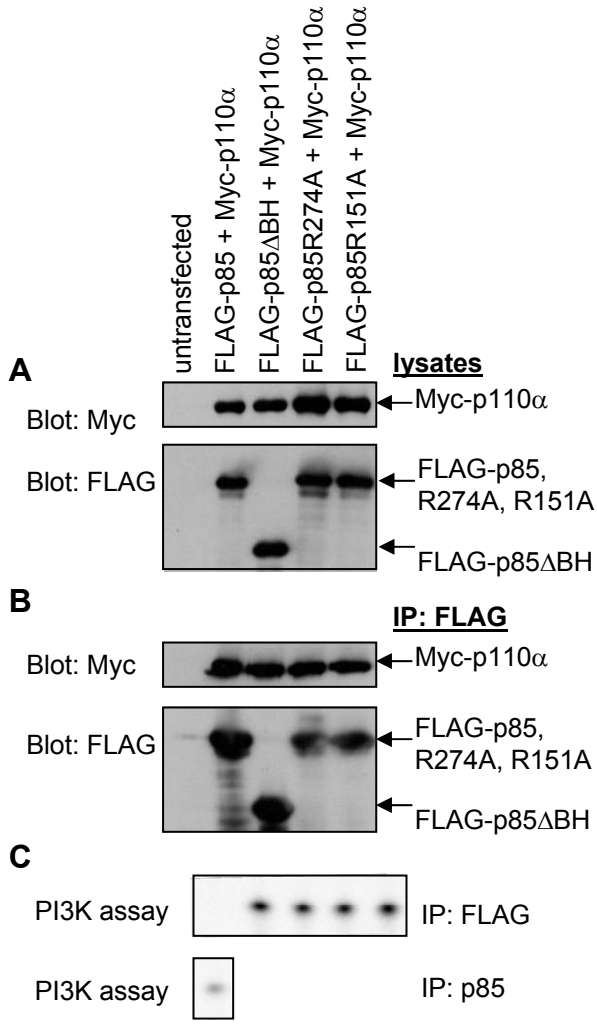


Figure 4.8 The p85 BH domain mutants retain their ability to bind to p110 and associate with PI3K activity. COS-1 cells were cotransfected with the indicated FLAG-p85 encoding plasmid together with one encoding Myc-p110α, or left untransfected as a control. A) Lysates (20 μg total protein) from each transfectant were probed with anti-Myc antibodies, stripped and reprobed with anti-FLAG antibodies in a Western blot analysis. B) Lysates (200 μg total protein) were immunoprecipitated (IP) with anti-FLAG antibodies and Western blots were probed with anti-Myc antibodies, stripped and reprobed with anti-FLAG antibodies. C) Anti-FLAG immunoprecipitates were assayed for associated PI3K activity. Radiolabelled PI3K lipid products were resolved by thin layer chromatography and visualized using a phosphorimager. As a positive control, an anti-p85 immunoprecipitate of lysates from untransfected NIH 3T3 cells was also assayed for associated PI3K activity. This is a typical result for at least three independent experiments.

results indicated that all of the FLAG-p85 proteins (wild type, p85ΔBH, p85R274A and p85R151A mutants) can associate with the Myc-p110α protein and PI3K activity.

NIH 3T3 cells were also transfected with wild type FLAG-p85 or mutants lacking the GAP activity encoded by the BH domain (FLAG-p85ΔBH and FLAG-p85R274A). Cell lines stably expressing these FLAG-p85 proteins were treated with PDGF-BB for various times. The PDGFR typically binds p85 in a PDGF-dependent manner, as shown by the presence of p85 in anti-PDGFR immunoprecipitates from PDGF treated NIH 3T3 cell lysates (Fig. 4.9A). Similarly, we found anti-PDGFR immunoprecipitates contained FLAG-p85 proteins (wild type p85, p85ΔBH and p85R274A) after PDGF stimulation (Fig. 4.9A). PDGFR immunoprecipitates from all of these cell lines also contained substantial amounts of associated PI3K activity after PDGF treatment (Fig. 4.9B). Thus, while p85ΔBH and p85R274A have severely decreased Rab5 GAP activity, they are still able to bind the p110 catalytic subunit of PI3K and associate with the PDGFR in a PDGF-dependent manner comparable to the wild type p85 protein.

4.3.1 Decrease in the rate of PDGFR degradation

To determine the effects of mutations of the p85 BH domain on PDGFR downregulation and cell signalling pathways, we characterized the NIH 3T3 cells stably expressing FLAG-p85, FLAG-p85ΔBH and FLAG-p85R274A (Fig. 4.10A). Cells were treated with PDGF for various times and the amount of PDGFR protein was analyzed using Western blot analysis (Fig. 4.10B). The lower band in the PDGFR blots is the immature incompletely glycosylated form of PDGFR. The PDGFR is both N-linked and O-link glycosylated (Bejcek *et al*., 1993). Untransfected NIH 3T3 cells stimulated with

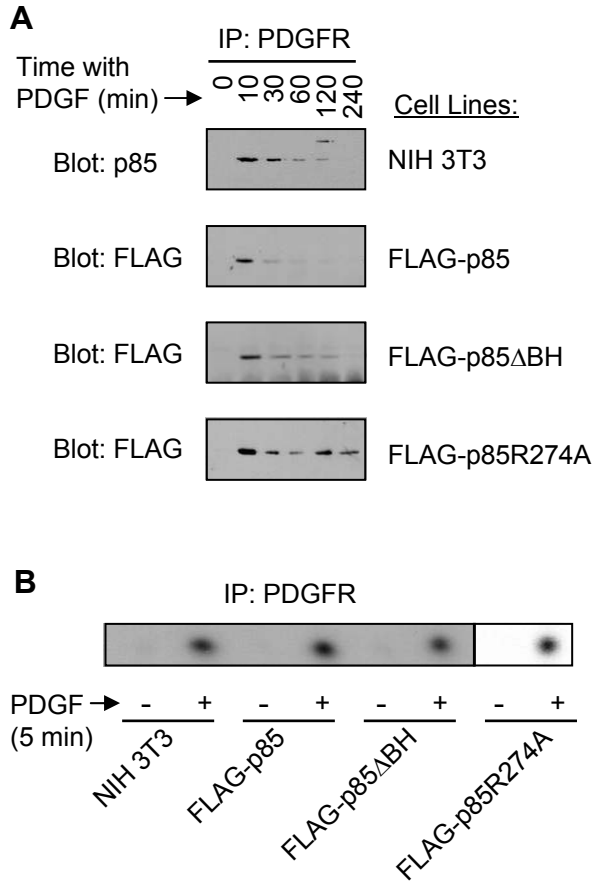


Figure 4.9 The p85 BH domain mutants retain their ability to bind to activated PDGFRs and do not prevent PDGF-dependent association of PI3K activity with the receptor. NIH 3T3 cells stably expressing the indicated FLAG-p85 proteins were stimulated for various times with PDGF BB. A) Lysates (200 μg) from each cell line were immunoprecipitated (IP) with anti-PDGFR antibodies and Western blots were probed with anti-FLAG antibodies (for cells expressing FLAG-p85, FLAG-p85ΔBH and FLAG-p85R274A), or with anti-p85 antibodies (for untransfected NIH 3T3 cells). B) Lysates from cells that had been stimulated with PDGF BB (5 min; +) or not (-) were immunoprecipitated with anti-PDGFR antibodies and were assayed for PDGFR-associated PI3K activity using radioactive PI4,5P. This is a typical result for at least three independent experiments.

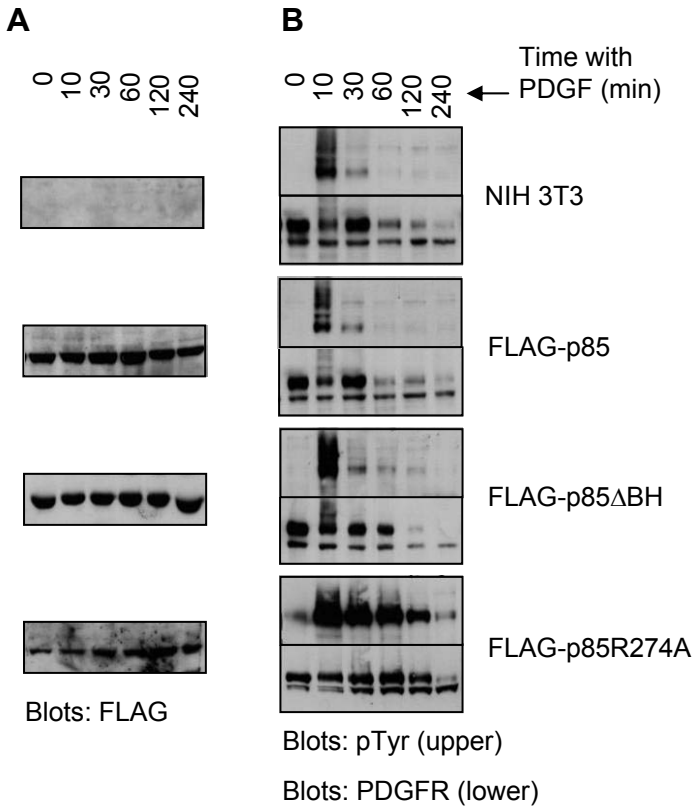


Figure 4.10 Overexpression of FLAG-p85ΔBH or FLAG-p85R274A slows the downregulation of the activated PDGFR. Untransfected NIH 3T3 cells, or cells stably expressing either wild type FLAG-p85, FLAG-p85ΔBH or FLAG-p85R274A, were stimulated with PDGF BB for the indicated times. Cell lysates (20 μg protein per lane) were analyzed by Western blot analysis with the indicated antibodies. A) The levels of expression for the transfected proteins. B) The upper panel is the amount of phosphorylated PDGFR and the lower panel is the amount of total PDGFR. Results are typical for at least three independent experiments.

PDGF for increasing times typically showed a reduction in PDGFR protein levels due to the degradation of receptor protein as part of the downregulation mechanism. By 60 minutes the majority of the receptor had been degraded (Fig. 4.10B, lower). Similar results were obtained for NIH 3T3 cells expressing wild type FLAG-p85. In contrast, cells expressing the FLAG-p85ΔBH or FLAG-p85R274A mutants showed a delay in the PDGFR degradation profile, with the majority of the receptor still present at 60 minutes or longer (Fig. 4.10B, lower). The corresponding anti-phosphotyrosine blots (Fig. 4.10B, upper) show enhanced tyrosine phosphorylation of a protein that likely corresponds to the PDGFR, most markedly in cells expressing p85R274A. Thus, mutation or loss of the p85 BH domain was sufficient to compromise its GAP activity, impair the ability of the PDGF receptor to be degraded and increase the half-life of the activated PDGFR. This suggests the loss of GAP activity by p85 decreased the targeting of the receptor from the early endosome to the late endosome and lysosome for degradation by altering the flow of receptor through the early endosome.

4.3.2 Changes to signalling from PDGFR

To assess if the enhanced activated PDGFR levels at the later time points gave rise to enhanced receptor signalling, the levels of activated Akt and activated MAPK were determined (Fig. 4.11). Cells expressing FLAG-p85ΔBH or FLAG-p85R274A showed minor increases in the phosphorylation of Akt (pAkt) during the PDGF stimulation time course, when compared to the parental NIH 3T3 or FLAG-p85 expressing cells (Fig. 4.11A). However, striking differences were observed for activated MAPK (Fig. 4.11B). Cells expressing FLAG-p85ΔBH or FLAG-p85R274A showed

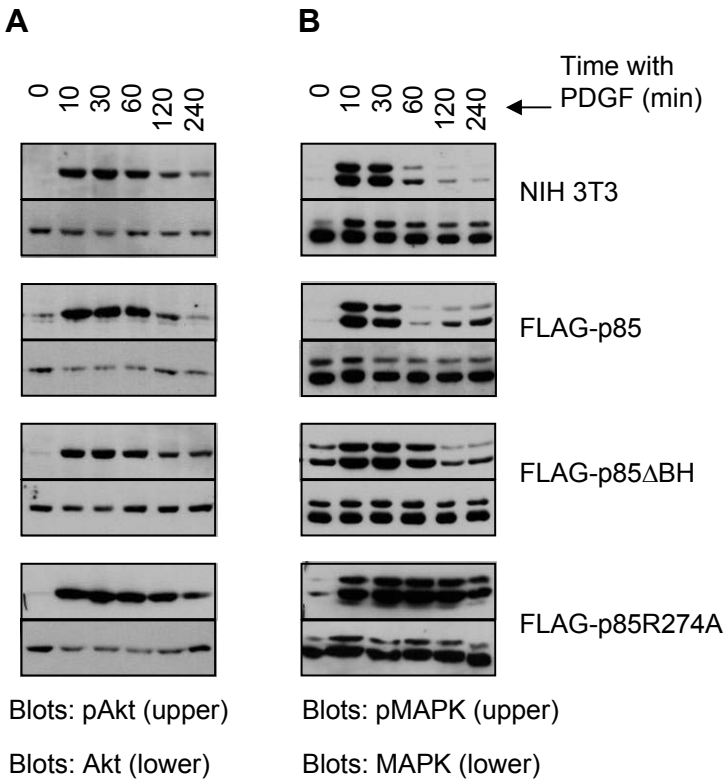


Figure 4.11 Expression of FLAG-p85ΔBH and FLAG-p85R274A increases signalling from the PDGFR. Untransfected NIH 3T3 cells or cells stably transfected with plasmids containing either FLAG-p85, FLAG-p85ΔBH or FLAG-p85R274A were stimulated with PDGF for the indicated times (min). Cell lysates (20 μg total protein) were probed with the indicated antibodies in a Western blot analysis. A) The upper panel is the amount of phosphorylated Akt in the cells and the lower panel is the amount of total Akt in the cells. B) The upper panel is the amount of phosphorylated MAPK in the cells and the lower panel is the amount of total MAPK in the cells. The results are typical for three independent trials.

enhanced MAPK activation even prior to PDGF treatment, and maintained high levels of phosphorylated MAPK (pMAPK) for 60 minutes or longer (Fig. 4.11B, upper). This result suggests that MAPK signalling pathways remain active while PDGFR levels were high in FLAG-p85ΔBH- or FLAG-p85R274A-expressing cells. These results are consistent with a previous report that suggested that MAPK signalling, but not PI3K signalling, is dependent upon internalized PDGFR (Chiarugi *et al*., 2002).

4.3.3 Increased rate of PDGFR recycling

The loss of GAP activity of p85 can have one of two effects on the cell. The first is to limit the amount of the inactive Rab protein bound to GDP needed for new rounds of Rab targeting to the membrane. Second, it can increase the overall levels of active Rab proteins bound to GTP. Which of these two effects happens depends on how the GAP protein functions in the cell. If the GAP acts only after the Rab has performed its function in membrane fusion, then the first case happens but if the GAP acts at any time on the Rab protein, then the second situation is more likely. Also, if there is more than one GAP protein for a Rab protein this could change the effects of the loss of the p85-encoded GAP activity.

There are several mechanisms that could account for the decrease in receptor degradation seen in the FLAG-p85R274A and the FLAG-p85ΔBH cells (Fig. 4.12). The regulation of receptor traffic through the early endosome may account for the decrease in receptor degradation. There are three points of receptor trafficking that may be perturbed by the p85 mutants that could account for the decrease in receptor degradation. The p85 mutants could cause a decrease in receptor entry into the early

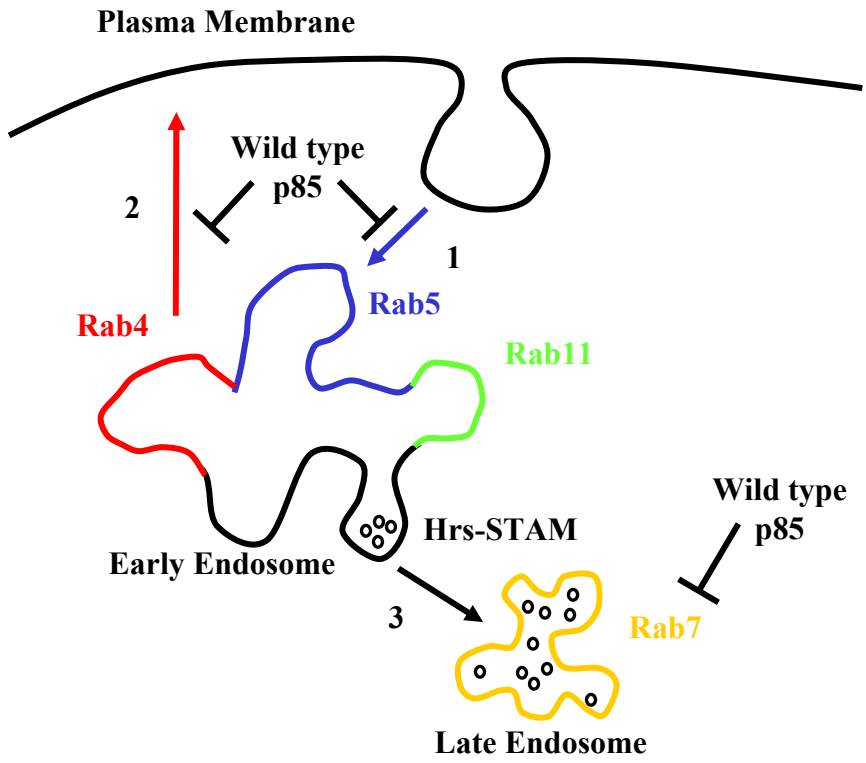


Figure 4.12 Possible mechanisms for decreased PDGFR degradation rates in p85R274A-expressing cells. p85 has GAP activity against Rab4, Rab5 and Rab7. Loss of this GAP activity could have several effects on receptor degradation. 1) The amount of Rab5 available for endocytosis could be reduced due to improper inactivation of Rab5-GTP. 2) The receptor could spend less time in the early endosome due to increased Rab4-GTP dependent recycling of the receptor with or without a change in Rab5-GTP dependent endocytosis. This could cause the receptor not to be mono-ubiquitylated by Cbl, or block the mono-ubiquitylated receptor from interacting with the Hrs-STAM complex to enter the MVB. 3) There could be malformation of the MVB or late endosome due to effects of p85R274A on Rab7.

endosome, increase the rate of receptor recycling or block the targeting of the receptor for degradation. The change in the rate of the receptors entering the early endosome, via a decrease in the rate of endocytosis due to a decrease in available Rab5-GDP, would result in a decrease in the number of receptors in the early endosome targeted for degradation. Conversely, if there was an increase in the rate of endocytosis due to more Rab5-GTP, then there would be an increased number of receptors in the early endosome. The increased level of receptors in the early endosome could cause an increase in receptor degradation or recycling. If there was an increase in receptor recycling due to more Rab4-GTP, then there would be less receptors in the early endosome to be targeted for degradation. If there was a decrease in receptor recycling due to a decreased availability in Rab4-GDP, there would be more receptors in the early endosome to be targeted for degradation. If p85R274A blocked receptor targeting for degradation, there would be a build up of receptor in the early endosome, which could cause the receptor to be recycled.

To determine which of the possible perturbments of the endosomal pathway is caused by p85R274A, a biotin-labelling endocytosis assay was used to measure the change in internalized biotin-labelled proteins when cells were stimulated by PDGF. Cell surface proteins were biotinylated and the cells were stimulated with PDGF to cause receptor endocytosis. Endocytosis was stopped by cooling the cells and the surface biotin was removed, allowing the amount of internalized biotinylated proteins to be measured. An initial experiment was performed to determine that the removal of surface biotin was successful (Fig. 4.13A). There also was an internal control in every experiment to determine if the removal of surface biotin was successful. This internal control was that the mean fluorescent intensity of the control sample that was not labeled

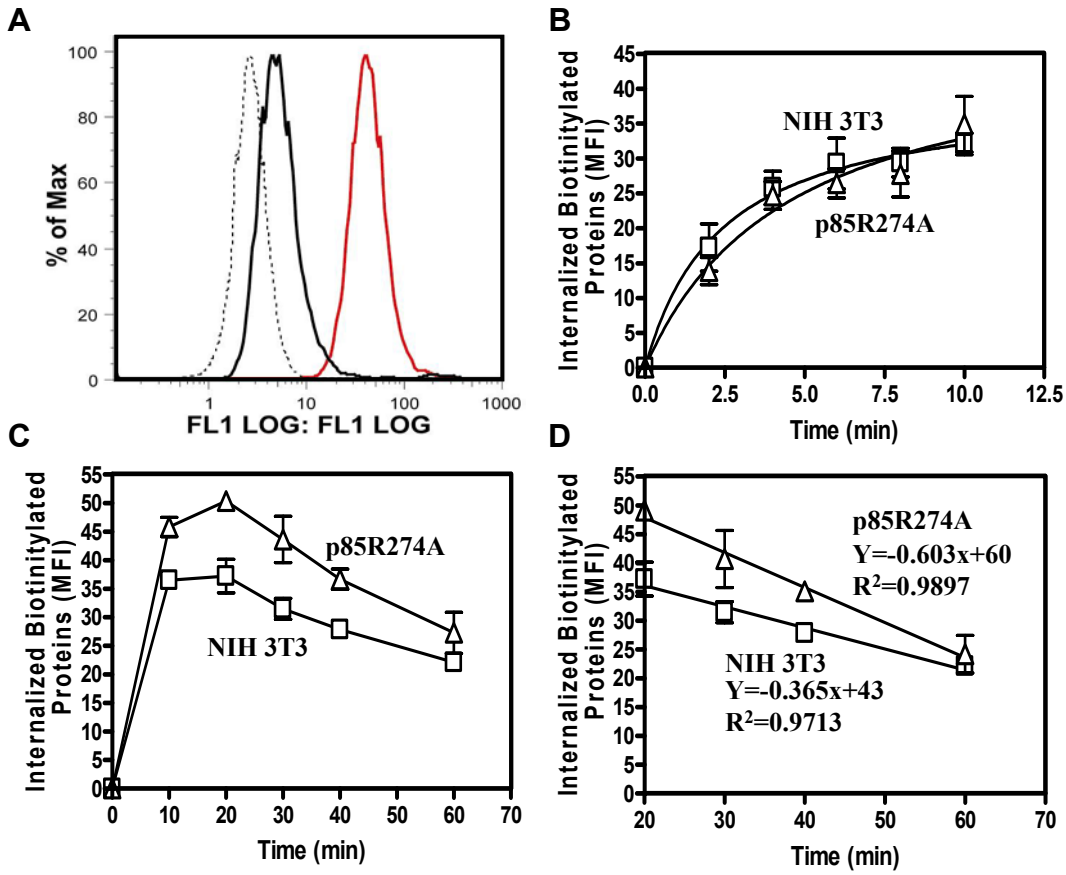


Figure 4.13 Internalization of biotin-tagged proteins in response to PDGF. The efficiency of stripping off the biotin tag is shown in panel A. The number of cells was plotted against the log scale of the fluorescent intensity of the cells. The dashed line are p85R274A-expressing cells that have not been biotinylated. The black line is p85R274A cells where the surface was biotinylated and then stripped off and the red line is the internalized biotin from p85R274A expressing cells at the 10 min time point of panel B. For panels B and C the NIH 3T3 cells (squares) or p85R274A-expressing cells (triangles) were surface-biotinylated and stimulated with 50 ng/ml PDGF for the indicated times. The surface biotin was stripped off and the amount of internalized biotinylated proteins was measured by flow cytometry. Panel D is the linear portion of panel C with a line drawn through the points and the equation of the line shown. The mean fluorescent intensity (MFI) results shown are from three determinations and the error is the SEM.

with biotin had similar mean fluorescent intensity of the zero time point sample. The biotin-labelling assay was used to measure the rate of internalized biotinylated proteins including the PDGFR, in response to PDGF stimulation. There was little difference in the rate of protein endocytosis between the FLAG-p85R274A-expressing and the parental NIH 3T3 cell lines during the initial 10 min of PDGF stimulation (Fig. 4.13B). This suggested that there was no change in the rate of receptor uptake during this initial 10 min of PDGF treatment but there was an increase in the bulk uptake of surface proteins over longer time points (Fig. 4.13C). This suggests that p85 may act as a Rab5 GAP on the plasma membrane which increases the amount of surface protein uptake when the GAP activity of p85 is compromised via the Arg274 to Ala point mutation.

The other two points in receptor trafficking that could be altered by the p85R274A mutant are the exit from the early endosome via either receptor recycling or targeting to the late endosome/ lysosome for degradation. To measure the rate of recycling, the amount of internalized protein was measured over 60 min of stimulation (Fig. 4.13C). Over that time frame, the NIH 3T3 cells had maximal protein uptake by 10 min that reached steady state equilibrium for 10 min compared to the p85R274A-expressing cell lines, which had maximal protein uptake by 20 min. After 20 min both cell lines showed a decrease in internalized protein. This decrease in internalized protein could be due to receptor recycling to the plasma membrane or receptor degradation in the lysosome.

The linear portion of Fig. 4.13C between the time points of 20 to 60 min was plotted and the equations of these lines were determined. The decrease in internalized protein from the cells over time was the rate of change or the slope of the line from Fig. 4.13D. The slopes for the NIH 3T3 and FLAG-p85R274A cell lines are -0.365 MFI/min

and -0.607 MFI/min, respectively (Fig. 4.13D). This suggested that the expression of p85R274A almost doubles the rate of loss of the internalized protein, which could be the receptor recycling rate. In the NIH 3T3 cells, the receptor half-life is approximately 60 min (Fig. 4.10C) so the decrease in internalized protein levels are probably due to both receptor recycling and degradation. In the p85R274A-expressing cells the receptor half-life is at least 2 hr (Fig. 4.10C) so the decrease in internalized protein levels over one hour (Fig. 4.13C-D) is most likely due to receptor recycling. This correlative experiment suggested that p85R274A expression increases the rate of receptor recycling, which causes a decrease in the rate of receptor degradation.

4.4 The Oncogenic properties of the arginine finger mutant p85R274A

Morphological differences were observed between the parental NIH 3T3 cells and the cells expressing wild type p85 and the cells with p85 mutants, p85ΔBH and p85R274A (Fig. 4.14). In contrast, the wild type p85-expressing cells had a similar morphology to the parental NIH 3T3 cells, suggesting that the altered morphologies were due to the BH domain mutations and not just due to the overexpression of the p85 protein. The different p85 and p85 mutant cells expressed similar levels of p85 protein, with the exception of p85ΔBH being expressed at levels less than 50% of p85 wild type and p85R274A clone 1 (Fig. 4.15). The p85ΔBH-expressing cells had a flattened and enlarged appearance compared to the NIH 3T3 cells due to cell spreading caused by cell adherence, whereas the p85R274A-expressing cells had a rounded appearance and seem smaller in size likely due to a loss of cell adherence (Fig. 4.14). The apparent decrease in cell size and the rounding of the cells due to loss of adherence are characteristic morphologic changes that frequently occur in transformed cells. Also, the p85R274A-

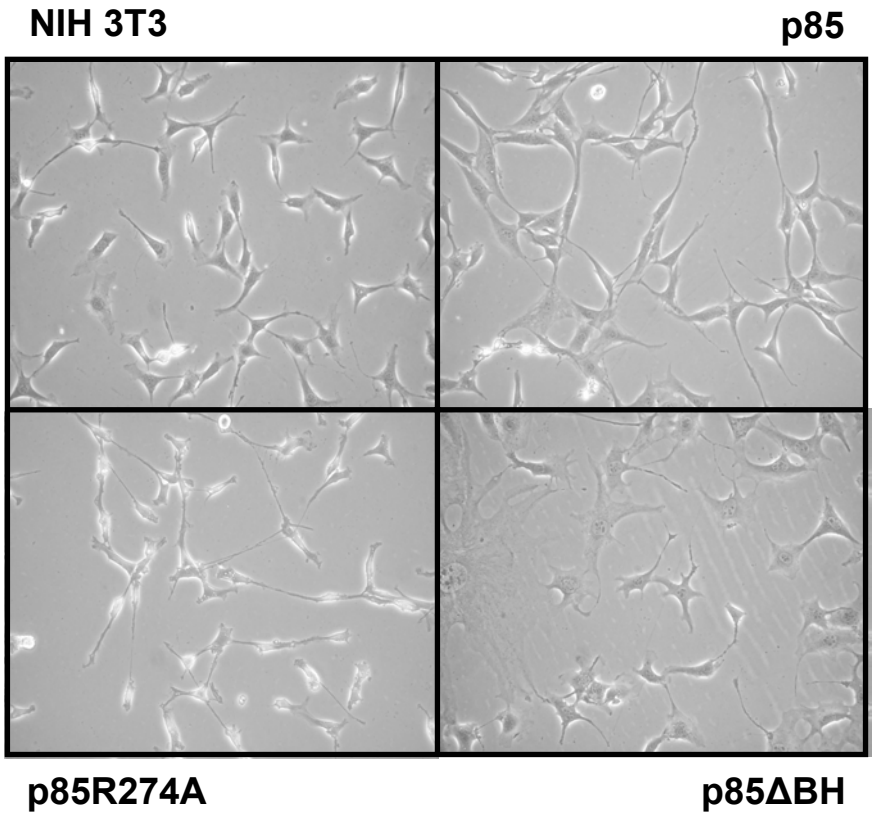


Figure 4.14 The differences in cell morphology of the stable FLAG-p85-expressing cell lines. The images are phase contrast, taken at 100x magnification.

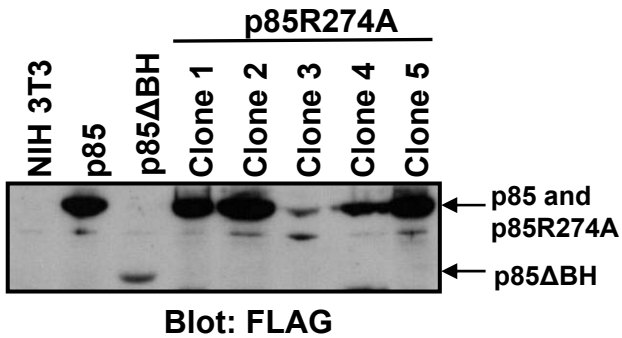


Figure 4.15 Levels of FLAG-p85 wild type and FLAG-p85 mutant protein expression in several clonal cell lines. Each lane contains 20 μg of a whole cell lysate that has been Western blots were probed with an anti-FLAG antibody followed by the appropriate secondary antibody.

expressing cells spontaneously formed foci on subconfluent plates under normal growing conditions, suggesting that they may have lost the ability to arrest cell growth via contact inhibition (data not shown). The subsequent experiments were carried out to determine if the p85R274A-expressing cells had transformed properties.

4.4.1 Changes in cell proliferation

In contrast to normal cells, transformed cells can proliferate in media containing reduced levels of growth and/or survival factors (Hanahan and Weinberg, 2000). To determine if there was a difference in the growth properties of the p85- and p85 mutant-transfected cell lines we generated, cells were grown in low serum media or serum-free media supplemented with PDGF. All of the p85-expressing cell lines continued to proliferate in media containing 2% serum over the course of 5 days, whereas the parental NIH 3T3 cells started to die after 24 hr under the same conditions (Fig. 4.16).

The proliferation of cells in serum-free media supplemented with PDGF showed a different result. The p85- and p85R274A-expressing cell lines continued to proliferate with just PDGF as a mitogenic signal. After 24 hr in the same media conditions, the parental NIH 3T3 and p85ΔBH-expressing cells appeared to arrest proliferation and enter a state of senescence (Fig. 4.17). The p85- and p85R274A-expressing cell lines exhibited a greater rate of proliferation with both above media conditions than the other cell lines, indicating that they have the ability to grow and survive in the presence of limited mitogenic stimuli.

Previous experimental results indicated that p85R274A-expressing cells have increased signalling via both the PI3K-Akt and the Ras-MAPK pathways when stimulated with PDGF (Section 3.2.5.1). Since these pathways drive cell cycle

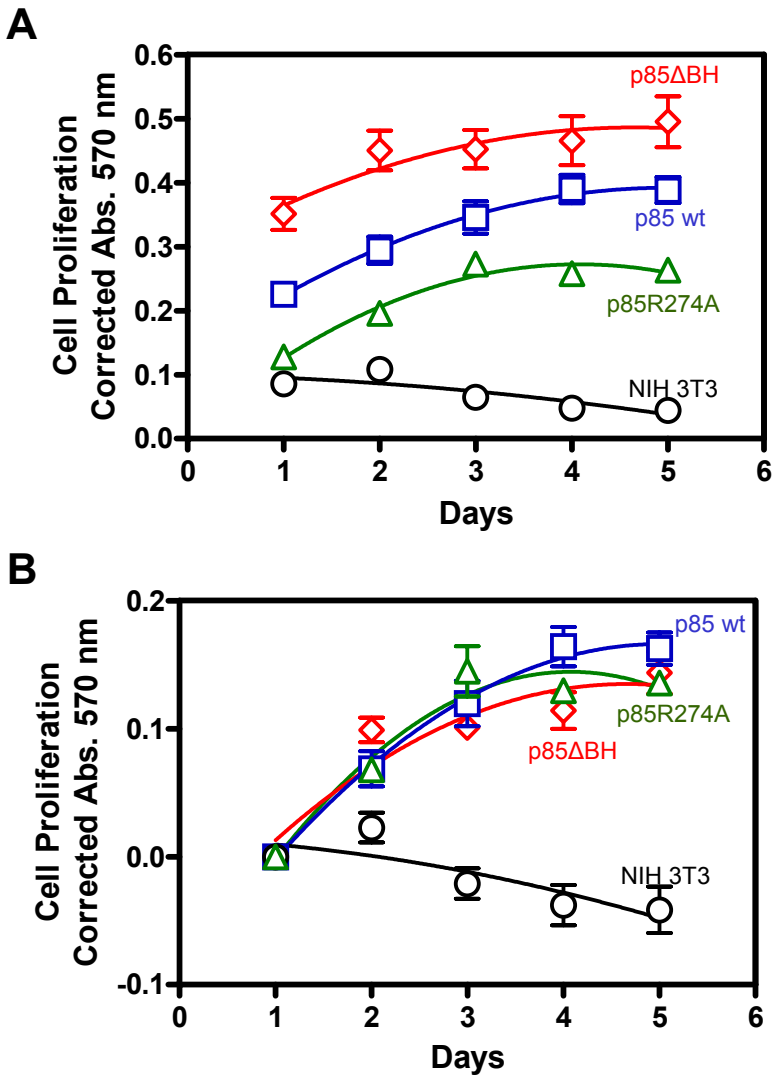


Figure 4.16 Cells expressing p85 and p85 mutants proliferate in low serum better than parental NIH 3T3 cells. Cell proliferation was measured for cells grown in media containing 2% fetal bovine serum over 5 days. The cells are either the parental NIH 3T3 (circles) or cells stably transfected with p85 (squares), p85ΔBH (diamonds) or p85R274A (triangles). Each day the amount of cell proliferation was measured with the addition of tetrazolium dye for 4 hrs. The formazan produced was measured at an absorbance of 570 nm. In panel A the absorbances for each cell line are plotted. In panel B the absorbance from day 1 was subtracted from the other days for each cell line to normalize the data. This made it possible to compare the rates of cell growth. The mean is plotted and error is expressed as the SEM for at least three independent experiments each with triplicate determinations.

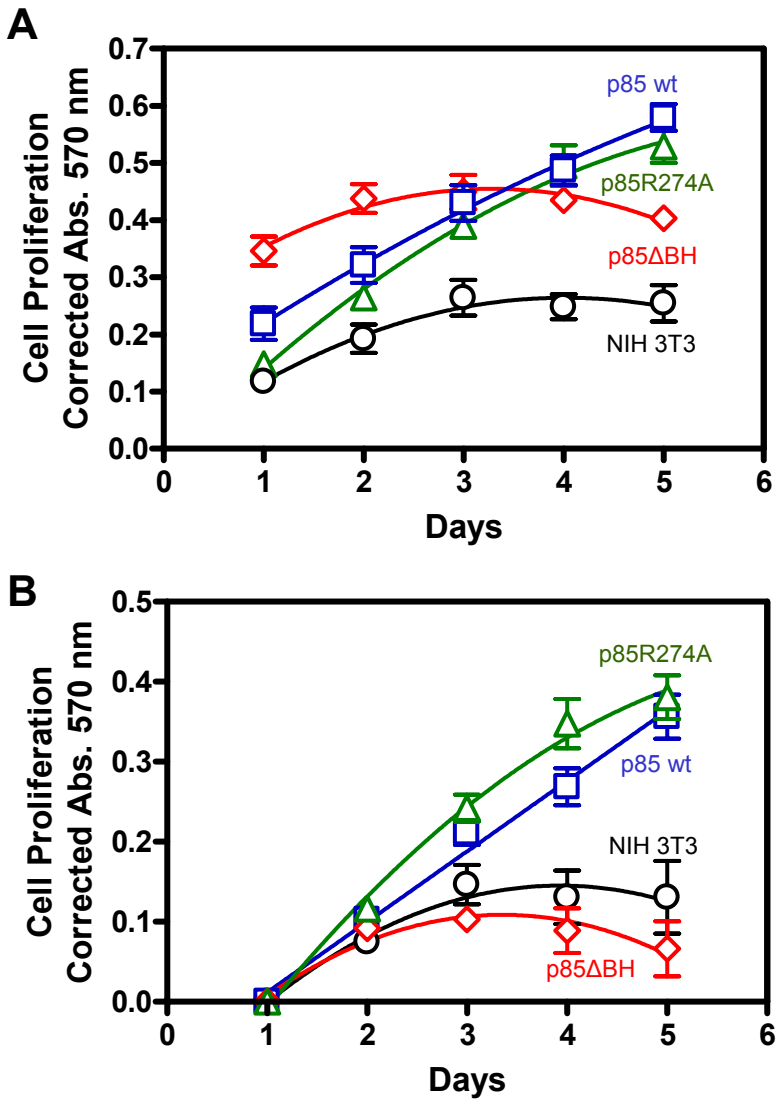


Figure 4.17 Cells expressing p85R274A and wild type p85 proliferate in response to PDGF better than cells expressing p85ΔBH and parental NIH 3T3 cells. Cell proliferation was measured for cells grown in serum-free media containing 50 ng/ml PDGF over 5 days. The cells are either the parental NIH 3T3 (circles) or cells stably transfected with p85 (squares), p85ΔBH (diamonds) or p85R274A (triangles). Each day the amount of cell proliferation was measured with the addition of tetrazolium dye for 4 hrs. The formazan produced was measured at an absorbance of 570 nm. In panel A the absorbances for each cell line are plotted. In panel B the absorbance from day 1 was subtracted from the other days for each cell line to normalize the data. This made it possible to compare the rates of cell growth. The mean is plotted and error is expressed as the SEM for at least three independent experiments each with triplicate determinations.

progression and promote cell survival, this provides a possible explanation for the enhanced cellular growth observed under these limiting conditions. The p85-expressing cells have not been shown to have increased activation of PI3K-Akt and the Ras-MAPK pathways when stimulated with PDGF (Section 3.2.5.1), but we have not tested other signalling pathways, which could be activated due to p85 overexpression that could increase the proliferation of these cells. The p85ΔBH-expressing cells have increased proliferation in low serum but not in the PDGF containing media. This is probably due to the loss of the entire BH domain and the two proline-rich flanking regions in this mutant. This is a large mutation that could have other affects on the normal function of p85 besides the loss of GAP activity.

4.4.2 Increased resistance to apoptosis

Another factor that contributes to the ability of a cell to proliferate is its susceptibility to apoptosis. Cells generally develop an increased resistance to apoptosis as part of tumour development (Hanahan and Weinberg, 2000). To determine if the p85- and p85 mutant-transfected cell lines showed changes in susceptibility to apoptosis compared to the parental NIH 3T3 cells as a result of oxidative stress, proliferating cells were treated for 4 hrs with 1 mM H_2O_2 to induce apoptosis (Fig. 4.18). All of the cell lines had approximately 75% viable cells without the hydrogen peroxide treatment. The wild type p85- and p85ΔBH-expressing cell lines were significantly more sensitive to hydrogen peroxide treatment than the control NIH 3T3 cells, demonstrating a decrease in cell viability to only 22% and 14%, respectively. The p85R274A-expressing and NIH 3T3 cells were more resistant to apoptosis than the other two cell lines and each had 53% viable cells after treatment with hydrogen peroxide. This confirms that although

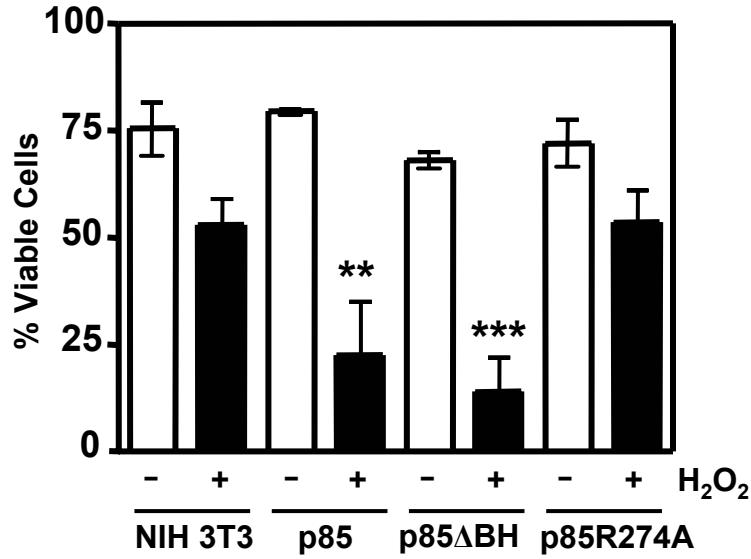
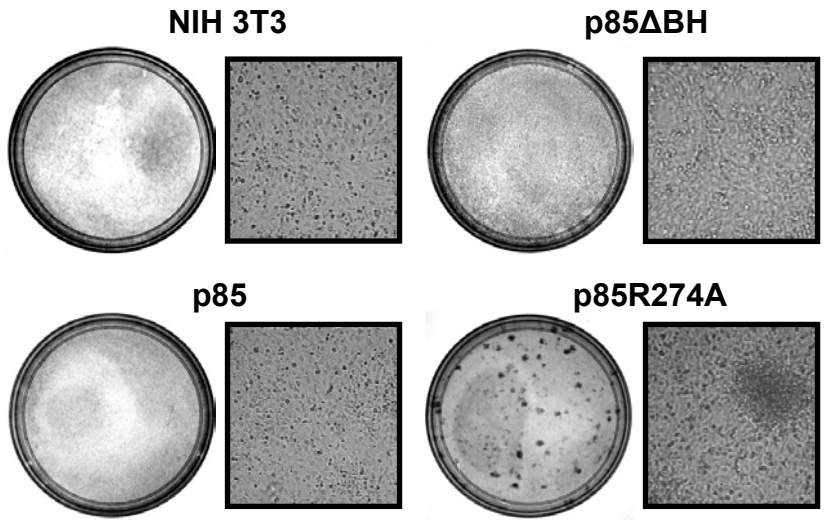


Figure 4.18 Apoptosis of the cell lines under conditions of oxidative stress. The parental NIH 3T3 cells and cells expressing different p85 proteins were treated with 1 mM H_2O_2 for 4 hrs (+) or left untreated (-) and the percent of viable cells was determined by FACS analysis after staining the cells with AnnexinV-Alexa488 and propidium iodide. The means of at least three independent experiments are shown and the error bars are the SEM. An ANOVA was done to determine the significance of the differences between wild type p85 and the p85 mutants. ** $p < 0.01$; *** $p < 0.001$.

the p85R274A-expressing cells proliferate under restrictive growth conditions, they are not highly apoptotic.

4.4.3 Loss of contact inhibition

An important characteristic of transformation frequently observed in cell monolayers is the loss of contact inhibition (Hanahan and Weinberg, 2000). The p85- and p85 mutant-transfected cell lines were tested for contact inhibition by growing the cells past 100% confluency and observing the presence of foci formed by the continued proliferation of cells after the cells reached confluency. The cells were plated to a density of 100 000 cells on a 10 cm plate and grown for 12 days, changing the media every 2 to 3 days. The formation of foci was then detected by two methods. The first was by counting the visible microscopic foci by light microscopy and the second involved staining of the monolayer with Giemsa stain and determining the number of macroscopic foci. The microscopic foci are foci that could be seen under 4x magnification and the macroscopic foci are foci that could be seen by the naked eye after Giemsa staining. Interestingly, several of the macroscopic foci could only be seen after the Giemsa staining. Also, different p85R274A clones formed different ratios of microscopic vs. macroscopic foci (Fig. 4.19). After 12 days, the parental NIH 3T3 and the p85ΔBH-expressing cells started to die and lift off the plate in small areas although the monolayer was still largely intact (Fig. 4.19). The p85-expressing cells reached 100% confluency, stopped growing and seemed to enter a state of senescence (Fig. 4.19). The p85R274A-expressing cells continued to proliferate after they reached 100% confluency and developed numerous foci (Fig. 4.19). To determine if the development of foci was a clone specific phenomenon, we tested four other p85R274A clones that



Cell Line	Macroscopic Foci	Microscopic Foci	Total Foci
NIH 3T3	0±0	0±1	0±1
p85 Wild Type	1±1	3±3	3±3
p85ΔBH	2±2	1±2	3±2
p85R274A Clone 1	78±47	21±9	99±47
p85R274A Clone 2	28±11	7±4	35±12
p85R274A Clone 3	109±56	3±1	112±56
p85R274A Clone 4	113±41	26±3	139±41
p85R274A Clone 5	34±6	9±6	43±8

Figure 4.19 p85R274A-expressing cells form foci in a contact inhibition assay. The indicated cell lines were plated (100 000 cells per 10 cm plate) and after 12 days of growth the cells were stained with Giemsa stain and viewed for foci formation. The left panel is an image of the entire 10 cm plate and the right panel is a microscopic image of a typical field of view. Below the images is a table of mean number of macroscopic and microscopic foci counted per plate and the error is the standard deviation. Similar results were observed in three independent experiments.

expressed various levels of p85R274A (Fig. 4.15). Each p85R274A-expressing cell line developed foci to some degree (Fig. 4.19) although there did not appear to be a direct correlation between the level of p85R274A expression and the number of foci observed.

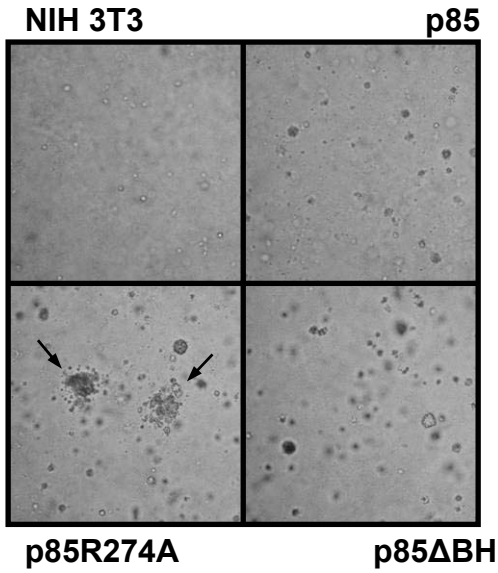
4.4.4 Loss of anchorage dependent growth

Another important factor in tumour formation is the ability of cells to grow without attachment to the extracellular matrix. To measure anchorage independent growth, the cells were seeded into soft agar. After 15 days the cells were visualized microscopically and counted. Only the p85R274A-expressing cells developed colonies (Fig. 4.20). Again, a total of five p85R274A clones were tested to ensure that colony formation was due to p85R274A expression and was not an aberrant effect in a single clonal cell line. After 15 days, all of the p85R274A-expressing clones developed colonies in soft agar (Fig. 4.20B). Initially, the p85ΔBH-expressing cell line looked as if it had also formed small colonies but these were in fact small clumps of cells that did not greatly increase in size over the duration of the experiment (Fig. 4.20A). These results indicated that p85R274A expression can cause cells to lose contact inhibition and anchorage dependence for growth and suggested that p85R274A has oncogenic potential.

4.4.5 Formation of tumours in nude mice

To assess the tumourigenicity of the p85R274A-expressing cells, their ability to form tumours in nude mice was examined. Several nude mice were subcutaneously injected with either 2.5 million NIH 3T3 or p85R274A-expressing cells and observed for tumour development (Fig. 4.21B). After 16 days the nude mice injected with p85R274A

A



B

Cell Line	Colonies
NIH 3T3	0 ± 0
p85	3 ± 0
p85ΔBH	1 ± 1
p85R274A clone 1	18 ± 8
p85R274A clone 2	29 ± 16
p85R274A clone 3	70 ± 9
p85R274A clone 4	26 ± 8
p85R274A clone 5	50 ± 23

Figure 4.20 The p85R274A-expressing cell lines form colonies in soft agar. Cells were plated in soft agar and grown for 15 days. A) Colonies are shown by arrows. The figure is a typical result of three independent experiments. B) The mean number of colonies formed per 10 000 cells plated with the error shown as standard deviation.

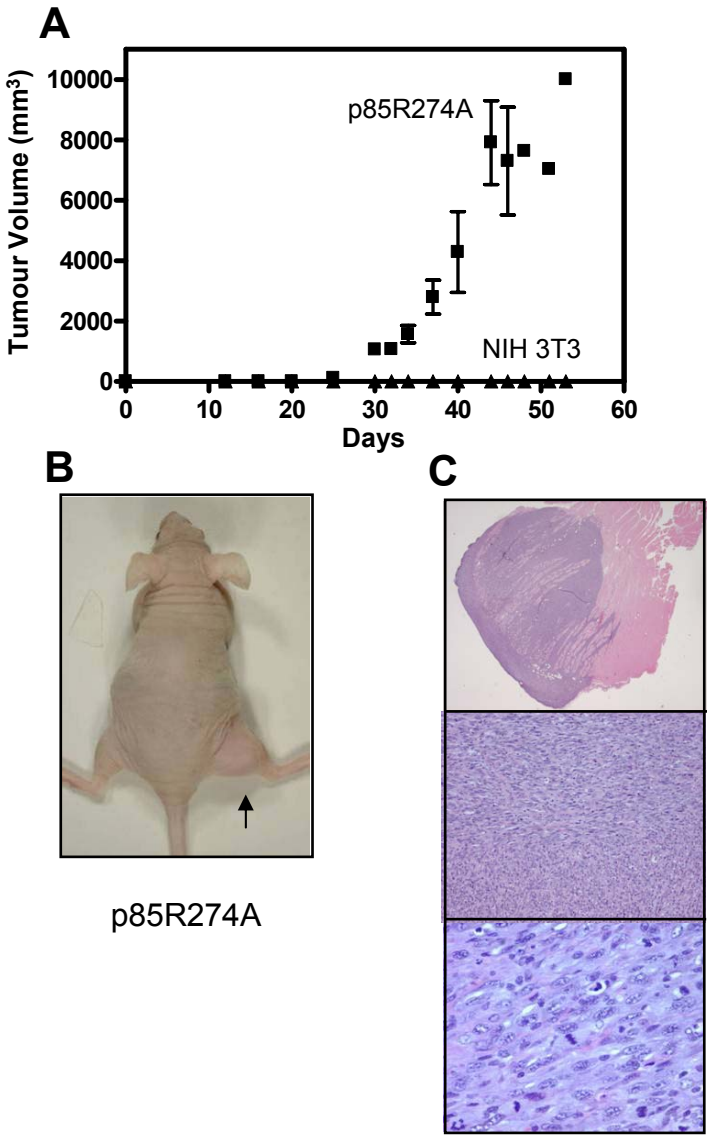


Figure 4.21 The p85R274A-expressing cells form tumours in nude mice. NIH 3T3 or p85R274A-expressing cells (2.5 x 10^6) were injected subcutaneously into the right flank of each of four nude mice. A) The average tumour volume with error being the SEM was plotted over time for mice injected with p85R274A-expressing cells (squares) or NIH 3T3 cells (triangles). B) A mouse injected with p85R274A-expressing cells with the tumour indicated by the arrow. C) Hematoxylin-eosin staining showing the architecture of a typical tumour. The top panel shows the tumor infiltrating the muscle (1x magnification). The middle panel shows the fascicles and spindle architecture of the tumour (10x). The bottom panel shows a typical high power (40x) field of view.

cells started to form tumours that rapidly grew in volume (Fig. 4.21A). Of the four mice injected with p85R274A cells, all of them formed tumours, whereas no tumours were observed in the four nude mice injected with NIH 3T3 cells. The tumours were excised and half of each tumour was paraffin-embedded, sectioned and stained with hematoxylin-eosin (Fig. 4.21C). The staining showed that the tumours exhibit a spindle cell pattern common to a fibrosarcoma, which is consistent for a tumour that is of fibroblast origin (Fig. 4.21C). The tumours were highly proliferative and well vascularized, with 50-60 mitotic events per 10 high-powered fields. The other half of the tumour was used to re-isolate the tumour cells in tissue culture. Of the four tumours formed, we successfully isolated and grew cells from two of them. The new cell lines continued to express FLAG-p85R274A (data not shown). These results clearly demonstrated the oncogenicity of cells expressing the p85R274A mutant.

5.0 DISCUSION

5.1 The interaction of p85 and Rab proteins

The first objective of this project was to determine if p85 interacts with Rab proteins, specifically Rab5. We determined that p85 and Rab5 interact directly by two different methods. Using an ELISA method, p85 was shown to interact similarly with Rab5 bound to GTP, GDP or with no nucleotide. A second method used to assess the interaction was a pull-down assay. The results showed that p85 bound best to Rab5 alone, then to Rab5-GTPγS, Rab5-GDP-AlF_4 and Rab5-GDP. Many GAP proteins bind preferentially to the GTP-bound form of their respective G protein, since GAP proteins act on the GTP-bound forms. However, there are GAP proteins that bind well to GDP-bound, GTP-bound, or G protein in the absence of nucleotide. Some examples of these latter GAP proteins include BNIP-2 binding to Cdc42 and the RhoGAP protein binding to GTP-bound and GDP-bound forms of Cdc42, Rac1 and RhoA (Low *et al*., 1999; Self and Hall, 1995). Thus, while some GAP proteins bind preferentially to the GTP-bound form of a G protein, this is not always the case. The p85 binding to Rab5 seems to follow the pattern of preferentially binding to Rab5-GTP then Rab5-GDP-AlF_4 and with somewhat less binding to Rab5-GDP and Rab5 alone.

The next objective was to determine if p85 has GAP activity towards Rab5. Again we used two different methods to measure GAP activity. Both the steady state and single turnover GAP assays showed that p85 has GAP activity towards Rab5. By testing

the various domains of p85 it was shown that the GAP activity was contained within the BH domain of p85. Furthermore, when the BH domain was removed from the protein, it no longer had GAP activity but it still bound to Rab5-GTP. This suggests that p85 is a GAP protein.

There was, however, an unusual quality with regard to the GAP activity of p85; p85 was shown to work in the μM concentration range whereas most GAP proteins work in the nM range. One possible explanation for this result is that p85 may only provide one of the functions that a GAP performs. GAP proteins perform two functions for stimulation of GTP hydrolysis. The first is the stabilization of the switch regions of the Rab protein and the second is the insertion of an arginine finger into the active site to stabilize the transition state of the reaction (Fig. 5.1)(Scheffzek *et al*., 1998). A previous study of the interaction of p85 with Cdc42 by another laboratory showed that although p85 had an arginine finger, p85 did not interact with the switch regions of Cdc42 (Fidyk and Cerione, 2002). This suggested that p85 might need a third protein to interact with the switch regions of the monomeric G protein to be fully active, with this trimeric complex increasing the binding affinity of p85 and Cdc42.

There are published examples of other GAP proteins that require a third protein in the complex to function optimally. RanGAP has a coactivator, Ran Binding Protein 1 (RanBP1), that interacts with the Ran G protein. The binding of RanBP1 causes a conformational change in Ran that removes the inhibition of RanGAP (Seewald *et al*., 2002; Seewald *et al*., 2003). Although RanBP1 increases GAP activity of RanGAP, they do not interact directly with each other but instead bind to different faces of Ran.

The formation of a trimeric protein complex for GAP activation also occurs with ADP-ribosylation factor 1 (Arf1) and ArfGAP. Arf1 GTPases regulate coatomer

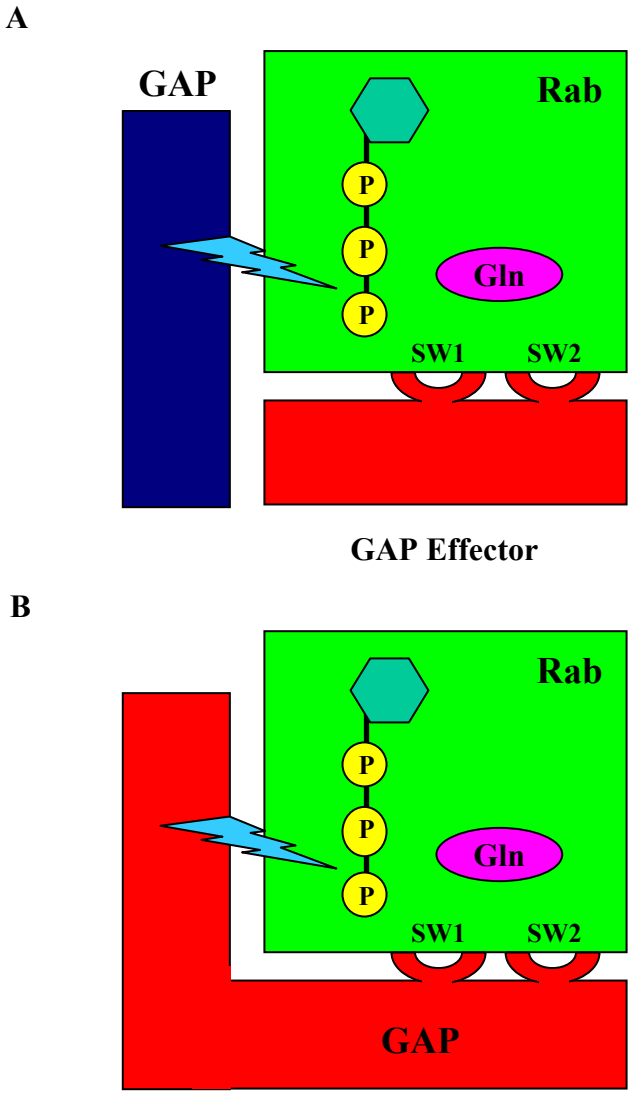


Figure 5.1 Requirements for Activation of Rab Hydrolysis of GTP. A) The GAP protein inserts the arginine finger (lighting bolt) and the Rab protein contains a critical glutamine, both of which are important for hydrolysis. A GAP effector protein interacts with the Rab protein switch regions (SW1 and SW2) to stabilize the active site for hydrolysis. B) The GAP protein supplies the arginine finger and interacts with the switch regions for the hydrolysis of GTP. Modified from Scheffzek, *et al.*, 1998.

(nonclathrin) recruitment during vesicle transport and so functionally are similar to the Rab GTPases (Chavrier and Goud, 1999; Goldberg, 1999; Szafer *et al*., 2000; Szafer *et al*., 2001). Arf1-GTP forms a stoichiometric complex with the coatomer protein and this interaction is required to maintain the stability of the vesicle coat (Chavrier and Goud, 1999; Goldberg, 1999; Szafer *et al*., 2000; Szafer *et al*., 2001). ArfGAP and Arf1 bind with low affinity and ArfGAP exhibits modest GAP activity in the absence of phospholipid vesicles or coatomer proteins. The relatively weak GAP activity of ArfGAP towards Arf1-GTP is enhanced 10-1000 fold by the presence of coatomer protein or phospholipids vesicles (Chavrier and Goud, 1999; Goldberg, 1999; Szafer *et al*., 2000; Szafer *et al*., 2001). The vesicle lipids and/or coatomer protein act as a bridge between the ArfGAP and Arf1. It has been suggested that a proximity effect increases the GAP activity since the tripartate ArfGAP:Arf1-GTP:coatomer complex, or colocalization of ArfGAP and Arf1-GTP to the same phospholipid vesicles, effectively creates a high local concentration of both proteins. The requirement for high local concentrations of both ArfGAP and Arf1-GTP ensures that the required levels of Arf1-GTP are sustained during the assembly of vesicle coats (Chavrier and Goud, 1999; Goldberg, 1999; Szafer *et al*., 2000; Szafer *et al*., 2001). The bridging of Arf1 and ArfGAP by either vesicles or coatomer changes the concentration needed for GAP activity from the μM range to the nM range. This suggests a model where the low affinity interactions between some G proteins and their respective GAP proteins are subject to additional regulation that increases their interactions. This regulation could be an increase in the local concentration at a subcellular location by endocytosis, or the interaction of the G protein and GAP with additional cellular factors, such as a third protein to form a trimeric complex.

A similar situation could exist for p85 where a third protein is needed to increase the interaction between p85 and the Rab5 protein. One possible candidate for a bridge protein is the p110β catalytic subunit of PI3K, since it interacts with both Rab5-GTP and p85 (Christoforidis *et al*., 1999b; Kurosu and Katada, 2001). We were unable to test p110β in a GAP assay because it is a highly unstable protein and we were unable to produce high quality, purified p110β. Another alternative that could increase the GAP activity of p85 is a high local concentration of p85 in the early endosome when the active receptor-p85 complex is endocytosed. This would allow p85 to have GAP activity towards Rab proteins in the early endosome. These two models are not mutually exclusive and either model could potentially explain how p85 functions as a Rab5 GAP in cells. Experiments designed to address these possible models for the regulation of p85 Rab GAP activity will be discussed in greater detail in the section describing future directions. It has previously been shown that p110β has a small effect on *in vitro* endosomal fusion assays (Christoforidis *et al*., 1999b). There was a small decrease in the percent of endosome fusion when an antibody for p110β was added to the assay, suggesting that class I PI3K may have some involvement in endosomal fusion reactions. In this assay transferrin was used as a cargo in the vesicle. Our model suggests that the GAP activity of p85 needs a high local concentration of p85 that could be supplied by receptor binding of p85 and the transferrin receptor does not bind p85 therefore this assay may not have shown the GAP activity of p85.

To determine whether p85 could act as a GAP for other G proteins, we tested its GAP activity towards several different Rab proteins. Our results showed that p85 has the ability to stimulate the GTPase activity of several, but not all of the Rab-family members tested, suggesting that the GAP activity of p85 is specific for a subset of Rab

proteins. Of the Rab GTPases tested, p85 displays the highest GAP activity towards Rab7 > Rab5 > Rab4 >> Rab6, with little or no activity towards Rab11. Rab7 is involved in the fusion of the late endosome with the lysosome (Bucci *et al*., 2000). Rab5 has been shown to function in membrane fusion events between endocytosing vesicles and early endosomes, as well as in homotypic fusions between early endosomes (Barbieri *et al*., 1996; Li, 1996). Rab4 is suggested to play a role in fast recycling (2-5 min) from early endosomes back to the plasma membrane, while Rab11 is believed to be important for slow recycling (15-30 min) of receptors, via intermediate recycling endosomes (Sheff *et al*., 1999). In contrast, Rab6 is associated with transport events in Golgi and trans-Golgi network membranes (Martinez *et al*., 1994).

We also found that p85 has GAP activity towards Cdc42 and Rac1, important regulators of actin structures. Previous reports showed that the BH domain of p85 could bind to both Cdc42 and Rac1 but had no GAP activity towards them using concentrations of p85 up to 1 μM, however, we used concentration of p85 up to 35 μM (Tolias *et al*., 1995; Wittmann and Waterman-Storer, 2001). It has been suggested that Cdc42 in particular plays a role in regulating the movement of vesicles by stimulating actin comet-tail formation (Ridley, 2001). It has been hypothesized previously that there may be multifunctional scaffolding proteins which can link structures involved in endocytosis to the actin cytoskeleton (Qualmann *et al*., 2000). We suggest that p85 may be an example of this since its GAP activity towards these Rho-family GTPases could allow the coordination of cell signalling by activated receptors with vesicle movement during receptor-mediated endocytosis.

To ensure p85 is a GAP protein and to determine the effects of its GAP activity, we mutated the BH domain of p85 to knockout its GAP activity. To do this there are

two parts of the p85 BH domain that could have been mutated; the region that interacts with the switch regions of the Rab protein or the arginine finger. The crystal structure of the BH domain was suggested to have a G protein binding cleft (Musacchio *et al*., 1996; Scheffzek *et al*., 1998). This binding cleft contains two arginines that could be the arginine finger and several other amino acids that could be involved in the interaction with the Rab5 switch regions (Fig. 5.2)(Musacchio *et al*., 1996; Scheffzek *et al*., 1998).

We decided to mutate the arginine finger because it would not involve mutating large, less well-defined surfaces of the p85 protein. There were two arginines that could be the arginine finger. Two separate mutants were generated, changing either arginine 151 or 274 to an alanine and the resulting protein products were tested for GAP activity. The p85R151A protein had approximately 65% of the GAP activity of wild type p85, while the p85R274A mutant had less than 5% GAP activity remaining. This suggested that arginine 274 is the arginine finger and supports the hypothesis that p85 is an actual GAP protein. Both p85 point mutants still bound to Rab5 although they bound preferentially to different nucleotide bound forms of Rab5. Similar to wild type p85, the p85R151A mutant bound to both Rab5-GDP and Rab5-GTP. In contrast, the p85R274A mutant bound only to the GDP bound form of Rab5. The inability of the p85R274A mutant to bind to Rab5-GTP may have contributed to the decreased GAP activity of this mutant. It is more plausible that the loss of GAP activity is due to the loss of the arginine finger required for stabilizing the hydrolysis reaction and that p85R274A does not bind stably to Rab5-GTP as a consequence of the lost arginine finger.

The three p85 mutants, p85ΔBH, p85R151A and p85R274A behaved normally with respect to several other p85 functions tested. They bound to p110 and formed functional complexes that had lipid kinase activity. Two mutants, p85ΔBH and

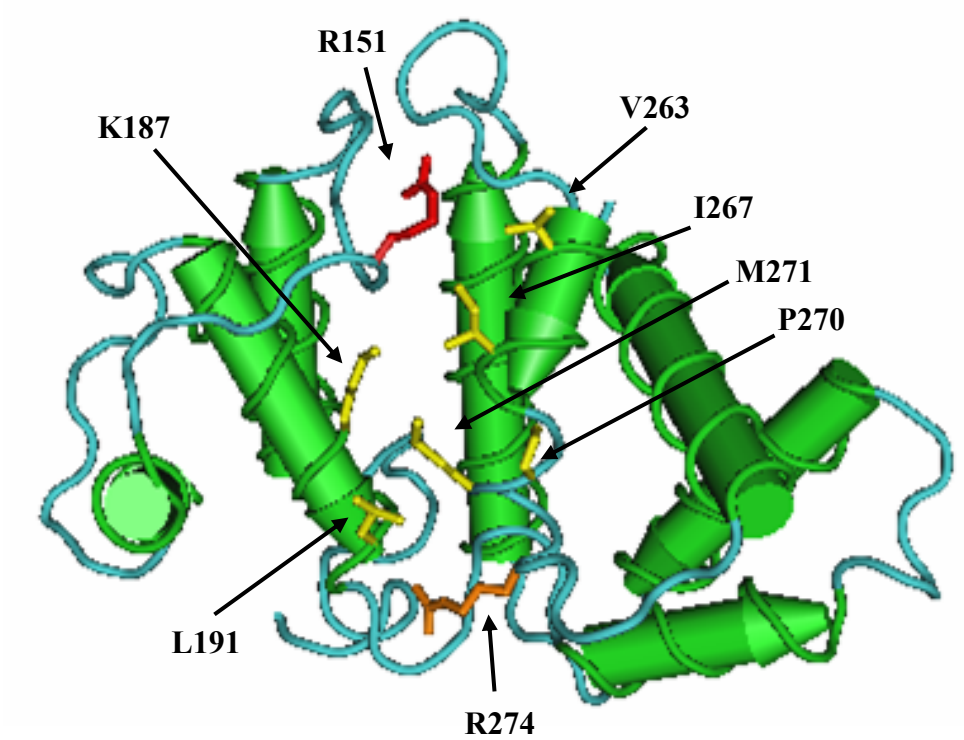


Figure 5.2 The Crystal Structure of the BH domain from Human p85. The coloured side chains within the possible Rab protein binding cleft are shown. The six amino acids shown in yellow are believed to interact with the Rab protein based on sequence homology to other GAP proteins. The red and orange amino acids are the possible arginine fingers. The red amino acid is Arg151 and the orange amino acid is Arg274. Modified from Musacchio, *et al.*, 1996.

p85R274A, were also tested for their ability to interact with the activated PDGFR and contribute to established signalling pathways. Coimmunoprecipitated PDGFR bound to each of these p85 mutants and receptor-associated PI3K activity was retained. Of significance, the p85R274A protein showed prolonged association with the receptor over the entire 4 hour time course of PDGF stimulation. In contrast, the wild type p85 and p85ΔBH proteins dissociated from the receptor by two hours of growth factor stimulation. This suggested that downregulation of the PDGFR may be impaired because the receptor was staying in an active p85-p110 associated state longer than normal.

To determine if there were changes in the downregulation of the PDGFR, we examined both total and activated receptor levels in cells stimulated with PDGF over the course of four hours. Cells expressing p85R274A and p85ΔBH both had a prolonged amount of receptor levels over the time course when compared to NIH 3T3 cells or cells expressing wild type p85. The PDGFR was also in an activated phosphorylated state. The expression of p85R274A had a more pronounced effect in delaying PDGFR degradation as well as sustaining receptor activation (i.e. tyrosine phosphorylation) and downstream signaling when compared to expression of p85ΔBH. While both of these mutated proteins retained approximately 5-8 % GAP activity, they displayed different preferences for binding to Rab5 nucleotide states and these binding preferences were distinct from the wild type p85 protein. As previously mentioned, while wild type p85 bound to both the inactive, GDP-bound and the active, GTP-bound Rab5, p85R274A bound only Rab5-GDP and p85ΔBH bound only Rab5-GTP. Differential binding preferences of the p85 mutants for the two nucleotide bound forms of Rab5 may explain

the difference in receptor degradation patterns seen between p85ΔBH- and p85R274A-expressing cells. It seems particularly evident that within the p85R274A-expressing cells, the receptor was not downregulated and degraded properly. This abnormal regulation affects the downstream receptor signalling pathways as well. The p85R274A- and p85ΔBH-expressing cells had prolonged and increased levels of phosphorylated Akt and greatly prolonged and increased levels of phosphorylated MAPK. These proteins are good indicators of activated PDGFR and are involved in several cellular functions such as cell survival and proliferation (Claesson-Welsh, 1994).

Cells expressing p85 mutants with defective GAP activity showed a corresponding decreased PDGFR downregulation and degradation. This suggested that the GAP activity of p85 regulates receptor degradation. Normally the p85 Rab GAP activity could decrease the levels of Rab5-GTP and Rab4-GTP in the cell, which decreases the rates of receptor endocytosis and recycling, respectively. There are two possible ways wild type p85 could regulate receptor trafficking into and out of the early endosome. The first is to delay receptor entry into the early endosome by decreasing the amount of Rab5-GTP on vesicles, which would decrease the rate of fusion of these vesicles with the early endosome. This could be important to slow receptor endocytosis to allow signalling by the activated receptor. The second is to delay receptor exit from the early endosome by decreasing the amount of Rab4-GTP on the early endosome, which would decrease the rate of receptor recycling.

Expression of p85 mutants defective for Rab GAP activity would be expected to have the opposite effects. To determine if the observed changes in receptor degradation were due to changes in the rate of endocytosis and/or recycling the rate of biotin-tagged surface protein internalization and the rate of internalized biotin-tagged protein loss from

the interior of the cell were measured. The rates of biotin-tagged protein endocytosis and recycling should correlate to the endocytosis and recycling of the PDGFR because upon PDGF stimulation of the cells the receptor is endocytosed. Both parental and p85R274A- expressing NIH 3T3 cells exhibited similar rates of internalization over 10 min of PDGF stimulation. This suggested that if p85 GAP activity had a role in the endocytosis of the receptor, it did not affect the rate of receptor internalization.

This does not preclude the possibility that p85 functions in the intermediate steps between receptor internalization and entry into the early endosome. It is thought that after Rab5-mediated endosomal fusion, Rab5 is released from the early endosome into the cytosol and moves back to the plasma membrane to carry out further fusion events and that this release of Rab5 from the early endosome could be rate limiting in endocytosis (Clague and Urbe, 2001). If the role of p85 GAP activity is to allow the release and re-utilization of Rab5, then knocking out this GAP activity may have little effect on the internalization of the receptor initially because the pool of cytosolic Rab5 would have to be depleted before internalization is affected.

The Rab GAP activity of p85 could regulate Rab function via the Rab4-mediated exit from the early endosome. To measure this, the rate of biotin-tagged internalized protein decrease from the interior of the cell was measured. Over the time period studied the majority of the internalized protein loss from the cell interior should be due to recycling and not degradation. Although in the NIH 3T3 cell line it is expected that some degradation of the receptor can occur. If p85 plays an important role in receptor recycling, knocking out its GAP activity should increase the amount of activated Rab4, which would increase the rate of receptor recycling. When protein recycling was measured, the rate was almost twice as fast in the p85R274A-expressing cells as

compared to the parental NIH 3T3 cells. This indicated that the GAP activity of p85 may regulate the rate of receptor recycling but not the rate of endocytosis. This is consistent with a report that the removal of the p85 binding sites from the PDGFR decreased receptor degradation (Hiles *et al*., 1992; Kapeller *et al*., 1993; Schu *et al*., 1993). Such manipulation would produce the end results similar to those caused by knocking out the GAP activity of p85, that being an increase in the rate of receptor recycling. The mechanism that the receptor uses for degradation is to target the receptor from the early endosome into the MVB (Raiborg and Stenmark, 2002). The data supports the hypothesis that loss of p85 GAP activity decreases the rate of receptor degradation by decreasing the amount of time the receptor spends in the early endosome. This would limit the amount of time the receptor could interact with the targeting mechanism for receptor degradation. Targeting to the MVB is dependent on two events: ubiquitylation of the receptor and interaction of the ubiquitylated receptor with the Hrs-STAM and ESCRT complexes (Haglund *et al*., 2003; Holler and Dikic, 2004; Komada and Soriano, 1999; Levkowitz *et al*., 1998; Raiborg and Stenmark, 2002).

5.2 Model for the Role of p85 in Receptor Endocytosis

When the PDGFR is activated, it recruits several adaptor proteins including p85 (Claesson-Welsh, 1994; Heldin *et al*., 1998). Shortly thereafter, the activated receptor complex is endocytosed and travels from the plasma membrane to the early endosome (Fig. 5.3)(Clague and Urbe, 2001; Miaczynska *et al*., 2004b; Sorkin and Von Zastrow, 2002). The early endosome contains at least 4 functional domains, each of which plays a different role in receptor function (Raiborg and Stenmark, 2002; Sonnichsen *et al*., 2000). The major domains are involved in regulating the entrance and exit of receptors

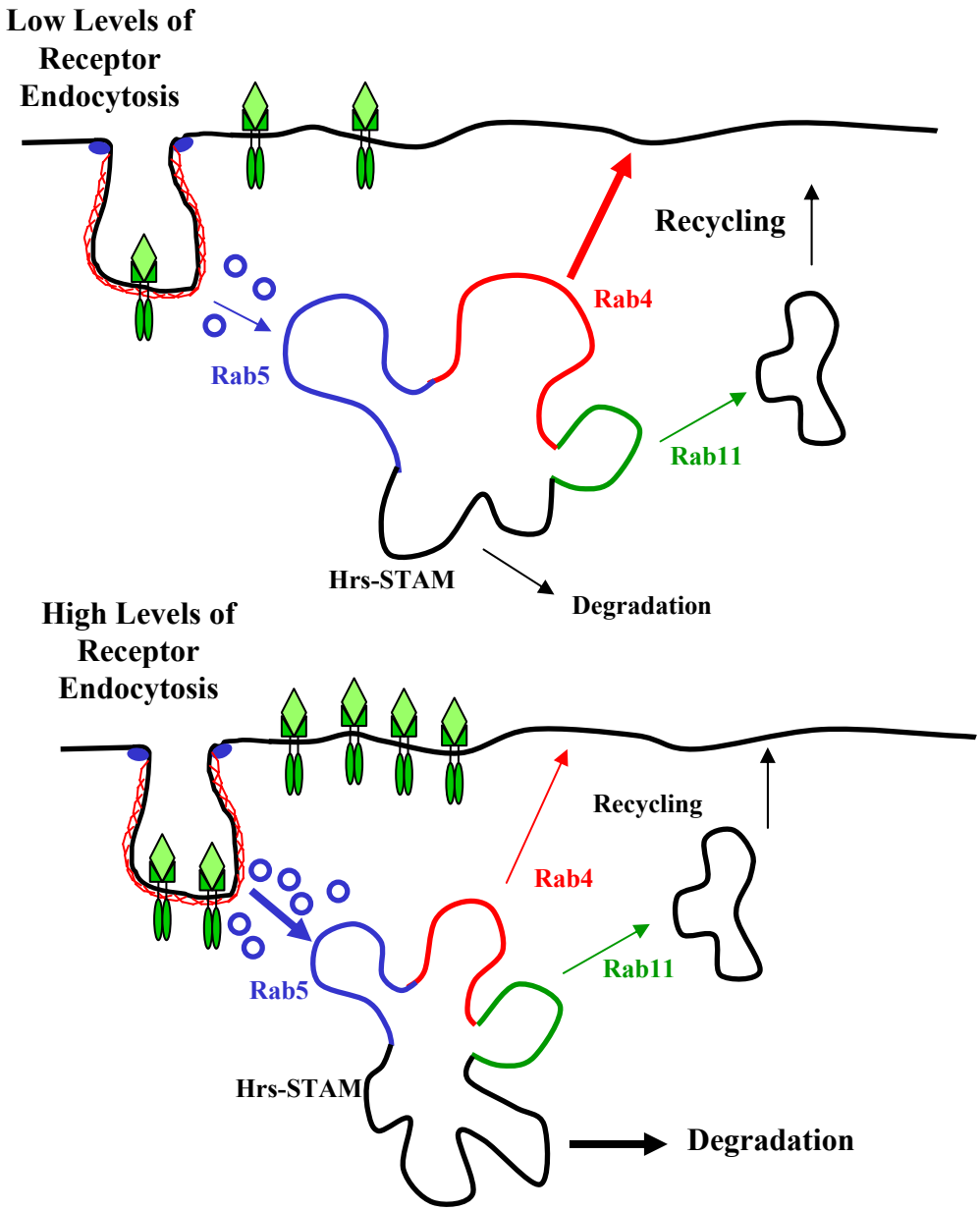


Figure 5.3 Model for the Involvement of p85 in Receptor Endocytosis. When there is a low level of PDGFR endocytosis (upper panel), there is a low level of p85 in the early endosome. This allows a greater amount of receptor recycling vs receptor degradation. When there is a high level of PDGFR endocytosis (lower panel), there is a high level of p85 in the early endosome. This decreases the size of the Rab4 and possibly the Rab5 domains on the early endosome. As a result, the Hrs-STAM containing domain would increase in size relative to the recycling domains, causing more receptors to be degraded.

from the early endosome. The Rab5 domain is involved in the entry of the receptor into the early endosome (Li, 1996; Somsel Rodman and Wandinger-Ness, 2000). There are two domains, Rab4 and Rab11, which are involved in the return of the receptor to the plasma membrane via recycling pathways (Maxfield and McGraw, 2004; Somsel Rodman and Wandinger-Ness, 2000). A Rab protein does not govern the fourth major domain but instead it may be regulated via the Hrs-STAM and ESCRT complexes. This domain targets receptors for degradation and forms the MVB (Raiborg and Stenmark, 2002).

The mechanism by which the receptor is delivered into and out of these different early endosomal domains is not well characterized. After the receptor enters the Rab5 domain, it most likely dissociates from the Rab5 domain and migrates to one of the other domains. This process could be directed, but it is more likely that the receptor migrates randomly to the adjacent domains through diffusion events influenced by the size of adjacent domains. A larger domain provides a greater surface area and therefore would have a higher chance to receive the diffusing receptor. The size of the domain could be regulated by the active state of the Rab protein that forms it. The GAP activity of p85 could regulate the size of domains on the early endosome, thus providing a possible mechanism for regulating the amount of receptor recycling vs degradation. As activated receptors enter the early endosome, the associated p85 levels would also increase, causing an increase in GAP activity towards Rab4 and possibly Rab5. This would then theoretically cause the Rab4 and Rab5 domains on the early endosome to decrease in size, allowing for an increase in receptor degradation because the majority of the endosomal surface area would be composed of the Hrs-STAM domain. This process would provide an ideal system to regulate the rate of receptor degradation. High levels

of receptor activation would result in more degradation, whereas low levels of receptor activation would instead favor the recycling system due to a relatively small Hrs-STAM domain. As the amount of p85 bound to the receptors entering the early endosome increases, the Rab4 recycling domain would decrease in size relative to Hrs-STAM degradation domain shifting receptor transport in favor of entry into the Hrs-STAM degradation domain. This would cause more receptors to be degraded. This proposed model of receptor endocytosis is consistent with the previous knowledge of endocytosis and assimilates the data provided in this thesis into the model of endocytosis. In the future directions several experiments have been outlined to further investigate this model.

5.3 The role of endocytosis in cancer

Many types of tumours have increased levels of growth factor receptors (Blume-Jensen and Hunter, 2001), which is known to drive aberrant cell proliferation and cell survival. The most common causes of the increased receptor levels are gene amplification and deregulation of receptor transcription (Blume-Jensen and Hunter, 2001). It has also been suggested that defects in receptor-mediated endocytosis and degradation pathways could contribute to elevated levels of activated growth factor receptors (Bache *et al*., 2004). The p85R274A-expressing cell line was shown to have increased levels of activated PDGFR and enhanced cell signalling. It was possible that these properties were indicative of a transformed phenotype for the p85R274A-expressing cell line. Preliminary evidence suggesting that the p85R274A-expressing cell line was transformed came from observations of spontaneous foci formation on subconfluent plates under normal growing conditions. In addition, the cells exhibited a

rounded morphology common to a loosely adherent transformed cell. Therefore we tested the p85R274A-expressing cell line for several of the hallmarks of tumourigenic cells. These hallmarks are the ability to proliferate with low concentration of growth signals, resistance to apoptosis, anchorage independent growth, loss of contact inhibition and formation of tumours in nude mice.

The changes in signalling in the p85R274A-expressing cells have direct effects on cell proliferation and cell survival. Our results showed that in contrast to parental NIH 3T3 cells, cells overexpressing wild type p85 and the p85 mutants (p85R274A and p85ΔBH) proliferated in low levels of mitogenic growth signals (i.e. 2% serum). When PDGF was used as the only mitogenic factor, the overexpression of wild type p85 and p85R274A allowed the cells to proliferate whereas the cells overexpressing p85ΔBH and the parental NIH 3T3 cells proliferated poorly. This suggests the overexpression of p85 or p85 mutants allows cells to proliferate in low mitogenic signals. One possible reason that the p85ΔBH expressing cells grew poorly in PDGF may be that the deletion of the large BH domain (amino acids 84-313) had additional effects that are important in PDGFR signalling, besides GAP activity.

The p85R274A-expressing cells showed more resistance to apoptosis than wild type p85- and p85ΔBH-expressing cells under conditions of oxidative stress. In fact, the p85R274A-expressing cells had the same resistance to oxidative stress as the normal parental NIH 3T3 cells. In contrast, the cells expressing wild type p85 and p85ΔBH underwent significant apoptosis in response to oxidative stress. The reason p85R274A-expressing cells do not have the same sensitivity to apoptosis as the cells overexpressing p85 could be due to the increased Akt signalling observed in the p85R274A-expressing cells, since it is well known that Akt signalling has anti-apoptotic effects (Song *et al*.,

2005). This data showed that p85R274A-expressing cells can proliferate in low levels of growth factors and are resistant to apoptosis when compared to cells expressing wild type p85 and p85ΔBH.

Other hallmarks of cell transformation mentioned above are the loss of contact inhibition and anchorage independent growth. We observed both of these traits in cells expressing p85R274A protein but not in cells expressing either p85 wild type or p85ΔBH. This suggested that the p85R274A-expressing cells are indeed transformed. An experiment used to determine the tumourigenicity of the p85R274A cells was to test their ability to form tumours in nude mice. The p85R274A-expressing cells did form tumours in nude mice and these tumours were highly proliferative and well vascularized. Together these results strongly suggest that the loss of GAP activity by the mutation of Arg 274 in p85 transforms the cells by increasing the levels of activated PDGFR in the cells.

5.4 Future directions

The mechanistic data presented here has led to a model for the involvement of p85 in the endocytosis of the PDGFR. Future experiments are required to test and further refine this model.

First, it would be valuable to determine the contribution of p110β to the GAP function of p85. Several different approaches have been examined to purify p110β from bacteria. Thus far, all of them have been unsuccessful due to the low-level expression of the protein and instability of the bacterially-expressed p110β. We know that p110β can be expressed successfully in mammalian cells. Therefore, it should be possible to express p85 with or without p110β in COS-1 cells and purify it by immunoprecipitation

of p85. It may then be possible to develop a GAP assay using the immunoprecipitates containing p85 with or without p110β. These could be used to help determine the effect of p110β on the GAP activity of p85.

There are also several immunofluorescence experiments that need to be performed. The above experiments have provided an approximate timeline of endocytosis, which can be used to help design efficient immunofluorescence experiments to determine the involvement of p85 in endocytosis. At early time points, under 10 min, it is important to determine if the PDGFR is entering the early endosome or is getting trapped somewhere between the plasma membrane and early endosome. This will help determine if p85 has GAP activity towards Rab5 in cells. At later time points, between 20 min and 2 hr, the location of the PDGFR also needs to be determined. If the proposed model is correct, then in parental NIH 3T3 cells the receptor should be distributed between the recycling and degradation pathways. At the 20 min mark, more receptor should be recycled and associated with Rab4 and at the 2 hr mark, more receptor should be degraded and associated with Rab7 or other late endosomal/lysosomal markers. In the p85R274A-expressing cells, less receptor should be associated with Rab7 at each time point and more should associate with Rab4 or localize to the plasma membrane. This will support the hypothesis that the expression of p85R274A is causing receptor recycling rather than the receptors building up in some endosomal compartment because of a blockage of receptor degradation. Immunofluorescence could also be used to determine if p85 colocalizes with both Rab5 and Rab4. This would help confirm that p85 is a GAP protein towards those Rab proteins.

The concept that the size of different early endosomal domains determines the targeting of receptors for recycling or degradation could be tested by looking at the domain size of Rab5, Rab4 and Hrs-STAM on early endosomes in both p85R274A-expressing cells and the parental NIH 3T3 cells over a PDGF stimulation time course of up to 60 min. In NIH 3T3 cells, the ratio of Rab5 and/or Rab4 to Hrs-STAM domain size should decrease the longer the cells are stimulated because of the increasing levels of p85 entering the early endosome. In contrast, in the p85R274A-expressing cells the ratio of Rab4 and/or Rab5 to Hrs-STAM domain size should stay the same or grow in size if the model is correct because the p85 would have no GAP activity.

Another experiment that would provide us with valuable information would be to determine the ubiquitylation state of the PDGFR in the NIH 3T3 cells vs the p85R274A-expressing cells over a time course of PDGF stimulation. This could indicate how the ubiquitylation of the receptor controls receptor degradation. If the p85R274A-expressing cells have increased levels of ubiquitylated receptor over the time course compared to NIH 3T3 cells that would mean that the receptor is being ubiquitylated but cannot enter the MVB. If the p85R274A-expressing cells have decreased levels of ubiquitylated receptor over the time course compared to NIH 3T3 cells, it would indicate that the receptor is not being ubiquitylated because the receptor may not be interacting with Cbl, which ubiquitylates the receptor. If the p85R274A-expressing cells have the same levels of ubiquitylated receptor as the NIH 3T3 cells at each time point, this would suggest that the receptor is being ubiquitylated and then deubiquitylated and recycled.

These experiments should help prove or disprove the model put forth and allow for the development of future models. These future models can then be used to develop

further experiments to increase our knowledge of how receptor-mediated endocytosis functions.

5.5 Conclusions

This research suggests a new model for how growth factor receptor endocytosis and degradation could be regulated. Although there has been much research into endocytosis over the past decade, there is still little known about how receptors are targeted from the early endosome to the lysosome for degradation. The p85 subunit of PI3K has a role in the regulation of the activity of Rab proteins of the early endosome, specifically Rab5 and Rab4. This regulation works by stimulation of the Rab protein to hydrolyze its GTP into GDP, deactivating Rab function. The inactivation of the Rab proteins then cause changes in the rates of endocytosis and recycling of the PDGFR. The inactivation of Rab5 causes a slowing of vesicle fusion with the early endosome. We believe this may be important in allowing the receptor time to activate downstream signalling proteins. The inactivation of Rab4 by p85 may also play an important role in receptor downregulation. Currently it is known that only a small percentage of receptors in the early endosome are targeted to the lysosome for degradation, while the rest are recycled back to the plasma membrane. The inactivation of Rab4 by the GAP activity of p85 would slow recycling of the receptor, allowing receptors to interact with proteins in the early endosome that could target the receptor to the lysosome for degradation. This temporal control of receptor recycling vs. receptor degradation by p85 could be an important point of regulation of the level of receptors. This could be a future target site for drugs to control receptors involved in cancer.

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