REGULATION OF PDGF RECEPTOR TRAFFICKING AND SIGNALLING BY THE RABGAP FUNCTION OF p85α

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by

Jennifer Nana Kyeiwa Nyarko

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Dr. Deborah H. Anderson
Cancer Cluster
Room 4D30.2 Health Science Building
107 Wiggins Road
University of Saskatchewan
Saskatoon, SK S7N 5E5

ABSTRACT

Activated receptor tyrosine kinases recruit many signalling proteins to initiate downstream cell proliferation and survival pathways, including phosphatidylinositol 3-kinase (PI3K), a heterodimer consisting of a p85 regulatory protein and a p110 catalytic protein. Our laboratory has previously shown the p85α protein also has *in vitro* GTPase activating protein (GAP) activity towards Rab5 and Rab4, small GTPases that regulate vesicle trafficking events for activated receptors. Expression of a p85α protein containing an arginine to alanine substitution at position 274 (p85R274A) that affects its GAP activity, caused sustained levels of activated platelet-derived growth factor receptors (PDGFRs), enhanced downstream signalling, and resulted in cellular transformation. Together with other data, this suggested that in p85R274A-expressing cells, PDGFRs are more rapidly trafficked through the endocytic pathway, which reduces opportunities for sorting events necessary for receptor degradation. Our laboratory has observed previously that p85 was capable of binding to both Rab5-GDP, as well as Rab5-GTP, which is an atypical characteristic of GAP proteins, whereas p110β had previously been reported to bind Rab5-GTP selectively. Based on these observations, this thesis project was designed to test the hypothesis that both proteins contributed GAP activity towards Rab5, with p85 providing a catalytic arginine residue (R274) and p110β providing switch stabilization functions specific to the GTP-bound state. To accomplish the thesis objective, cells expressing individual p85 defects (lacking GAP activity, R274A; or lacking p110-binding ability through deletion of residues 478-513, Δ110) were compared to cells expressing a double mutant missing both functions. Stable clonal NIH 3T3 cell lines were generated and selected in G418 and clones expressing similar levels of FLAG-tagged p85 wild type or mutants compared to the control cell lines (NIH 3T3, FLAG-vector control, p85 wild type, and p85R274A) were chosen for analysis. A time-course of PDGF stimulation showed that cells expressing p85R274A or p85Δ110+R274A have sustained phosphorylation levels of the PDGFR, reduced rates of PDGFR degradation and sustained MAPK/Erk signalling. Contrary to the cellular transformation previously reported for p85R274A-expressing cells, expression of p85Δ110+R274A did not lead to cellular transformation. These divergent results suggest that p85-associated p110 serves two functions. As the catalytic subunit of PI3K, one function is the localized generation of $PI3,4,5P_3$ lipids at the plasma membrane for Akt activation, and possibly during receptor endocytosis where it could impact MAPK/Erk activation/deactivation kinetics and cell transformation. These

results support a second function for p110 in the regulation of PDGFR activation/deactivation kinetics and PDGFR half-life, both strongly influenced by alterations in PDGFR trafficking. This suggests that p110β may regulate PDGFR trafficking by providing Rab5-GTP switch stabilization that complements the catalytic arginine residue (R274) within p85, and that p85α and p110β work together as a Rab5 GAP.

The role of PDGFR in the localization of the RabGAP function of p85 to specific subcellular compartments was also examined. It was hypothesized that PDGFR may help localize the RabGAP function of p85 to vesicles containing Rab5 or Rab4 through the binding of p85 to phosphorylated tyrosine residues on activated PDGFR. Stable cell lines expressing individual p85 defects (lacking GAP activity, R274A; or lacking PDGFR-binding ability through site-directed mutation of residues 358 and 649 from arginine to alanine, ΔR; or a double mutant missing both functions) demonstrated that p85R274A or p85ΔR+R274A expression leads to sustained PDGFR activation and signalling, and to delayed PDGFR degradation in response to PDGF stimulation. The sustained signalling observed resulted in cellular transformation in cells expressing p85R274A or p85ΔR+R274A. The data suggests that PDGFR does not play a role in the localization of the RabGAP activity of p85.

The findings of this study elucidates important non-canonical functions of the PI3K heterodimer and contributes to our understanding of how specific mutations in both p85 and p110β within regions implicated in the regulation of RabGAP activity can alter signalling events and lead to enhancement of tumour-associated phenotypes.

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This work is dedicated to my parents,
Emmanuel Leslie Nyarko and Esther Naa Adarku Nyarko

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ABD	Adaptor binding domain, p85 binding domain
AEBSF	4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride
AP2	Adaptor protein 2
APS	Ammonium persulfate
BCR	Breakpoint cluster region
BH	BCR homology domain
BSA	Bovine serum albumin
Cbl	Casitas B-lineage Lymphoma
DMEM	Dulbecco's Modified Eagle Medium
E. coli	*Escherichia coli*
EEA1	Early endosomal autoantigen 1
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EGTA	Ethyleneglycol-bis (B-aminoethylether) N,N,N,N, tetraacetic acid
Eps15	EGFR pathway substrate clone 15
ER	Endoplasmic reticulum
Erk	Extracellular regulated kinase
ESCRT	Endosomal sorting complex required for transport
FBS	Fetal bovine serum
G418	Geneticin
GAP	GTPase activating protein
GEF	Guanine nucleotide exchange factor
GDF	GDI-displacement factor
GDI	Guanosine nucleotide dissociation inhibitor
GDP	Guanosine diphosphate
GLUE	GRAM-like ubiquitin binding in EAP45
GPCR	G protein-coupled receptor
GRAM	Glucosyltransferases, Rab-like GTPase activators and Myotubularins

Grb2	Growth factor receptor-bound protein 2
GSK3	Glycogen synthase kinase 3
GTP	Guanosine triphosphate
HECT	Homologous to the E6-AP carboxy terminus
HEPES	N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid)
Hrs	Hepatocyte growth factor-regulated tyrosine kinase substrate
Ig	Immunoglobulin
IgG	Immunoglobulin G
IgG-AC	Immunoglobulin G, agarose-conjugated
IP	Immunoprecipitation
iSH2	InterSH2 Domain, p110 binding domain
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
MEM	Minimum essential medium
MVB	Multivesicular body
NaPPi	Sodium pyrophosphate
Na_3VO_4	Sodium orthovanadate
NF-κB	Nuclear factor-kappa B
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with Tween-20
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PDK1	Phosphoinositide-dependent protein kinase 1
Pfu	*Pyrococcus furiosus*
PH	Pleckstrin homology domain
PI3K	Phosphatidylinositol-3'-kinase
PI	Phosphatidylinositol

PI3P	Phosphatidylinositol-3-phosphate
PI4P	Phosphatidylinositol-4-phosphate
$PI4,5P_2$	Phosphatidylinositol-4,5-bisphosphate
$PI3,4,5P_3$	Phosphatidylinositol-3,4,5-trisphosphate
PLC-γ	Phospholipase C-γ
PS	Phosphatidylserine
PTB	pTyr binding domain
PTEN	phosphatase and tensin homologue deleted on chromosome 10
pTyr or pY	Phosphotyrosine
RBD	Ras-binding domain
RING	Really interesting new gene
RTK	Receptor tyrosine kinase
S6K	p70 S6 kinase
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SH2	Src homology 2
SH3	Src homology 3
Shc	Src homologous and collagen-like
SNARE	Soluble N-ethylmaleimide sensitive factor attachment protein receptor
Sos	Son of Sevenless
STAM	Signal/transducer molecule
STAT	Signal transducer and activator of transcription
TEMED	N,N,N',N'-Tetra-methylethylenediamine
Tris	Tris(hydroxymethyl)aminomethane
TLC	Thin layer chromatography
Tween-20	Polyoxyethylenesorbitan monolaurate
Ub	Ubiquitin
UBA	Ubiquitin associated domain
UIM	Ubiquitin interacting motif
Vps	Vacuolar protein sorting

1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 Signal Transduction Pathways

Cells interact with and respond to stimuli generated from their external environment through a process referred to as signal transduction. The stimuli may include either scarcity or availability of hormones, nutrients, or oxygen (Schuller, 1991). Communication of information between the exterior and interior parts of a cell directs and regulates the many signal transduction pathways (also referred to as 'signalling cascades') that lead to normal cellular functions. Cellular responses to these signals include either amplifying or diminishing events that lead to, for example, cell proliferation and survival (McKay and Morrison, 2007).

Signal transduction pathways are initiated by an interaction between the external stimulus usually referred to as the ligand, and receptors that are expressed on the plasma membrane (Biarc *et al*., 2011; Citri and Yarden, 2006; Dengjel *et al*., 2009; Madhani, 2001; McKay and Morrison, 2007). Examples of these cell surface receptors include receptor tyrosine kinases (RTKs) and G-protein coupled receptors (GPCRs). The information is transferred across the membrane through the receptor to intracellular effector molecules. These effector molecules then activate a cascade of signalling events that culminate in the stimulation of cell proliferation, survival and differentiation (Aaronson, 2005; Heldin and Westermark, 1999; Lemmon and Schlessinger, 2010).

Activation of signalling within the cell is regulated by a variety of factors including post-translational modifications and compartmentalization of both receptors and intracellular effector molecules. The most common post-translational modification for the activation or deactivation of these proteins is phosphorylation of tyrosine, threonine or serine residues (Burnett and Kennedy, 1954; Cozzone, 1988; Olsen *et al*., 2006; Pawson and Scott, 2005; Walton and Dixon, 1993). Signalling proteins can also be modified by ubiquitination, prenylation, myristoylation, glycosylation, methylation or acetylation (Biarc *et al*., 2011; Yang and Seto, 2008). This can result in a change in binding affinity or specificity of the receptor for the ligand, or changes in the conformation or localization of the protein. Ultimately, these changes determine whether the protein is active or not, and can thus enhance or terminate a signal.

Due to a protein's role in cell proliferation and survival, it is very important for every step within a signal transduction pathway be tightly regulated. The control of synthesis and degradation regulates protein levels; whereas the initiation, duration and intensity of signalling

mediated by the protein are regulated by post-translational modifications and compartmentalization. Deregulation of any of these processes can lead to pathological conditions, including cancer, diabetes and heart diseases (Robertson *et al*., 2000; Tonks, 2006).

Cancer has been classically referred to as a group of diseases that results from the abnormal regulation of cell proliferation. The transformation of a normal cell into a tumour cell is a multistage process that may be initiated by external agents (such as tobacco products, viruses, X-rays, UV radiation, and chemicals) as well as genetic factors, both inherited and non-inherited (Croce, 2008; Danaei *et al*., 2005; Merlo *et al*., 2006). Among these genetic factors are the over-expression of wild type or constitutively active cell surface receptors; as a result of gene amplification, increased gene expression, and more recently defects in receptor trafficking and degradation (Bache *et al*., 2004; Bacus *et al*., 1990; Haeder *et al*., 1988; Liu and Tsao, 1993; Peschard and Park, 2003; Tripathy, 2005). Alterations in the structure and function of the intracellular signalling molecules have also been shown to lead to certain malignancies, including carcinomas, sarcomas, leukemias and lymphomas (Blume-Jensen and Hunter, 2001). More insight into the regulation of signal transduction is therefore necessary for the development of appropriately targeted therapeutics for cancer patients.

1.2 Receptor Tyrosine Kinases (RTKs)

A group of cell surface receptors that have been implicated in cancers are the receptor tyrosine kinases (RTKs). RTKs are a large family of plasma membrane spanning receptors with an intracellular tyrosine kinase domain (Alvarez *et al*., 2006; Gavi *et al*., 2006; Hunter, 2000; Schlessinger, 2000). This family includes the well-characterized epidermal growth factor receptor (EGFR) as well as the platelet-derived growth factor receptor (PDGFR). The typical structure of RTKs includes an N-terminal extracellular ligand binding domain, a single α-helical transmembrane domain and a C-terminal intracellular domain that contains an intrinsic tyrosine kinase domain (Cowan-Jacob, 2006; Garrett *et al*., 2002; Garrett *et al*., 1998; Li and Hristova, 2010; Ogiso *et al*., 2002). The tyrosine kinase domain also contains regulatory sites that are phosphorylated by the receptor itself or other kinases in order to fully activate the receptor signalling pathway (Hubbard *et al*., 1998; Hunter, 1998; Pawson and Scott, 2005). Members of the RTK family are subdivided into 20 groups according to their ligand specificity as well as structural differences in their extracellular and tyrosine kinase domains. The subfamilies include,

but are not limited to, EGFR, PDGFR, insulin receptor (IR), insulin-like growth factor receptor (IGFR), vascular endothelial growth factor receptor (VEGFR), fibroblast growth factor receptor (FGFR) and nerve growth factor receptors (NGFR) (Aaronson, 2005; Aaronson, 1991; Alvarez *et al*., 2006; Lemmon and Schlessinger, 2010; Robinson *et al*., 2000). These RTKs might have cell-dependent expression patterns and certainly respond to specific growth factor ligands, all of which contribute to the cell's ability to test its environment at any given time and, once it has consolidated all of the input signalling data, to respond in an appropriate manner.

The RTK binds to its ligand, the growth factor, to activate the cascade of signalling events. Binding of the growth factor to the extracellular domain of receptors causes a conformational change within the receptor that unmasks the dimerization region (Alvarez *et al*., 2006; Heldin and Ostman, 1996; Heldin *et al*., 1982; Lemmon and Schlessinger, 1994; Ogiso *et al*., 2002). This leads to dimerization of the receptor either to itself (e.g. homodimerization) or another subfamily member (e.g. heterodimerization) (Burgess *et al*., 2003; Garrett *et al*., 1998; Schlessinger, 2002). In the absence of ligand, RTKs generally exist as monomers. Exceptions to this include the insulin receptor (IR) and the insulin-like growth factor receptor (IGFR) subfamily members. These RTKs exist as constitutive heterodimers of α and β chains linked by disulfide bonds (Garrett *et al*., 1998; Ottensmeyer *et al*., 2000). Upon binding of the ligand, e.g. insulin or the insulin-like growth factor, two individual α/β-chain heterodimers are brought into close enough proximity that dimerization between two α chains occurs and signalling is initiated. Another exception to the canonical ligand binding and dimerization mechanism exhibited by RTKs is ErbB2, a member of the EGFR subfamily, which has a constitutively exposed dimerization region and is able to bind other receptors independent of growth factors (Burgess *et al*., 2003; Ferguson, 2008; Garrett *et al*., 2003).

Upon ligand binding and dimerization, the receptor undergoes further conformational changes within the tyrosine kinase domain that relieves the inhibition on the activation loop and allows for trans-autophosphorylation of the kinase domain (Alvarez *et al*., 2006; Bae and Schlessinger, 2010; Ferguson, 2008; Heldin and Westermark, 1999). Other tyrosine residues within the intracellular domain are then phosphorylated by the receptor. These phosphorylated tyrosine residues serve as docking sites for intracellular signalling molecules that contain a Src homology 2 (SH2) or phosphotyrosine binding (PTB) domain, and activate several distinct signal transduction pathways including; the phosphatidylinositol 3'-kinase (PI3K)/Akt and the

Ras/MAPK pathway (Biarc *et al*., 2011; Cross *et al*., 2000; Pawson, 1992, 1994, 2002; Pawson and Scott, 2005; Schlessinger, 2000; Ueki *et al*., 2002). These signal transduction pathways play various roles in cell survival. The PI3K/Akt and the Ras/MAPK pathways are described in greater detail in Sections 1.3.1 and 1.3.2.

Signalling from the RTK is regulated by phosphorylation and dephosphorylation of residues within the receptor by the action of kinases (catalyze phosphorylation events) and phosphatases (catalyze dephosphorylation events) (Burnett and Kennedy, 1954; Hunter, 1998; Olsen *et al*., 2006; Pawson and Scott, 2005; Tonks, 2006; Walton and Dixon, 1993). Just as importantly, RTK signalling can also be terminated by internalization of the activated ligand-receptor complex followed by either deactivation and recycling of the receptor or degradation of the receptor by the lysosomal pathway (Holler and Dikic, 2004; Mukherjee *et al*., 1997; Sigismund *et al*., 2005; Sorkin and Von Zastrow, 2002). Internalization and trafficking of the ligand-receptor complex is referred to as endocytosis and has been reviewed in Section 1.4.

The mechanisms for regulating growth factor receptor function are similar for most RTKs. The following discussion will focus on the regulation and function of the receptor for the platelet-derived growth factor, and will be compared to other RTKs as needed.

1.2.1 Platelet-derived Growth Factor (PDGF) Ligand and its Receptor

The platelet-derived growth factor (PDGF) is a major mitogen for fibroblasts, connective tissue and smooth muscle cells, and activates a cascade of signalling events that are important for mitosis (hence 'mitogen') and that involve the stimulation of cell growth and changes in cell shape and motility (Alvarez *et al*., 2006; Heldin *et al*., 1982; Schlessinger, 2000). PDGF belongs to the cystine knot superfamily of growth factors. The cystine knot superfamily derives its name from the fact that all its members have six cysteine residues involved in homo- and/or heterodimeric disulfide bond formation (McDonald and Hendrickson, 1993; Vitt *et al*., 2001). The PDGF subfamily is made up of four gene products located on four different chromosomes; *PDGF-A* (chromosome 7), *PDGF-B* (chromosome 22), *PDGF-C* (chromosome 4) and *PDGF-D* (chromosome 11) (Betsholtz *et al*., 1986; Deuel *et al*., 1981; LaRochelle *et al*., 2001; Li *et al*., 2000b; Ross *et al*., 1986; Uutela *et al*., 2001). There is about 25% amino acid sequence identity between all four forms of PDGFs, and among the individual PDGFs, PDGF-A and PDGF-B share 50-60% sequence identity, while PDGF-C and PDGF-D share 42-50% identity (Alvarez *et*

al., 2006; Fredriksson *et al*., 2004; Heldin and Westermark, 1999; LaRochelle *et al*., 2001). All forms of PDGF have a C terminal growth factor domain. PDGF chains C and D differ from chains A and B by the presence of an N-terminal C1r/C1s urchin endothelial growth factor-like protein and bone morphogenic protein 1 (CUB) domain (Reigstad *et al*., 2005).

PDGFs exist and function as five distinct dimers; namely, PDGF-AA, PDGF-AB, PDGF-BB, PDGF-CC and PDGF-DD (Figure 1.1) (Reigstad *et al*., 2005; Tallquist and Kazlauskas, 2004). Both PDGF-A and PDGF-B can form homodimers or can form a heterodimer involving the A and B chains. In contrast, PDGF-C and PDGF-D are only able to form homodimers. PDGF dimerization occurs within the endoplasmic reticulum resulting in inactive precursor PDGF dimers (Fredriksson *et al*., 2004; Tallquist and Kazlauskas, 2004). The precursor PDGF-AA, PDGF-AB and PDGF-BB dimers are further processed by proteolytic cleavage during protein maturation and exocytic events within the trans-Golgi network, and are secreted in their biologically active forms (Fredriksson *et al*., 2004; Tallquist and Kazlauskas, 2004). On the other hand, cleavage of the CUB domain and activation of PDGF-CC and PDGF-DD dimers occur after secretion of inactive dimers into the extracellular environment (Fredriksson *et al*., 2004; Tallquist and Kazlauskas, 2004). The main site of PDGF synthesis is within the megakaryocytes, bone marrow precursor cells that differentiate into platelets. In quiescent cells, PDGF is stored within the alpha granules of platelets. PDGF can also be synthesized by fibroblasts, smooth muscle cells, endothelial cells, macrophages, glial cells and astrocytes (Heldin and Westermark, 1999; Linder *et al*., 1979).

Dimeric forms of PDGF bind to the receptor and initiate signal transduction pathways. Two structurally related isoforms of the PDGF receptor (PDGFR) has been discovered to date, PDGFRα and PDGFRβ (Claesson-Welsh, 1994a, b). These genes for the α and β isoforms are located on chromosomes 7 and 22, respectively. PDGFRα is a 170 kDa protein and the β isoform is 180 kDa. They are glycoproteins with sites for both N-linked and O-linked glycosylation. Like all other RTKs, PDGFRs contain an extracellular domain, a transmembrane domain and a tyrosine kinase domain (Figure 1.1). The N-terminal extracellular domain contains five immunoglobulin-like domains, and there is 30% amino acid sequence identity between the extracellular domains of the α and β isoforms (Claesson-Welsh, 1994b). Immunoglobulin-like (Ig-like) domains 1 to 3 are linked by disulfide bridges and are thought to be important for ligand-receptor binding, with domain 2 being the most essential domain for PDGFRα and

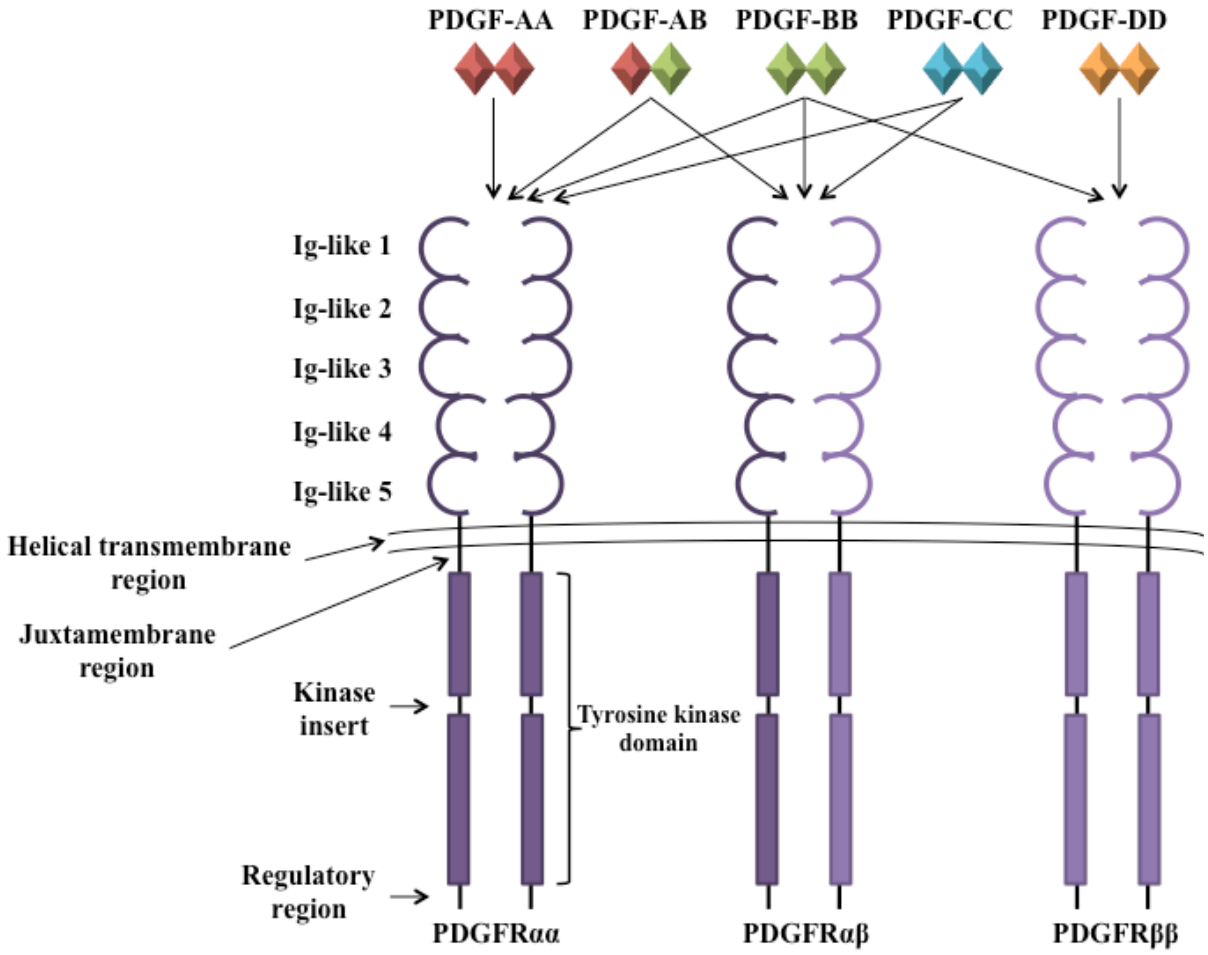

Figure 1.1: Homo- and heterodimers of PDGFR and the ligands that bind to them. The PDGF subfamily is made up of four gene products located on four different chromosomes; PDGF-A (chromosome 7), PDGF-B (chromosome 22), PDGF-C (chromosome 4) and PDGF-D (chromosome 11). PDGFs exist and function as five distinct dimers; namely, PDGF-AA, PDGF-AB, PDGF-BB, PDGF-CC and PDGF-DD. There are two structurally related isoforms of the PDGFR, namely PDGFRα and PDGFRβ. PDGFRs contain an extracellular domain, a transmembrane domain and a tyrosine kinase domain. The N terminal extracellular domain contains five immunoglobulin-like domains. The PDGF dimers have different affinities for the three isoform specific 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.

domains 2 and 3 both being essential for PDGFRβ (Claesson-Welsh, 1994a; Heldin *et al*., 1998; Heldin and Westermark, 1999). Ig-like domain 4 plays a role in stabilization of receptor homo-/heterodimers (Heldin *et al*., 1998; Heldin and Westermark, 1999). The C-terminal intracellular region of PDGFR can be further divided into, (i) the region between the cytoplasmic side of the plasma membrane and the tyrosine kinase domain referred to as the juxtamembrane domain (about 49 amino acids), (ii) the catalytically active tyrosine kinase domain is characterised by a kinase domain separated into two regions by a non-catalytic sequence of 100 hydrophilic amino acids referred to as the kinase insert, and (iii) the C-terminal tail or regulatory region. Studies have shown that between the α and β isoforms of the PDGFR there exists an 83% amino acid sequence identity between the juxtamembrane domains, 87% between the first half of the tyrosine kinase domain, 74% between the second half of the tyrosine kinase domain, 35% between the kinase insert and 27% between the C-terminal tail (Claesson-Welsh, 1994a, b).

Upon binding of the ligand, the PDGFRα and PDGFRβ isoforms interact to form the following homo- or heterodimers: PDGFRαα, PDGFRαβ and PDGFRββ (Figure 1.1). These receptor dimers have different affinities for the ligand dimers (Figure 1.1). The combination of receptor dimers and the various ligand dimers allows for different signalling responses. PDGF-AA dimers bind exclusively to PDGFRαα, whereas PDGF-DD binds exclusively to PDGFRββ. PDGF-AB and PDGF-CC both bind PDGFRαα and PDGFRαβ receptors (Figure 1.1) (Claesson-Welsh, 1994a, 1996; Fredriksson *et al*., 2004; Reigstad *et al*., 2005). As mentioned earlier, dimerization of receptors induces a conformational change within the activation loop of the receptor kinase domain that exposes the ATP binding site, which leads to receptor activation *via* trans-autophosphorylation. Trans-autophosphorylation occurs on the tyrosine residue 849 for PDGFRα and tyrosine residue 857 for PDGFRβ isoforms (Claesson-Welsh, 1994a, b). Following receptor activation, numerous tyrosine residues within the intracellular region are autophosphorylated by the receptor itself or phosphorylated by other intracellular signalling proteins. For instance, PDGFRβ is autophosphorylated at tyrosine residues 562, 581, 589, 716, 740, 751, 763, 771, 775, 778, 1009 and 1021, and can be phosphorylated by the Src-like nonreceptor tyrosine kinase Abl at residues 686, 934 and 970 (Claesson-Welsh, 1994b; Srinivasan *et al*., 2009). The phosphorylated tyrosine residues provide sites for recruiting signalling molecules to the receptor. A representation of cellular proteins and the tyrosine phosphorylated residue that they bind to is illustrated in Figure 1.2 (Claesson-Welsh, 1994a, b).

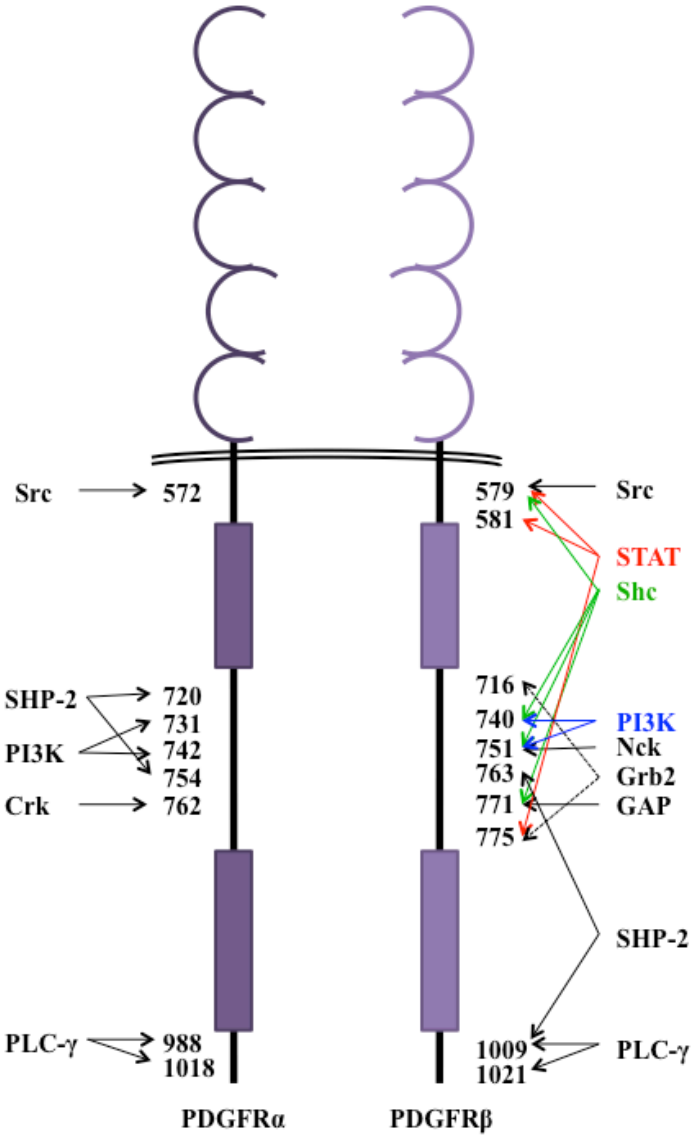

Figure 1.2: The two PDGFR isoforms utilize different phosphorylated tyrosine residues as docking sites for downstream signalling molecules. Following receptor activation, signalling molecules are able to interact with the receptor through the binding sites created by phosphorylated tyrosine residues. The PDGFRα is phosphorylated on at least 8 sites and is known to bind to 5 different protein families. The PDGFRβ binds to SH2 or PTB domains of at least 9 different protein families. This figure represents a summary of some cellular proteins that bind to phosphorylated PDGFRα or PDGFRβ, and the tyrosine phosphorylated residue that they bind to.

The differences in amino acid sequences in the intracellular region of both α and β receptors may account for the recruitment of specific downstream substrates and differential activation of PDGF-mediated signalling pathways. Two examples of these signalling pathways are described in Sections 1.3.1 and 1.3.2 (the PI3K/Akt and the Ras/MAPK pathways).

The outcome of signal transduction pathways initiated by PDGF is controlled by the tyrosine phosphorylation signature of the isoforms, as well as by their expression pattern within different cell types. PDGFR α and β are expressed in fibroblasts, neurons, vascular smooth muscle cells, kidney mesangial cells and pericytes (Alvarez *et al*., 2006; Heldin and Westermark, 1999). Platelets and myoblasts exclusively express PDGFRα while mammary epithelial cells, macrophages, liver cells and myeloid hematopoietic cells express the β isoform (Alvarez *et al*., 2006).

Both receptor isoforms play important roles in proliferation, migration, membrane ruffles, cytoskeletal rearrangements, turnover of phosphatidylinositols and inositol phosphatases and calcium fluxes (Claesson-Welsh, 1994a). Involvement of PDGFRα in cell migration and the turnover of phosphatidylinositols is purported to be cell type-dependent (Claesson-Welsh, 1994a). PDGFRα, but not PDGFRβ, is required for the timing of cell differentiation during oligodendrocyte maturation (Claesson-Welsh, 1994a), while PDGFRβ is involved in angiogenesis (Claesson-Welsh, 1994a).

1.3 Signalling pathways associated with the Platelet-derived Growth Factor (PDGF)

Signalling pathways/cascades initiated by PDGF are numerous and diverse. Examples include the Ras/MAPK pathway and the Phosphatidylinositol 3'-kinase (PI3K)/Akt pathway (Alvarez *et al*., 2006; Cross *et al*., 2000; Fruman *et al*., 1998; McKay and Morrison, 2007). Signalling from PDGF activates anti-apoptotic cascades that ultimately result in cell growth, differential and survival (Datta *et al*., 1999). The Ras/MAPK pathway and the PI3K/Akt pathway are reviewed below.

1.3.1 The Ras/MAPK Signalling Pathway

Receptor activation through phosphorylation events creates signalling scaffolds on the cytoplasmic domain of PDGFR that help to recruit binding partners and/or substrates. For example, phosphorylated tyrosine residues (pTyr) 579, 740, 751 and 771 within the PDGFR are

all possible binding sites for the Src homologous and collagen (Shc) adapter protein (Alam *et al*., 2009; Claesson-Welsh, 1994a; Finetti *et al*., 2009; Heldin *et al*., 1998). Binding of Shc – *via* its phosphotyrosine binding (PTB) domain – to the PDGFR initiates the Ras/MAPK signalling cascade. Shc also contains a Src homology 2 (SH2) domain that may play a role in the binding of Shc to the receptor (Figure 1.3) (Alam *et al*., 2009; Finetti *et al*., 2009). Both PTB and SH2 domains have recognition motifs for interacting with phosphorylated tyrosine residues within specific motifs (Alam *et al*., 2009; Finetti *et al*., 2009). The activated PDGFR phosphorylates Shc on Tyr317 (Heldin *et al*., 1998). This pTyr317 residue recruits the Growth factor receptor-bound protein 2 (Grb2) adaptor *via* its own SH2 domain (Egan *et al*., 1993; Li *et al*., 1994; Reebye *et al*., 2012). Grb2 can also be recruited directly to the activated receptor (e.g. EGFR) through its SH2 domain (Claesson-Welsh, 1994a; Giubellino *et al*., 2008; Heldin *et al*., 1998; Jang *et al*., 2009; Li *et al*., 1994). Grb2 exists as a complex with Son of Sevenless (Sos), the guanine nucleotide exchange factor (GEF) for the G-protein Ras (Heldin *et al*., 1998; Heldin and Westermark, 1999; Li *et al*., 1994). Binding of Grb2 to Shc therefore relocalizes Sos closer to its target, the GDP-bound Ras (Bos et al., 2007; Egan et al., 1993; Margarit et al., 2003; Pierre et al., 2011; Reebye et al., 2012; Rojas et al., 2011; Sondermann et al., 2004; Vigil et al., 2010). Ras alternates between a guanosine diphosphate (GDP)-bound "off" state and a guanosine triphosphate (GTP)-bound "on" state by the control of GTP hydrolysis (Fehrenbacher and Philips, 2009; Rojas *et al*., 2012; Rojas *et al*., 2011; Roskoski, 2010). Sos catalyzes the exchange of GDP for GTP resulting in an active conformation for the Ras protein (Ahearn *et al*., 2012; Freedman *et al*., 2006; Margarit *et al*., 2003; Sondermann *et al*., 2004). Conversely, Ras can be deactivated by hydrolyzing GTP to GDP, which is enhanced by GTPase activating proteins (GAP) (Ahearn *et al*., 2012; Bos *et al*., 2007; Vigil *et al*., 2010). Regulation of small monomeric G-proteins such as Ras is discussed in greater detail in Section 1.4.1.3.1.

Ras recruits and activates the serine/threonine protein kinase activity of Raf (Avruch *et al*., 2001; Barnard *et al*., 1995; Fehrenbacher and Philips, 2009; Leicht *et al*., 2007; Rojas *et al*., 2012; Roskoski, 2010). Raf contains a Ras binding domain (RBD) and, as the name suggests, the RBD binds to Ras. Raf can be phosphorylated by several other kinases to fully activate its activity (Matallanas *et al*., 2011; Maurer *et al*., 2011; Roskoski, 2010). In turn, Raf phosphorylates the mitogen activated protein kinase (MAPK)/Erk kinase (MEK), e.g. on serine

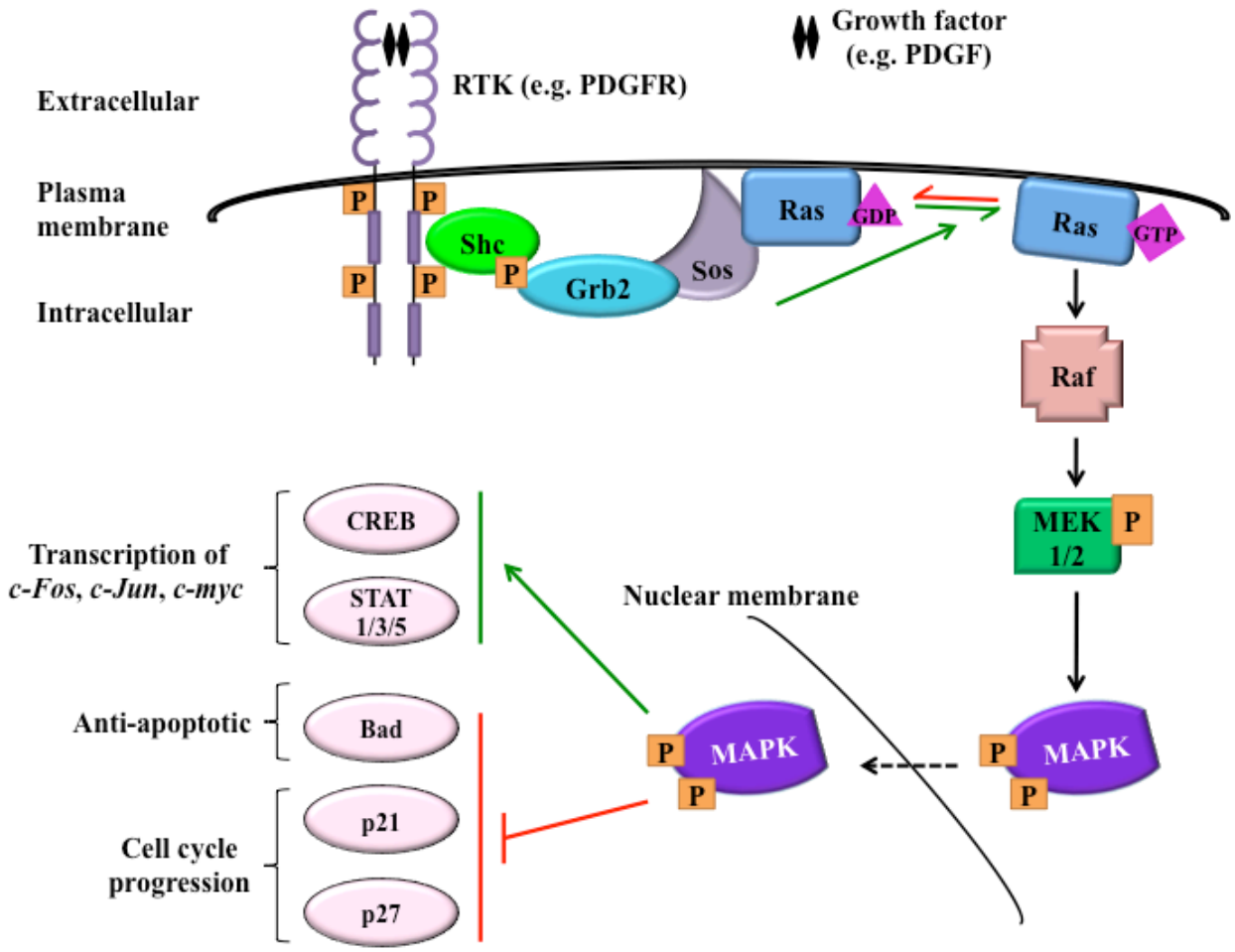

Figure 1.3: Ras/MAPK Signalling Cascade induced by PDGF. The binding of PDGF to PDGFR induces receptor dimerization and initiates signalling events that result in activation of Ras and a cascade kinase phosphorylation of MEK and MAPK/Erk. Binding of the Shc adapter protein to the PDGFR initiates the Ras/MAPK signalling cascade. The Grb2/Sos complex is recruited by Shc, relocalizing Sos closer to its target, Ras. Activation of Raf induces activation of Raf, which in turn phosphorylates and activates MEK. MEK activates MAPK/Erk by phosphorylation. MAPK/Erk translocates to the nucleus and regulates transcription of genes involved in cell survival such as CREB, Bad or p21.

218 and 222 for MEK1, and serine 222 and 226 on MEK2 (Keshet and Seger, 2010; Sebolt-Leopold and Herrera, 2004; Shaul and Seger, 2007; Yoon and Seger, 2006; Zheng and Guan, 1994). The role of MEK in this signalling cascade is that of a dual specificity tyrosine and serine/threonine kinase which is capable of phosphorylating threonine 183 and tyrosine 185 on MAPK1, and threonine 202 and tyrosine 204 in MAPK3 (Keshet and Seger, 2010; Payne *et al*., 1991; Rubinfeld and Seger, 2005; Shaul and Seger, 2007). When MAPK/Erk is phosphorylated, it is activated and it can, in turn, phosphorylate cytoplasmic and nuclear proteins, as well as transcription factors by translocating to the nucleus (McKay and Morrison, 2007; Plotnikov *et al*., 2011; Rubinfeld and Seger, 2005; Yoon and Seger, 2006). Some transcription factors regulated by the Ras/MAPK signalling cascade include c-Fos, c-Jun, c-myc and cAMP response element-binding protein (CREB) (De Luca *et al*., 2012; Plotnikov *et al*., 2011; Yoon and Seger, 2006). Phosphorylation of these intracellular targets by the Ras/MAPK signalling cascade elicits either activating or inhibitory events that contribute to the transcriptional regulation of genes required for entry into the cell cycle and survival of the cell (Plotnikov *et al*., 2011; Yoon and Seger, 2006).

1.3.2 The Phosphatidylinositol 3'-kinase (PI3K)/Akt Signalling Pathway

The PI3K/Akt signalling cascade is activated by class IA PI3Ks. PI3Ks phosphorylate phosphatidylinositols (PI) on the 3′ hydroxyl position of the inositol ring. An in-depth description of the PI3K family can be found in Section 1.3.2.1, below. Class IA PI3Ks exist as a heterodimeric complex consisting of a p85 regulatory subunit and a p110 catalytic subunit. In quiescent cells, p85 inhibits the kinase activity of the p110 subunit (Cuevas *et al*., 2001; Yu *et al*., 1998b). The p85 regulatory subunit of PI3K has SH2 domains with which it can bind directly to the PDGFR on tyrosine residues 740 and 751 (Franke *et al*., 1995). The receptor-p85 interaction relieves the inhibitory effects of p85 on p110 and brings p110 close to its lipid substrates phosphatidylinositol-4,5-bisphosphate ($PI4,5P_2$) located within the membrane lipid bilayer (Figure 1.4) (Cuevas *et al*., 2001; Yu *et al*., 1998b).

$PI3,4,5P_3$, generated by PI3K, is a second messenger that recruits and activates proteins that contain a pleckstrin homology (PH) domain, such as the serine/threonine kinases 3-phosphoinositide dependent protein kinase-1 (PDK1) and Akt (also known as protein kinase B, PKB) (Burgering and Coffer, 1995; Coffer *et al*., 1998; Franke *et al*., 1997; Franke *et al*., 1995).

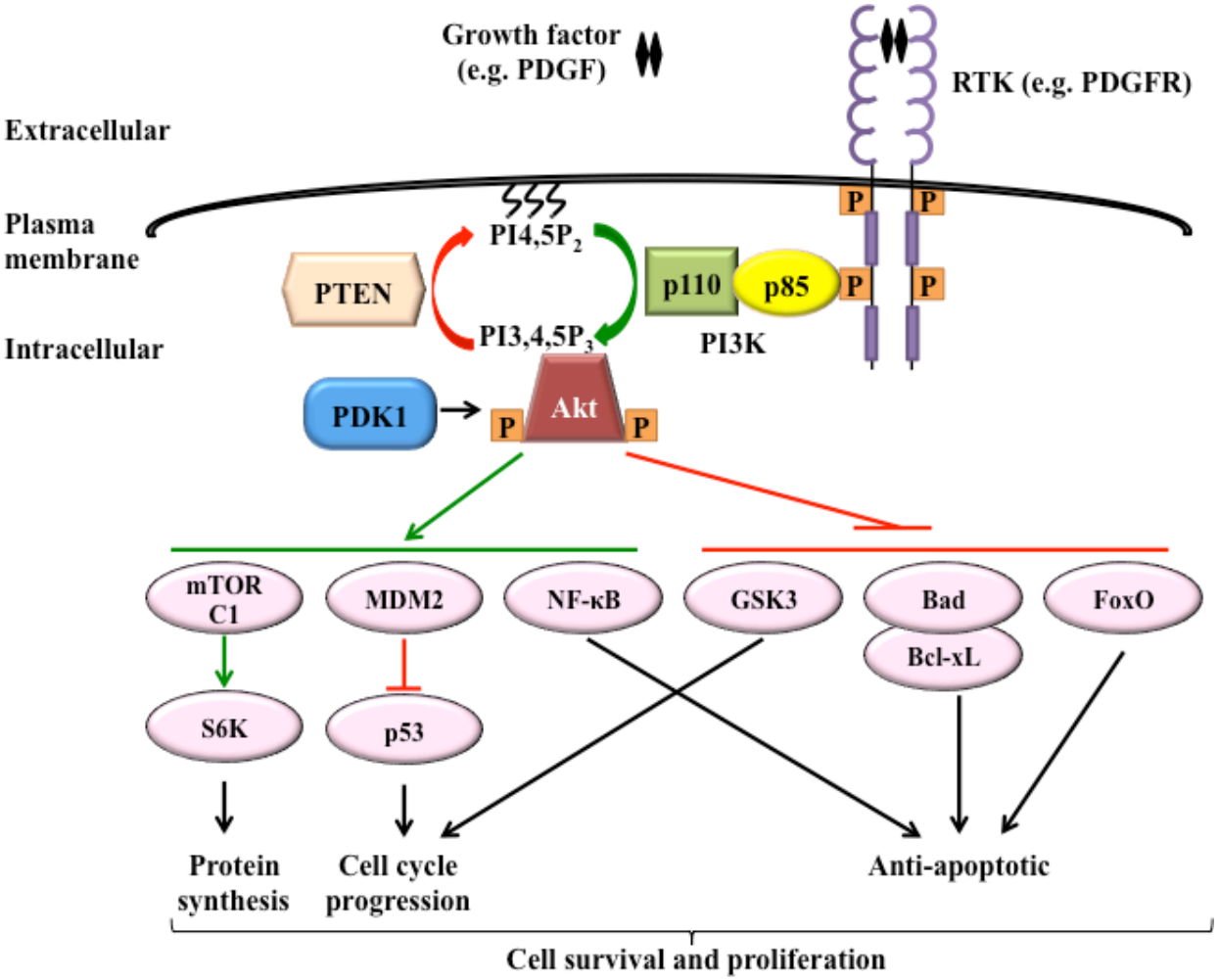

Figure 1.4: PI3K/Akt Signalling Cascade induced by PDGF. The binding of PDGF to PDGFR induces receptor dimerization and initiates signalling events that result in activation of PI3K and the generation of the $PI3,4,5P_3$ second messenger. $PI3,4,5P_3$ recruits Akt and PDK1 and results in the full activation of Akt. Akt phosphorylates and activates genes involved in cell survival. Activation of mTORC1, and eventually S6K, culminated in activation of protein synthesis. Activation of MDM2, a negative regulator of p53, promotes cell cycle progression. Phosphorylation of GSK3, Bad/Bcl-xL or FoxO inhibits their activities and drives the cell towards cell cycle progression and cell survival. PTEN counteracts the effects of PI3K by dephosphorylating $PI3,4,5P_3$ at the 3′ position.

PDK1 phosphorylates and activates Akt on threonine 308 (Thr308) (Le Good *et al*., 1998). Complete activation of Akt requires phosphorylation of serine 473 (Ser473) either by autophosphorylation or by the mammalian target of rapamycin complex (mTORC). When both Thr308 and Ser473 are phosphorylated, Akt then dissociates from the plasma membrane and phosphorylates numerous cytoplasmic and nuclear substrates, including glycogen synthase kinase 3 (GSK3), p70 ribosomal S6 protein kinase (S6K) and Bad, which are responsible for cell cycle entry, protein synthesis and anti-apoptotic events, respectively (Pullen *et al*., 1998). Akt signalling is central to a host of pathways with extensive crosstalk at various levels of the signal amplification cascade. For example, p110 can bind to Ras and activate the Ras/MAPK signalling pathway. Growth signals initiated by the PI3K/Akt pathway can be attenuated by phosphatase and tensin homologue protein (PTEN), a protein phosphatase that dephosphorylates $PI3,4,5P_3$ at the 3′ position. This event hinders the recruitment of Akt to the plasma membrane and inhibits full activation of Akt and any associated downstream signalling.

1.3.2.1 Phosphatidylinositol 3′-kinase (PI3K): Classification and subunits

PI3Ks are heterodimers comprised of regulatory and catalytic components and are grouped into three classes based on the specific components of the heterodimer, on the specific substrates that they target and on upstream stimuli that activate the PI3K complex (Table 1.1) (Domin and Waterfield, 1997). Class I PI3Ks phosphorylate the 3′ position of $PI4,5P_2$ and form heterodimers consisting of a regulatory and a catalytic subunit (Domin and Waterfield, 1997). They are further divided into class IA and IB depending on whether they are activated by RTKs or G-protein coupled receptors (GPCRs) (Domin and Waterfield, 1997). PI3Ks belonging to class IA form heterodimeric complexes consisting of catalytic subunit ranging in size from 110 to 120 kDa and a regulatory subunit ranging in size from 50 to 85 kDa. There are three class IA catalytic subunit isoforms expressed by separate genes; p110α, p110β, and p110δ expressed by *Pik3ca*, *Pik3cb*, and *Pik3cd* respectively (Hiles *et al*., 1992; Hu *et al*., 1993; Vanhaesebroeck *et al*., 1997). These subunits are structurally identical and consist of protein domains such as; (i) an adapter binding domain (ABD) with which it binds the regulatory subunit, (ii) an N-terminal Ras binding domain (RBD) for binding p21ras-GTP (Ras), (iii) a protein kinase-C homology (C2) domain for lipid binding, (iv) a helical domain, and (v) a C-terminal lipid kinase domain (Figure 1.5) (Holt *et al*., 1994; Rodriguez-Viciana *et al*., 1996).

Table 1.1: PI3K classification

Class	Regulatory subunit(s)	Catalytic subunit(s)	Substrate(s)	Product(s)
IA	p85α p55α p50α p85β p55γ	p110α p110β p110δ	Phosphatidylinositol-4,5-bisphosphate ($PI4,5P_2$)	Phosphatidylinositol-3,4,5-trisphosphate ($PI3,4,5P_3$)
IB	p101	p110γ	$PI4,5P_2$	$PI3,4,5P_3$
II	None	C2α C2β C2γ	Phosphatidylinositol (PI) and Phosphatidylinositol-4-phosphate (PI4P)	Phosphatidylinositol-3-phosphate (PI-3-P) and Phosphatidylinositol-3,4-bisphosphate ($PI3,4P_2$)
III	p150	Vps34	PI	PI3P

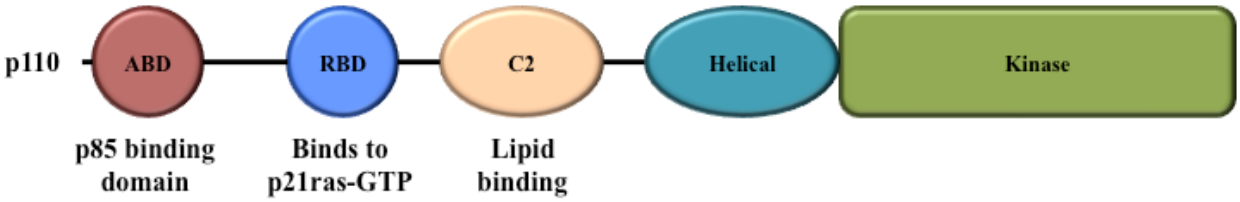

Figure 1.5: The p110 subunit of PI3K class IA. Domain structure of class IA p110 subunits. All three isoforms have the same domain structures consisting of an adaptor protein binding domain (ABD), a Ras binding domain (RBD), a protein kinase-C-homology 2 (C2) domain, a helical domain, and a catalytic domain.

There are five regulatory subunits referred to as the p85 regulatory subunits because the larger p85α and p85β isoforms were characterized first (Figure 1.6) (Vanhaesebroeck *et al*., 2005). They include p85α, p55α, p50α, p85β and p55γ and are able to dimerize with any of the p110 isoforms (Antonetti *et al*., 1996; Dhand *et al*., 1994; Pons *et al*., 1995). It is now known that the p85α and its splice variant isoforms, p55α and p50α, are products of the *Pi3kr1* gene, while p85β and p55γ are products of the *Pi3kr2* and *Pi3kr3* respectively. p85α and p85β are homologous to each other and contain; (i) an N-terminal SH3 domain, (ii) two proline rich domains, (iii) a GTPase activating protein (GAP) domain (also referred to as a breakpoint cluster region homology (BH) domain, (iv) two SH2 domains and (v) an inter-SH2 (iSH2) domain (Figure 1.6) . The shorter forms p55α, p50α and p55γ lack the SH3 and GAP domains (Antonetti *et al*., 1996; Pons *et al*., 1995). p55α and p55γ have 35 unique amino acids at the N-terminus while the p50α isoform has only five amino acids within this same region (Antonetti *et al*., 1996; Pons *et al*., 1995).

Class IA heterodimers consisting of p110α are activated by RTKs, while those containing p110β are activated by both RTKs and GPCRs. Both p110α and p110β are ubiquitously expressed, in contrast to the p110δ subunit that is expressed exclusively in haemopoietic cells of the immune system. Class IB PI3Ks act downstream of signals induced by GPCRs and consist of a single regulatory subunit p101 dimerized to a single p110γ catalytic subunit. They are expressed in platelets and neutrophils.

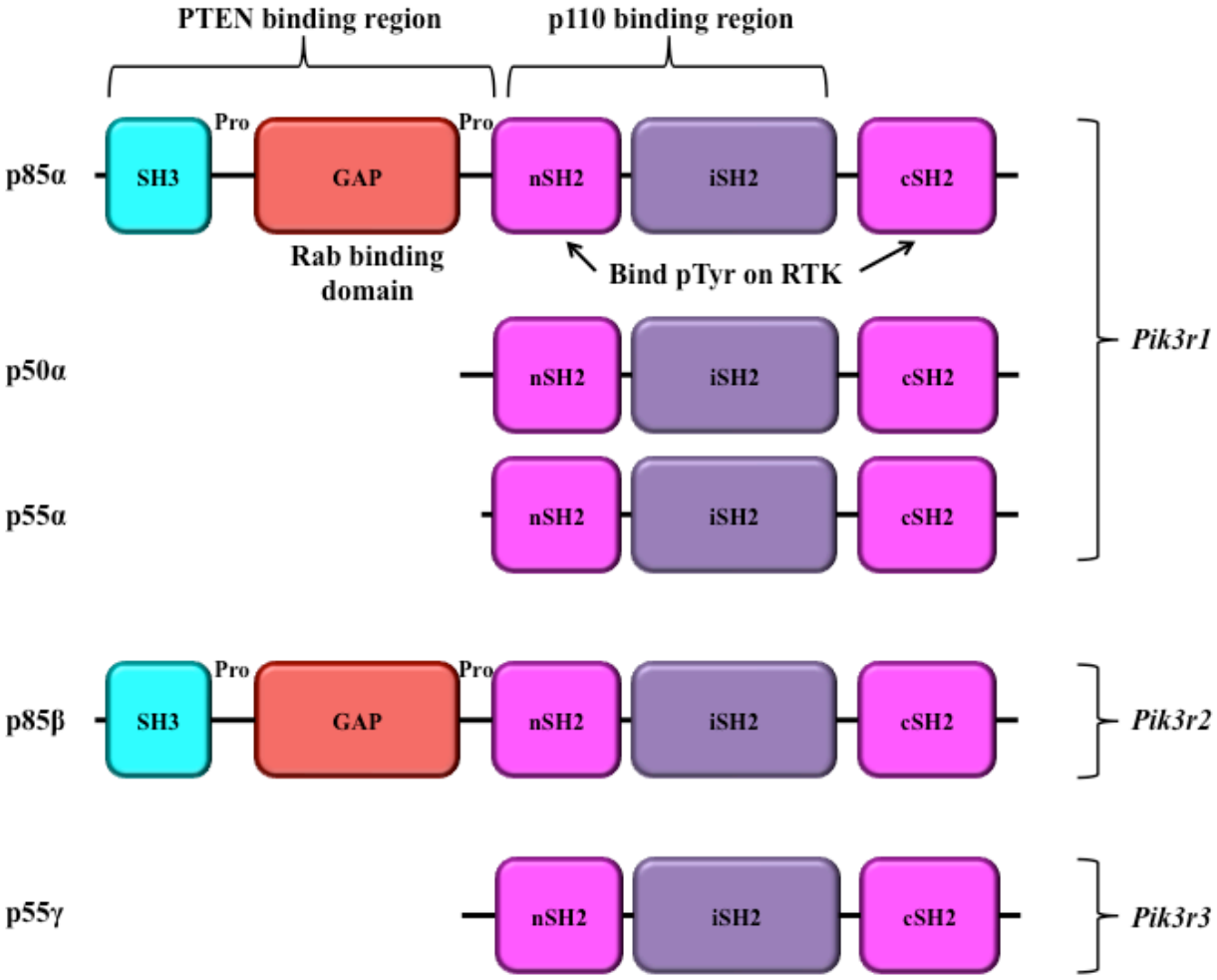

Figure 1.6: The p85 regulatory subunit family of the PI3K class IA. Domain structure of p85 regulatory subunit family. p85α, p55α and p50α are encoded by *Pik3r1*, while encodes *Pik3r2* encodes p85β and *Pik3r3* encodes p55γ . The smaller isoforms lack the N-terminus SH3 - GAP region.

Class II PI3Ks lack a regulatory subunit and consist solely of a carboxy-terminal C2 (CalB) domain. There are three isoforms namely, C2α, C2β and C2γ (Table 1.1). Class II PI3Ks generate both PI3P and PI3,4P_2. Class III PI3Ks is a complex consisting of a Vps34 catalytic subunit and a p150 regulatory subunit. They produce PI3P that is very important in the endocytic pathway (discussed in Section 1.4).

1.3.2.2 The role of p85 in PI3K signalling and trafficking of receptor

The canonical function of the p85 subunit of PI3K has been to regulate the catalytic subunit, p110. The p85 regulatory subunit of PI3K is essential for the activation of the PI3K signalling cascade. Stability of the p110 catalytic subunit of PI3K is dependent on heterodimeric association with p85 and *vice versa*. Even though p85 and p110 classically exist as heterodimers, there are instances where a pool of 'free' p85 generated by an increase in expression of p85 has been observed. 'Free' p85 exists as p85-p85 homodimers by forming weak interactions between the SH3 domain of one p85 subunit and the first proline rich domain of the other p85 subunit, and between the GTPase activating protein (GAP) domains of p85 (Figure 1.7). Homodimers of p85 are in part responsible for regulation of PI3K signalling by concealing the SH3-proline rich-GAP region of p85 in quiescent cells and only expose these domains in response to growth factor stimulation. Upon growth factor stimulation, p85 homodimers dissociate, making them available to bind other intracellular molecules. For instance, 'free' p85 can be co-immunoprecipitated with X-box-binding protein 1 (XBP-1) in response to insulin (Park *et al*., 2010). The interaction between the GAP domain of p85 and XBP-1 was shown to be necessary for the nuclear translocation of XBP-1.

To form the p85-p110 heterodimer, various contacts are made between the C-terminal half of p85 and the entire structure of p110. This not only stabilizes p110, but also regulates its function (Figure 1.7). In order to regulate the function of p110, the first (i.e. N-terminal) SH2 domain (nSH2) of p85 interacts with the calcium binding-homology (C2), helical and kinase domains of p110. The inter-SH2 (iSH2) domain of p85 and the C2 domain of p110 also make contacts. Additionally, the second, more C-terminal SH2 domain (cSH2) of p85 can form contacts with the kinase domain of p110β, but not the p110α isoform. All of these serve to obstruct domains required for p110 activity and thus inhibit the catalytic kinase activity of PI3K. Steady levels of

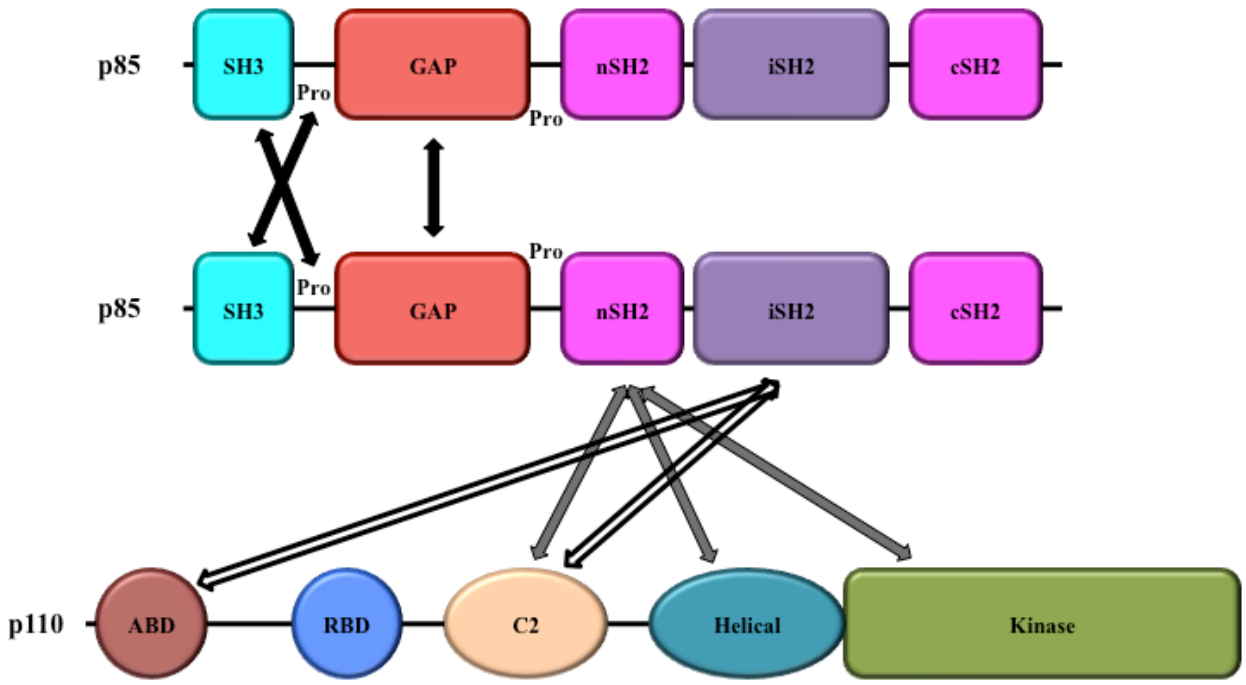

Figure 1.7: Intermolecular charge-charge interactions between p85 homodimers and p85-p110 heterodimers. p85 dimerizes with another p85 subunit by forming direct interactions between the SH3 domain of one p85 subunit and the first proline rich domain of the other p85 subunit. Contacts between the GAP domains are also necessary for homodimerization of p85. The nSH2 domain of p85 interacts with the p110 subunit of PI3K by making contacts with the C2, helical and kinase domain of p110. An additional regulatory contact is made between the iSH2 domain of p85 and the C2 domain of p110. p85 exerts its inhibitory effects on p110 through these nSH2 and iSH2 contacts. The iSH2 domain of p85 also interacts with the ABD domain of p110, which is important for stabilizing p110. cSH2 (p85) – kinase domain (p110) interactions occur with heterodimers containing p110β, but not the p110α isoform. It should be noted that p85 in p85-p110 heterodimers does not interact with another p85 as illustrated in the diagram. SH3, Src homology 3 domain; Pro, proline rich domain; GAP, GTPase activating protein domain; nSH2, N terminal Src homology 2 domain; iSH2, inter-SH2 domain; cSH2, C terminal Src homology 2 domain; ABD, adaptor binding domain; RBD, ras binding domain; C2, calcium binding-homology domain.

the p110 protein are maintained by stabilizing interactions made between the iSH2 domain of p85 and the adaptor binding domain (ABD) domain of p110.

The subcellular localization and specific function of p85 can be controlled by phosphorylation of serine and tyrosine residues (Mellor *et al*., 2012). A summary of residues that are phosphorylated and their respective associated biological outcomes can be seen in Table 1.2. p85 can also be modified by Casitas B-lineage Lymphoma (Cbl)-dependent ubiquitination (Bulut *et al*., 2013; Mellor *et al*., 2012). Cbl-b-dependent ubiquitination of p85 initiates the cascade of events that result in internalization and down-regulation of the erythropoietin receptor (EpoR) through endocytosis (Bulut *et al*., 2013), and can also prevent the formation of a complex between p85, CD28 and T-cell receptor that is essential for the PI3K-dependent activation of Vav-1, a GEF for Rac (Fang and Liu, 2001; Fang *et al*., 2001; Han *et al*., 1998; Ma *et al*., 1998). p85 is an important signalling node in numerous signal transduction pathways. It acts as a scaffolding protein to enhance crosstalk between signalling pathways and receptor endocytosis and trafficking (Mellor *et al*., 2012). During RTK-mediated PI3K signalling, p85 binds to the RTK (e.g. PDGFR) *via* its phosphotyrosine-dependent SH2 domain and this action relieves the inhibitory effects exerted by p85 on p110. p110 is then brought into proximity to its lipid substrates and fully activates the PI3K signalling pathway. Alternatively, the PI3K pathway can be activated through phosphorylation independent interactions between the proline rich domain of p85 and some intracellular signalling molecules including members of the Abl-Scr family (non-receptor/cytoplasmic tyrosine kinases) (Kapeller *et al*., 1994; Pleiman *et al*., 1994), Crk (adaptor protein that can bind E3 ubiquitin ligase Cbl) (Gelkop *et al*., 2001; Sattler *et al*., 1996) and Growth factor receptor-bound protein 2 (Grb2) (Wang *et al*., 1995; Weinger *et al*., 2008). The antagonist to PI3K signalling, PTEN, can be activated by binding of PTEN to the GAP domain of p85 (Anderson, 2010; Chagpar *et al*., 2010). This mechanism functions as a negative feedback loop that down-regulates signalling events initiated by PI3K (Mellor *et al*., 2012). A phosphotyrosine independent interaction between the SH2 domain of p85 and A-Raf (serine/threonine protein kinase) can also inhibit PI3K activity (Fang *et al*., 2002; King *et al*., 2000).

The regulatory p85 subunit can transiently or constitutively increase local concentrations of signalling molecules by sequestering intracellular proteins to locations where they are of the most importance. Specifically, the GAP domain of p85 interacts with Rab5 to enhance the

Table 1.2: p85 posttranslational modification and the effect on cellular signalling. (Mellor *et al*., 2012)

Phosphorylation of p85		
Amino acid modified [Domain]	**Kinase involved**	**Effects on signalling**
Ser 83 [Pro rich region]	Protein kinase A (PKA)	(i) Serves as a binding site for the regulatory subunit of PKA in the cAMP/PKA pathway. (ii) Increases binding between PI3K and Ras resulting in PI3K activation, induced by the cAMP/PKA pathway.
Ser 608	p110	Significant reduction in PI3K activity.
Tyr 508 [i-SH2]	PDGFR Interleukin (IL)-8 Granulocyte/Macrophage colony-stimulating factor (GM-CSF)	Not known.
Tyr 688 [c-SH2]	Abl and Src family	(i) Modifies the SH2 binding potential of p85, and relieves its inhibitory effect on p110, hence activating PI3K. (ii) Promotes de novo p85-p110 heterdimerization.

De-phosphorylation of p85		
Amino acid modified	**Phosphatase involved**	**Effects on signalling**
Ser 608	Protein phosphatase 2C	Leads to increased PI3K activity by reversing the action of p110.
Tyr residues	Src homology-2 domain containing phosphatase-1 (SHP-1) CD148	Reverses phosphorylation induced by the Src family.

hydrolysis of GTP to GDP (Chamberlain *et al*., 2004). The p85 subunit of PI3K has RabGAP activity towards Rab5 and Rab4 (Chamberlain *et al*., 2004). Rab5 is important for the internalization of activated RTKs whereas Rab4 mediates events leading up to RTK recycling (reviewed in Section 1.4.1.3). p85 regulates the "on" GTP-bound and "off" GDP-bound states of Rab5 and Rab4, thereby, regulating intracellular receptor trafficking. Expression of a p85 protein in which a critical arginine is substituted for an alanine (R274A), which significantly abolishes its RabGAP activity, results in a prolonged activation of PDGF receptor and sustained downstream signalling especially in the MAPK/Erk pathway. This implies a role for p85α in receptor trafficking and down-regulation (Chamberlain *et al*., 2004).

Expressing this RabGAP defective p85R274A mutant in cells results in the transformation of these cells (Chamberlain *et al*., 2008). These cells appear more rounded than control cells expressing wild type p85 and also spontaneously form foci on subconfluent plates (Chamberlain *et al*., 2008). The p85R274A-expressing cells form large colonies when cultured in a medium of soft agar (Chamberlain *et al*., 2008). These results suggest that disruption of the RabGAP activity of p85 in cells leads to a loss of contact inhibition and of anchorage dependence growth, which are characteristic of transformed cells. In order to investigate the tumorigenic properties of these transformed cells, p85R274A-expressing cells were injected subcutaneously into nude mice. By day 16, tumours had started developing and they continued to grow rapidly (Chamberlain *et al*., 2010). Further examination of the tissues revealed that the tumours were invasive and highly proliferative (Chamberlain *et al*., 2010). These results indicate that p85 plays essential roles in the regulation of PDGFR intracellular trafficking and signalling which is not limited to roles in PDGFR signalling.

Other roles for p85 in receptor signalling and trafficking have been reported in the literature (Mellor *et al*., 2012). During β-integrin signalling, p85 binds Caspase 8 *via* a phosphotyrosine-dependent SH2 domain and Caspase 8 sequesters p85 to prevent its Rab5 regulatory function (Torres *et al*., 2008). Signalling initiated by integrins leads to the association of the SH3 domain of p85 with focal adhesion kinase (FAK), which in turn interacts with Crk-associated substrate, $p130^{CAS}$ to activate pathways culminating in a change in the adhesive properties of the cell (Guinebault *et al*., 1995). Furthermore, binding of the SH2 domain of p85 to phosphorylated tyrosine residues on β-catenin is important for cell adhesion (Woodfield *et al*., 2001). Interaction between the SH3 domain of p85 and $p130^{CAS}$ important for cell migration has

also been observed (Li *et al*., 2000a). During dynamin-dependent receptor endocytosis, the SH3 domain of p85 binds to dynamin and this interaction has been shown to be important for the vesicle scission process, one of the early steps of endocytosis (Gout *et al*., 1993; Scaife *et al*., 1994). A phosphotyrosine-independent association between the SH2 domain of p85 and Ankyrin 3 has been reported to be involved in PDGFR trafficking and degradation *via* the endocytic pathway (Ignatiuk *et al*., 2006). The iSH2 domain of p85 also binds α/β-tubulins, proteins that function in microtubule-dependent vesicle trafficking to regulate budding or fusion of vesicles (Kapeller *et al*., 1995).

1.3.2.3 PI3K signalling and cancer

As discussed previously, the PI3K/Akt signalling pathway is activated in response to binding of a growth factor to its RTK. The PI3K/Akt signalling pathway can be down-regulated by PTEN as well as by endocytosis of the receptor ligand complex. Deregulation of these pathways has been associated with cancers. In several types of human cancers, including those of the breast, lung and brain, RTKs or components of the signal transduction pathways that they activate are either over-expressed or mutated (Bache *et al*., 2004; Blume-Jensen and Hunter, 2001). Overexpression of RTKs resulting from gene amplification, increased gene expression, or defects in receptor trafficking and degradation can activate a given pathway, even in the absence of ligand (Bache *et al*., 2004; Blume-Jensen and Hunter, 2001). The RTK, PDGFRβ, has been reported to be overexpressed in glioblastomas and some skin cancers (Fleming *et al*., 1992; Hagerstrand *et al*., 2006; Shimizu *et al*., 1999; Sjoblom *et al*., 2001). Overexpression of another RTK, EGFR, has been implicated in a number of cancers including that of the breast (Arteaga, 2003; Sebastian *et al*., 2006). RTKs or intracellular signalling molecules may also initiate tumorigenesis due to defects such as mutations, frame-shifts, deletions or chromosomal translocations (Bache *et al*., 2004; Blume-Jensen and Hunter, 2001). PDGFRβ chromosomal translocation generates a fusion-PDGFR kinase (e.g. TEL-PDGFR) and has been implicated in chronic myelomonocytic leukemias (Apperley *et al*., 2002; Baxter *et al*., 2003; Golub *et al*., 1994; Hidalgo-Curtis *et al*., 2010; Rodrigues and Park, 1994; Steer and Cross, 2002). PTEN is mutated and inactivated in glioblastomas, for example, resulting in constitutive Akt signalling and thus, the formation of tumours (Li *et al*., 1997). Some cancers have been shown to develop

as a result of mutations in proteins involved in multivesicular body formation and sorting (e.g. Tsg101) (Bache *et al*., 2004; Jiang and Beaudet, 2004; Katzmann *et al*., 2002).

Mutations within the p85 regulatory subunit and the p110 catalytic subunit of PI3K are of particular interest to this project. The most widely reported mutations have been those discovered in the p110α catalytic subunit. These mutations have been reported in a variety of cancers (Gabelli *et al*., 2010; Samuels and Waldman, 2010; Zhao and Vogt, 2008). The most frequent mutations in cancer include E542K and E545K in the helical domain of p110α, as well as H1047R in the kinase domain (Gabelli *et al*., 2010; Ikenoue *et al*., 2005; Miled *et al*., 2007; Samuels and Velculescu, 2004; Samuels *et al*., 2004). However, mutations can occur in all other domains of p110α except the Ras-binding domain. These mutations are gain-of-function mutations that result in constitutively active p110α and thus, enhanced Akt signalling in the absence of growth factor, cell survival and the development of tumours (Vogt *et al*., 2010). Mutation of glutamic acid 542 or 545 to lysine relieves the inhibitory effects exerted by the nSH2 domain of p85 (Huang *et al*., 2007a; Samuels and Velculescu, 2004). The histidine 1047 to arginine mutation causes a conformational change in p110α that enhances p110α's ability to interact with the membrane and lipid substrates within the membrane (Huang *et al*., 2007a; Mandelker *et al*., 2009).

Recently, mutations in p85α have been reported to lead to the development of glioblastomas, colorectal, urothelial, ovarian, colon, breast and pancreatic cancers (Ikenoue *et al*., 2005; Jaiswal *et al*., 2009; Ross *et al*., 2013). The most frequently mutated sites include Arg348, Gly376 and Leu380 in the nSH2 domain, and Lys459, Asp560, Asn564 and Arg574 in the iSH2 domain (Cancer Genome Atlas Research, 2008; Ikenoue *et al*., 2005; Jaiswal *et al*., 2009). These mutations result in increased p110α activity due to a disruption of inhibitory contacts made between p85 and p110 (Ikenoue *et al*., 2005; Jaiswal *et al*., 2009; Samuels and Velculescu, 2004). A recent study, using urothelial cancers as a model, discovered novel mutations within the GAP (E137K, R262T, K288Q), nSH2 (N377K), iSH2 (R481W, R557P) and cSH2 (T645I) domains of p85α (Ross *et al*., 2013). It has been shown that GAP domain mutants do not alter p85α inhibition and stabilization of p110α, suggesting that the oncogenicity of these mutations is independent of p110 catalytic functions (Ross *et al*., 2013). The mechanism of cancer development in GAP domain mutants remains unknown.

1.4 Endocytosis

Communication of vital signals as well as the import and export of essential molecules across membranes is facilitated by signal transduction pathways, transport channels within membranes and by endo- and exocytosis (Conner and Schmid, 2003). Endocytosis allows for the uptake of molecules in the extracellular environment or the internalization of molecules embedded in the membrane (Conner and Schmid, 2003; Marsh and McMahon, 1999; Mukherjee *et al*., 1997; Scita and Di Fiore, 2010). Exocytosis on the other hand is important for the secretion of molecules to the outside of the cell.

Endocytosis can be grouped into two processes depending on the size and type of material that is internalized. In the first type, referred to as phagocytosis, large particles including bacteria and apoptotic bodies are completely engulfed by cells such as macrophages (Conner and Schmid, 2003; Mukherjee *et al*., 1997). The second process, termed pinocytosis, involves the uptake of extracellular fluid carrying smaller particles including solutes, proteins and nutrients (Conner and Schmid, 2003; Mukherjee *et al*., 1997). Pinocytosis is further classified into macropinocytosis, clathrin-mediated endocytosis, caveolin-mediated endocytosis, or clathrin/caveolin-independent endocytosis on the basis of the mechanism of vesicle formation as well as cargo specificity (Conner and Schmid, 2003; Le Roy and Wrana, 2005; Mayor and Pagano, 2007; Mukherjee *et al*., 1997). The first step in the endocytic pathway is the recognition and internalization of the particle of interest. This involves the invagination of plasma membrane, which leads to the scission of the membrane to form a vesicle within the cell. All types of endocytosis follow the same path irrespective of the molecules being internalized and the method of internalization. After internalization and vesicle formation, the cargo is trafficked within the cell and delivered to the lysosome, the endoplasmic reticulum, the trans-Golgi network, or to the plasma membrane (Sigismund *et al*., 2012).

1.4.1 RTK-mediated Endocytosis

Signal transduction regulation is necessary for maintaining homeostasis of the cell. RTK signalling is regulated by clathrin-mediated endocytosis, caveolin-mediated endocytosis (also referred to as clathrin-independent endocytosis), or clathrin/caveolin-independent endocytosis (e.g. Flotillin-dependent endocytosis) (Aguilar and Wendland, 2005; Anderson, 1998; Conner and Schmid, 2003; Doherty and McMahon, 2009; Le Roy and Wrana, 2005; Mayor and Pagano,

2007; Sorkin and von Zastrow, 2009). Endocytosis is not only important for regulation of signal by reducing the pool of receptors available for signalling, but also for the turnover of RTKs and the maintenance of plasma membrane composition (Le Roy and Wrana, 2005; Sorkin and von Zastrow, 2009).

1.4.1.1 Clathrin-mediated endocytosis

The mechanism of clathrin-mediated endocytosis has been studied more extensively than the other forms of endocytosis, which are consequently also less well understood. Clathrin is a protein lattice made up of triskelion (three clathrin heavy chains tightly bound to three clathrin light chains and all joined together to make a three-legged structure) that assembles into polyhexagonal cages (Brodsky *et al*., 2001; Edeling *et al*., 2006; Kirchhausen, 2000; McMahon and Boucrot, 2011). These polyhexagonal cages coat and support the curvature of the plasma membrane within invaginated pits. Clathrin-mediated endocytosis begins with the recruitment of clathrin, adapter proteins and accessory protein to the plasma membrane (Figure 1.8) (Brodsky *et al*., 2001; McMahon and Boucrot, 2011; Owen *et al*., 2004). RTKs contain internalization recognition motifs for adapter proteins that aid in their recruitment to the budding site (Sorkin, 2004). Adaptor protein 2 (AP-2) is one such adapter protein (Traub, 2003). It is a heterotetrameric protein complex that binds to phosphatidylinositol-4,5-bisphosphate ($PI4,5P_2$) within the plasma membrane and to the RTK (Mousavi *et al*., 2004; Sigismund *et al*., 2012; Takei and Haucke, 2001; Traub, 2003). Clathrin binds to AP-2 and assembles into the lattice. Other assembly or accessory proteins include EGFR pathway substrate clone 15 (Eps15) and epsin 1/2 in the case of EGFR endocytosis (McMahon and Boucrot, 2011). These proteins drive curvature of the membrane and promote invagination of plasma membrane (Ford *et al*., 2002; Ungewickell and Hinrichsen, 2007). Eps15 and epsin 1/2 bind to each other, to AP-2 and to clathrin. They can also associate with the receptor by binding to ubiquitin on the receptor *via* ubiquitin interaction motifs (UIM) or ubiquitin associated (UBA) domains in Eps15 and epsin 1/2 (Chen and De Camilli, 2005; Polo *et al*., 2003; Polo *et al*., 2002). In some cases ubiquitin acts as an endocytic signal (Acconcia *et al*., 2009). Ubiquitination has been shown to be important for PDGFR internalization and degradation, whereas it is not required for EGFR internalization (Duan *et al*., 2003; Huang *et al*., 2007b; Mori *et al*., 1993; Stang *et al*., 2004).

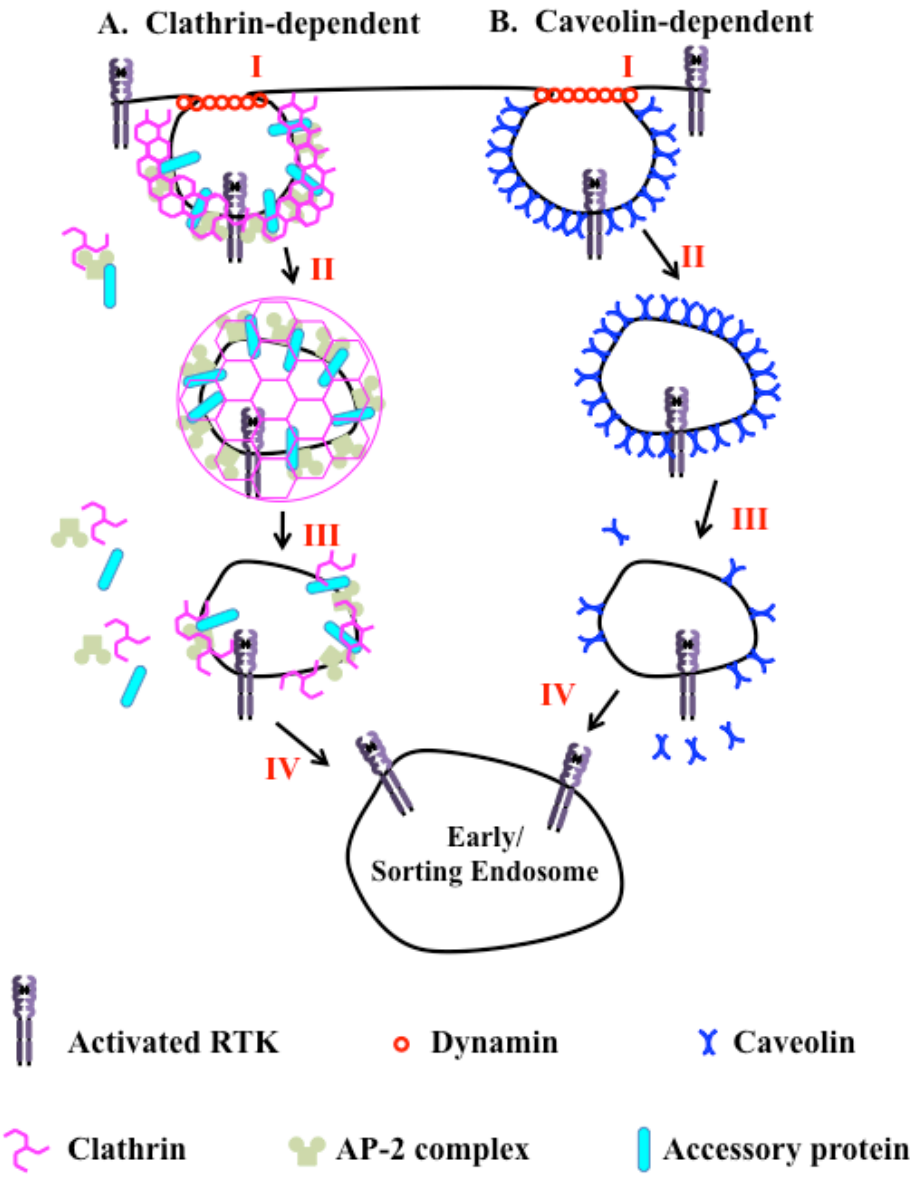

Figure 1.8: Clathrin and Caveolin modes of RTK internalization. I) During internalization of activated RTK, the budding vesicle is coated with clathrin, AP-2 and accessory proteins in the case of clathrin-mediated endocytosis. The pits are coated with caveolin during caveolin-mediated endocytosis. Dynamin facilitates the scission of the budding vesicles in both routes of internalization. II) The vesicles are coated with either clathrin or caveolin. III) Uncoating occurs as the vesicles move along the actin cytoskeleton to traffic the RTK within the cells. IV) The vesicle fuses with the early/sorting endosome and cargo is delivered to the early/sorting endosome or further intracellular trafficking events.

Ubiquitination is the conjugation of the 76 amino acid ubiquitin (Ub) polypeptide to a single lysine residue in a protein (monoubiquitination) or to multiple lysine residues within the protein (multiubiquitination) (Hershko and Ciechanover, 1998; Komander, 2009). A chain of ubiquitin molecules can also be conjugated to a single lysine residue and is denoted as polyubiquitination. Ubiquitin can be conjugated onto any one of the seven lysine residues within the ubiquitin molecule. Polyubiquitination can, therefore, be classified as Lys48-, 63-, 6-, 11-, 27-, 29-, or 33-linked polyubiquitination (Komander, 2009). Some studies have demonstrated that ubiquitin can sometimes by conjugated onto a cysteine residue (Cadwell and Coscoy, 2005; Hensel *et al*., 2011; Okumoto *et al*., 2011). Lysine 48-polyubiquitination usually promotes the proteosomal degradation of proteins, while mono- and multiubiquitination ultimately result in lysosomal degradation of proteins (Di Fiore et al., 2003; Haglund et al., 2003a; Haglund et al., 2003b; Hicke, 2001; Komander, 2009; Marmor and Yarden, 2004; Sigismund et al., 2004; Umebayashi, 2003). There are three steps in the ubiquitination process: (i) ATP-dependent conjugation of Ub to an E1 Ub activating enzyme; (ii) Transfer of Ub to an E2 Ub conjugating enzyme; and (iii) Transfer of Ub to an E3 Ub ligase and the subsequent conjugation of Ub onto the substrate (Figure 1.9) (Hershko and Ciechanover, 1998; Komander, 2009). There are two types of E3 Ub ligase; the "homologous to the E6-AP carboxy terminus" (HECT) and the "really interesting new gene" (RING) domain containing E3 ligases (Urbe, 2005). With HECT E3 ligases including Nedd4, Ub is transferred from the E2 enzyme to the HECT E3 enzyme before it is conjugated onto the substrate (Komander, 2009; Urbe, 2005; Wang et al., 2007). RING E3 ligases on the other hand act as adapter proteins by forming a bridge between the E2 enzyme and the substrate (Komander, 2009; Thien and Langdon, 2005; Urbe, 2005). In this case Ub is transferred directly from E2 to the substrate. An example of a RING E3 ligase is Cbl (d'Azzo et al., 2005; Fang et al., 2001; Komander, 2009; Thien and Langdon, 2005; Urbe, 2005). Cbl associates with PDGFR on the phosphorylated tyrosine at position 1021 within the receptor tail and monoubiquitinates the receptor (Miyake *et al*., 1998; Reddi *et al*., 2007; Thien and Langdon, 2001, 2005; Tsygankov *et al*., 2001). Monoubiquitination serves as the signal for internalization of the PDGFR.

Recruitment of AP-2, clathrin, Eps15, epsin 1/2 and Rab5 to the site of activated receptor triggers coat assembly, membrane invagination and ultimately scission of the vesicle. In order to

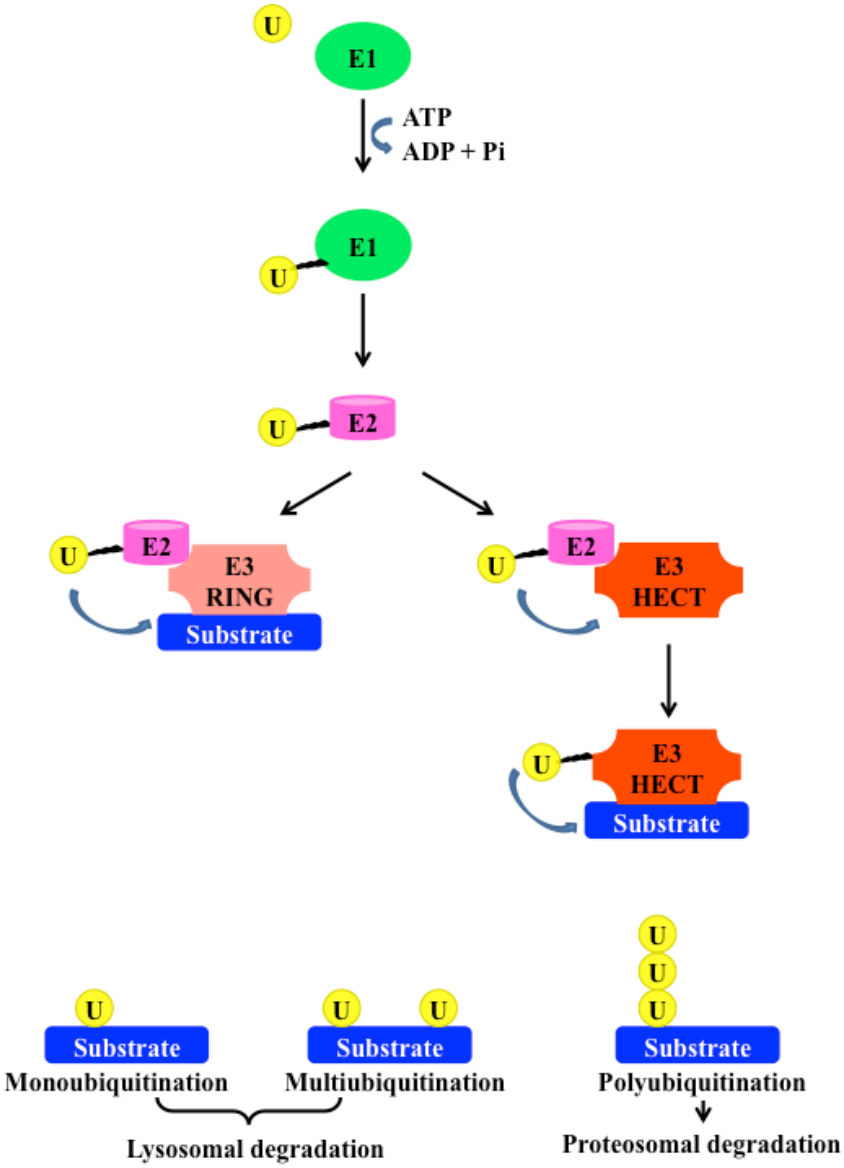

Figure 1.9: RTK Ubiquitination. The 3 steps involved in ubiquitination. ATP-dependent conjugation of Ub to an E1 Ub activating enzyme. Transfer of Ub to an E2 Ub conjugating enzyme. Transfer of Ub to an E3 Ub ligase and the subsequent conjugation of Ub onto the substrate. Conjugation with one ubiquitin moiety (monoubiquitylation), conjugation with multiple ubiquitin monomers (multiubiquitylation) or the formation of a ubiquitin chain branched at internal lysines within ubiquitin (polyubiquitylation).

facilitate scission of the vesicle, the large GTPase dynamin is loaded around the point of closure of the invaginating vesicle, the neck (Conner and Schmid, 2003; Mettlen *et al*., 2009; Pucadyil and Schmid, 2009; Sweitzer and Hinshaw, 1998). Dynamin interacts with $PI4,5P_2$ in the membrane and organizes into polymers to form a helical ring or collar (Pucadyil and Schmid, 2009; Ramachandran *et al*., 2009). The activity of dynamin is enhanced by its ability to hydrolyse GTP (Pucadyil and Schmid, 2009; Song and Schmid, 2003). The action of dynamin and other proteins, including actin and myosin, result in constriction of the neck and detachment of the vesicle from the membrane into the cytosol. The budded vesicle travels along the actin cytoskeleton and fuses with the early/sorting endosome, disassembling its coat in the process.

1.4.1.2 Caveolin-mediated endocytosis

Caveolin is a hairpin-shaped protein that embeds into the membrane lipid bilayer (Anderson, 1998; Doherty and McMahon, 2009; Miaczynska and Zerial, 2002). Caveolin-mediated endocytosis involves the assembly of caveolin at specific locations within the plasma membrane referred to as the caveolar raft (Figure 1.8) (Lajoie and Nabi, 2010; Mayor and Pagano, 2007; Nabi and Le, 2003; Parton and Richards, 2003). These rafts are analogous to lipid rafts (Nabi and Le, 2003; Parton and Richards, 2003). Lipid rafts are highly ordered, tightly packed microdomains within the plasma membrane that are concentrated with proteins, cholesterol and glycosphingolipids (Lajoie and Nabi, 2010; Nabi and Le, 2003; Parton and Richards, 2003). Lipid rafts are central to signal transduction as they serve as hubs for all the essential players important for any given pathway (Anderson, 2006; Lajoie and Nabi, 2010; Nabi and Le, 2003; Parton and Richards, 2003; Simons and Toomre, 2000). They also play essential roles in receptor trafficking (Anderson, 2006; Lajoie and Nabi, 2010). Insertion of caveolin into the membrane as well as oligomerization of caveolin molecules into a spiral-like coat may contribute to membrane curvature and invagination of pits during caveolin-mediated endocytosis (Anderson, 1998). As with clathrin-mediated endocytosis, dynamin causes the scission of the budding vesicle coated with caveolin. The budded vesicle uncoats and fuses with the early/sorting endosome. Caveolin-mediated endocytosis plays a dual role of allowing for the uptake of lipid molecules and secondly, the regulation of signalling events (Anderson, 1998; Simons and Toomre, 2000).

It has been reported that RTKs, e.g. EGFR, can follow different routes for internalization of activated receptor complexes depending on factors such as ligand availability, cholesterol and ubiquitination state (Sigismund *et al*., 2008; Sigismund *et al*., 2005). Under limited EGF conditions, EGFR is internalized *via* the clathrin-dependent pathway (Sigismund *et al*., 2005). Conversely, EGFR is internalized using both clathrin-dependent and clathrin-independent pathways when EGF is abundant (Sigismund *et al*., 2005). The internalization route taken contributes to differential signalling effects (Sigismund *et al*., 2008; Sorkin and von Zastrow, 2009). For instance, when cells expressing the transforming growth factor β receptor (TGFβR) utilize the clathrin-dependent pathway it results in a continued activation of downstream signalling events (Di Guglielmo *et al*., 2003). However, TGFβR is ubiquitinated and degraded when cells use the caveolin-mediated pathway (Di Guglielmo *et al*., 2003). The ubiquitination state of the receptor may also play a role in determining the route taken, though ubiquitin-dependent internalization appears to be caveolin specific (Huang *et al*., 2006; Sigismund *et al*., 2005; Umebayashi, 2003).

1.4.1.3 RTK intracellular trafficking

Signal transduction continues to occur from activated receptor complexes internalized into the early endosome and early/sorting endosome (Burke *et al*., 2001; Cavalli *et al*., 2001; Wang *et al*., 2004). The early/sorting endosome is so named because from this compartment the receptor is either deactivated and recycled to the plasma membrane for further signal transduction, or ubiquitinated and sorted into a lysosomal degradation pathway (Figure 1.10) (Lemmon and Traub, 2000; Rosenfeld *et al*., 1984). There are different schools of thought on the issue of how different endosomes form and mature. It is postulated that one endosome matures into another simply by disassembly of marker proteins of the previous endosome due to a change in the lipid composition of the membrane, which allows a new set of proteins specific to the 'new' endosome to accumulate on the membrane (Christoforidis *et al*., 1999a; De Camilli *et al*., 1996; Poteryaev *et al*., 2010; Rink *et al*., 2005). Another theory is that the early/sorting endosome is pre-existing and vesicles from the plasma membrane fuse with it. Then, vesicles that become recycling endosomes or late endosomes bud off from the early/sorting endosome (Vonderheit and Helenius, 2005).

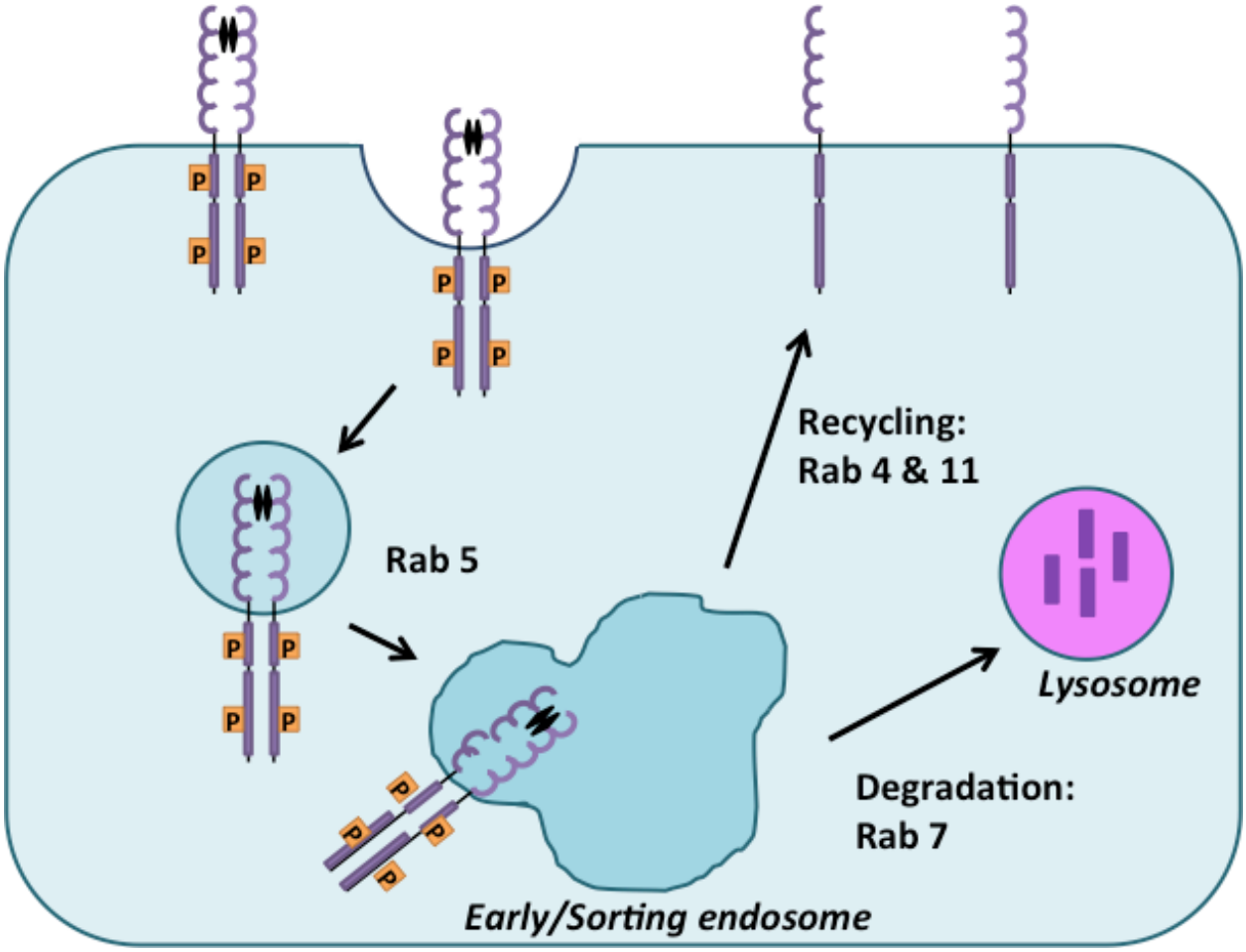

Figure 1.10: Rab GTPase proteins regulate vesicle trafficking events. Rab5 mediates the transfer of receptor-ligand cargo to the early/sorting endosome. Most of the receptor complexes are recycled either by a slow Rab4- and Rab11-mediated pathway or a fast Rab4-mediated pathway. A small fraction of the receptor-ligand complexes are internalized into multivesicular bodies within late endosomes and finally degraded in the lysosome *via* a Rab7-dependent pathway.

Internalized vesicles are loaded with the small GTPase, Rab5, which mediates homotypic vesicle fusion events between the internalized vesicle and the early sorting endosome (Figures 1.10 and 1.11) (Bucci *et al*., 1994; Gorvel *et al*., 1991; Wickner, 2010). A discussion of Rab proteins can be found in Section 1.4.1.5. Rab5 binds to the p85 regulatory subunit of PI3K on the activated receptor complex (Figure 1.11) (Chamberlain *et al*., 2004; Christoforidis *et al*., 1999a). The activated receptor complex binds and recruits Rin1, a Rab5 guanine nucleotide exchange factor (GEF) responsible for generating active Rab5 (Rab5-GTP) (Figure 1.11) (Barbieri *et al*., 2004; Barbieri *et al*., 2003; Kong *et al*., 2007). p85 acts as a GTPase activating protein (GAP) for Rab5-GTP that enhances GTP to GDP hydrolysis, resulting in the inactivation of Rab5 (Chamberlain *et al*., 2004). However, another Rab5 GAP, RN-Tre – that negatively regulates the endocytosis of EGFR – is recruited to the plasma membrane by the presence of Rab5 bound to p85 (Lanzetti *et al*., 2000; Martinu *et al*., 2002). During vesicle fusion and tethering, Rab5 recruits effector molecules such as the early endosomal auto-antigen 1 (EEA1) and Rabenosyn-5 to the site of membrane fusion (Figures 1.11 and 1.13) (Callaghan *et al*., 1999a; Christoforidis *et al*., 1999a; Mills *et al*., 1998; Nielsen *et al*., 2000; Rubino *et al*., 2000; Shin *et al*., 2005; Simonsen *et al*., 1998). Parallel coiled-coil homodimers of EEA1 bind to both Rab5 and PI3P on different endosomes to form the tether (Figures 1.11 and 1.13) (Callaghan *et al*., 1999b). EEA1 interacts with the membrane of one endosome *via* its C-terminal FYVE domain and with Rab5 on the other endosome through its N-terminal zinc finger (Mishra *et al*., 2010). FYVE domain containing proteins specifically bind to PI3P, a lipid product of the class III p150–Vps34-PI3Ks. Much like EEA1, Rabenosyn-5 is a FYVE zinc finger protein (Nielsen *et al*., 2000). Other effector molecules recruited by Rab5 to the site of membrane fusion include the lipid phosphatases, PI-4-phosphatase and PI-5-phosphatase, that use $PI3,4,5P_3$ as a substrate to generate PI3P on the early endosome (Shin *et al*., 2005). Tethering of two endosomes by EEA1 allows for the formation of a soluble NSF (N-ethylmaleimide-sensitive factor)-attachment protein receptor (SNARE) complex (McBride *et al*., 1999; Wickner, 2010). SNAREs are a group of membrane-anchored proteins that organize into a complex to facilitate membrane fusion and endocytic trafficking of RTKs (Figure 1.11) (Woodman, 2000; Xu *et al*., 2013). SNARE complex formation is not specific to the fusion of membranes between the early endosome and the early/sorting endosome. It is a requirement for membrane fusion at different steps within the endocytic trafficking pathway.

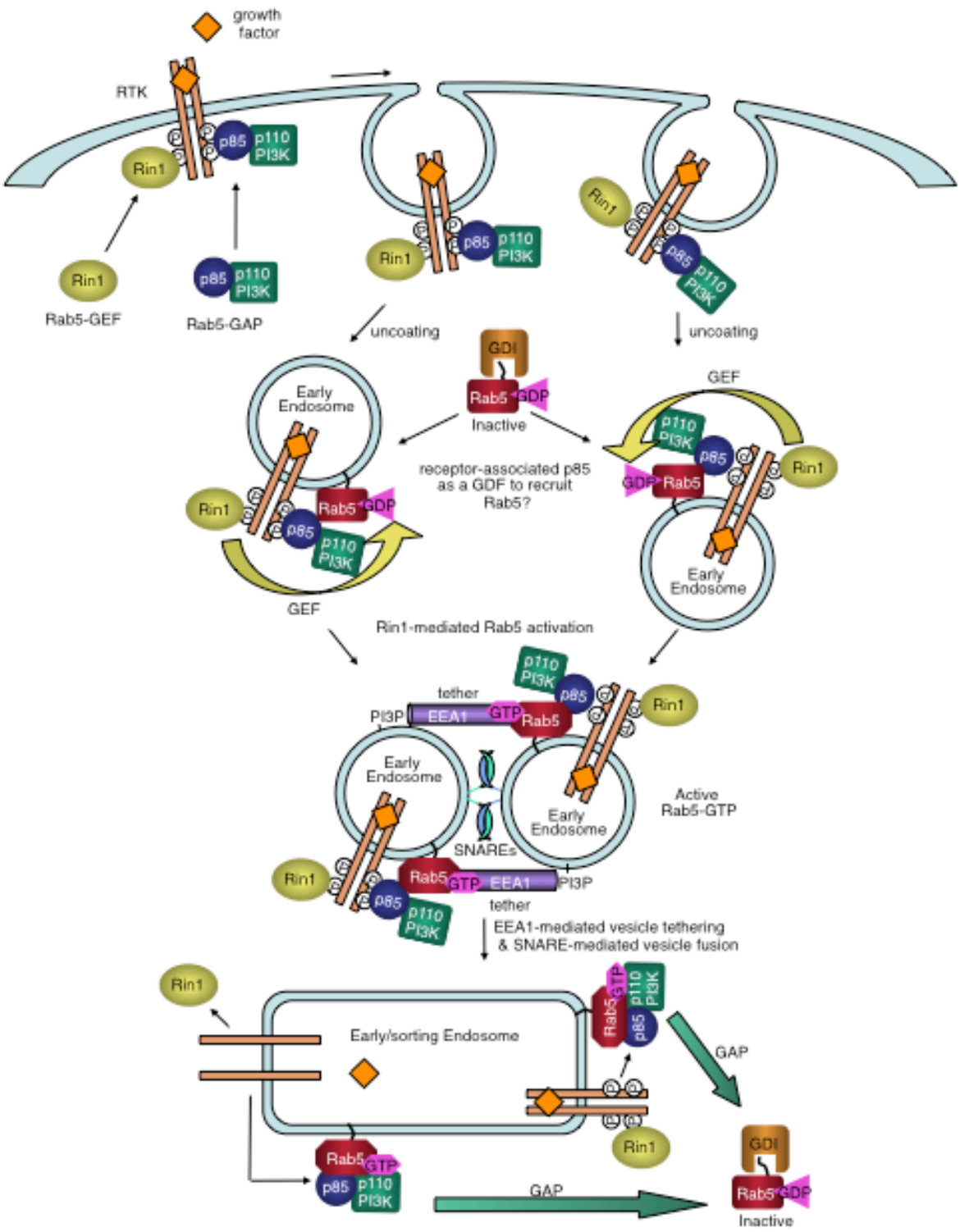
growth factor
RTK
p85
p110 PI3K
Rin1
Rab5-GEF
Rab5-GAP
uncoating
GDI
Rab5 GDP
Inactive
Early Endosome
receptor-associated p85 as a GDF to recruit Rab5?
GEF
Rin1-mediated Rab5 activation
tether
PI3P
EEA1
GTP
Rab5
Active Rab5-GTP
SNAREs
EEA1-mediated vesicle tethering & SNARE-mediated vesicle fusion
Early/sorting Endosome
GAP
Inactive

Figure 1.11: Model for receptor-associated proteins in the regulation of Rab5-mediated processes. RTKs activated by growth factor binding become tyrosine phosphorylated and recruit Rin1 (a Rab5 guanine nucleotide exchange factor (GEF)) and p85α–p110β (a possible Rab5 GDI-displacement factor (GDF) and a Rab5 GTPase activating protein (GAP)). RTK complexes are internalized into early endosomal vesicles by clathrin-dependent and clathrin-independent processes, which involve dynamin-dependent scission from the plasma membrane and the subsequent uncoating steps (not shown). The ability of p85 to bind Rab5-GDP suggests that RTK-associated p85 could act as a GDF to identify the appropriate vesicle membranes for Rab5 recruitment. The RTK-associated Rin1 is a Rab5 GEF that generates active Rab5-GTP suitable for interactions with many effector proteins. One effector protein is EEA1, which forms a tether between two early endosomes by binding to Rab5-GTP on one endosome and PI3P lipids on a second endosome, to facilitate SNARE-mediated vesicle fusion. Rab5-GTP is no longer required within the larger early/sorting endosome. We propose that p85α–p110β dissociates from the RTK, up-regulating its GAP activity towards Rab5-GTP to promote the formation of inactive Rab5-GDP, and allowing for GDI binding and membrane release. (Reproduced from (Mellor *et al*., 2012)). A general discussion of Rab protein regulation can be found in section 1.4.1.5.

As discussed above, receptor complexes can be delivered from the early/sorting endosome to either a recycling endosome to be transported back to the plasma membrane, or can be delivered to a late endosome as part of a lysosomal degradation pathway (Figure 1.10). Most receptor complexes are recycled either by a slow Rab4- and Rab11-mediated pathway or a fast Rab4-mediated pathway (Sheff *et al*., 1999; Stenmark, 2009; Takahashi *et al*., 2012). A small fraction of the receptor-ligand complexes are internalized into multivesicular bodies (MVBs) within late endosomes and finally degraded in the lysosome *via* a Rab7-dependent pathway. It has been postulated that 'new' endosomes may form from the budding off of specific domain within the early/sorting endosome. In support of this hypothesis, Rab microdomains have been reported to exist within early/sorting endosomes for Rab5, Rab4, and Rab11 (Miaczynska, 2013; Miaczynska and Zerial, 2002; Zerial and McBride, 2001). The specific Rab proteins, together with the appropriate endosome markers, are loaded onto the early/sorting and those sections bud off to generate recycling endosomes. The balance between recycling and degradation is regulated by the ubiquitination status of the RTK, other sorting signal proteins, or the level of RTK phosphorylation (Miaczynska, 2013). Specifically, loss of the T-cell protein tyrosine phosphatase (TC-PTP) leads to an increase in PDGFRβ, but not PDGFRα, phosphorylation resulting in a shift towards rapid recycling rather than degradation (Miaczynska, 2013).

1.4.1.4 Late endosomal sorting

Activated RTKs can transduce a signal during the initial phases of endocytosis and can continue to do so until the receptors are either dephosphorylated and recycled or sequestered into multivesicular bodies within the late endosome (Cavalli *et al*., 2001; Miaczynska, 2013). A small fraction of the receptor-ligand complexes are targeted for degradation in the lysosome *via* a Rab7-dependent pathway. One of the theories of endosome formation argues for the disassembly of marker proteins of the early/sorting endosome, for instance, and the assembly of recycling endosome markers that converts an early/sorting endosome into a recycling endosome (Poteryaev *et al*., 2010; Spang, 2009). Early/sorting endosomes are converted into late endosomes by the assembly of Rab7 into a microdomain within the membrane of the early/sorting endosome (Cavalli *et al*., 2001). Mon1 regulates the conversion of early to late endosomes by displacing Rabex-5 and the recruitment of the homotypic fusion and vacuole protein sorting (HOPS) complex (Poteryaev *et al*., 2010; Spang, 2009). Rabex-5 is a Rab5 GEF (Lippe *et al*., 2001). Displacement of this protein results in less activation of Rab5. The HOPS complex contains Vps39, a GEF for Rab7, which is crucial for Rab7 activation (Rink *et al*., 2005). A Rab5 GAP is thought to be recruited to deactivate Rab5 and promote guanine nucleotide dissociation inhibitor (GDI)-mediated membrane extraction of Rab5 to convert the vesicle from a Rab5-positive early endosome into a Rab7-positive late endosome (Rink *et al*., 2005).

Markers of the late endosome include the presence of PI3P in the membrane, lysobisphosphatidic acid, hydrolytic enzymes within its lumen and a decrease in pH to 5-6 (Marmor and Yarden, 2004; Teis and Huber, 2003). Receptor complexes on the membrane of the late endosome are completely engulfed into the luminal space where there are sequestered into multivesicular bodies (MVBs). As mentioned earlier, RTKs such as the PDGFR that are degraded as part of receptor down-regulation associate with the Cbl E3 ubiquitin ligase (Levkowitz *et al*., 1998; Miyake *et al*., 1998; Thien and Langdon, 2001; Tsygankov *et al*., 2001). Monoubiquitination is an absolute requirement for sorting of RTKs into the MVBs of the late endosomes (Haglund *et al*., 2003a; Haglund *et al*., 2003b; Hicke, 2001; Ravid *et al*., 2004). MVB biogenesis takes place through interactions between ubiquitin on mono- or multiubiquitinated receptors and the ubiquitin interacting motif (UIM) or the ubiquitin-conjugating enzyme E2 variant (UEV) domain or the ubiquitin-associated (UBA) domain of

members of the Endosomal Sorting Complex Required for Transport (ESCRT) protein complexes (Hicke and Dunn, 2003; Polo *et al*., 2002; Raiborg and Stenmark, 2009).

The ESCRT machinery consists of four complexes; ESCRT 0, ESCRT I, ESCRT II and ESCRT III (Figure 1.12) (Hurley and Emr, 2006; Katzmann *et al*., 2002; Raiborg and Stenmark, 2009). The ESCRT machinery operates on the basis of a 'hand off' model of MVB biogenesis. ESCRT 0 is a heterodimer of the hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) and the signal-transducer adapter molecule (STAM) (Raiborg and Stenmark, 2009). It associates with the membrane of the late endosome by binding to PI3P through the FYVE domain of Hrs (Hanson *et al*., 2009; Henne *et al*., 2011; Raiborg and Stenmark, 2009). ESCRT 0 localizes to microdomains on the membrane that are characterized by flat clathrin coats (Raiborg *et al*., 2006). MVB formation starts with the recognition and binding of ubiquitin on the receptor by the UIMs of Hrs (Bache *et al*., 2003; Henne *et al*., 2011; Hirano *et al*., 2006; Katzmann *et al*., 2003). Hrs then recruits ESCRT I *via* interactions between ESCRT I and the four amino acid motif of Hrs; the PSAP motif (Clague and Urbe, 2003). ESCRT I is a heterotetramer consisting of Tsg101.

Tsg101 is thought to be the main subunit required for the function of ESCRT I as knock-down studies show a dysregulation of degradation of EGFR (Babst *et al*., 2000). Studies on the other subunits are lacking. The UEV domain of Tsg101 binds to ubiquitinated receptor. ESCRT I recruits ESCRT II to the site of MVB biogenesis. Both ESCRT I and ESCRT II are important for sorting of the receptor. ESCRT II interacts with ubiquitin through its GRAM-like ubiquitin binding in EAP45 (GLUE) domain (Henne *et al*., 2011; Slagsvold *et al*., 2005).

Finally, ESCRT II recruits ESCRT III, which is a filament-like oligomer responsible for membrane invagination, scission of budding MVBs and disassembly of the ESCRT complex (Hanson *et al*., 2009; Raiborg and Stenmark, 2009; Saksena *et al*., 2009). Deubiquitination precedes inclusion of receptor within the MVBs. ESCRT III recruits the deubiquitinating enzyme Doa4, which catalyzes the removal of ubiquitin on all cargo associated with the budding MVB (Amerik *et al*., 2000; Henne *et al*., 2011). ESCRT III constricts the area of the MVB to be enclosed and pinches it off in an ATP-dependent manner (Hanson *et al*., 2009; Saksena *et al*., 2009). All the components of the ESCRT complex are dissociated and MVB biogenesis is complete. The late endosome containing MVBs fuse with the lysosome. Cargo is transferred and completely degraded.

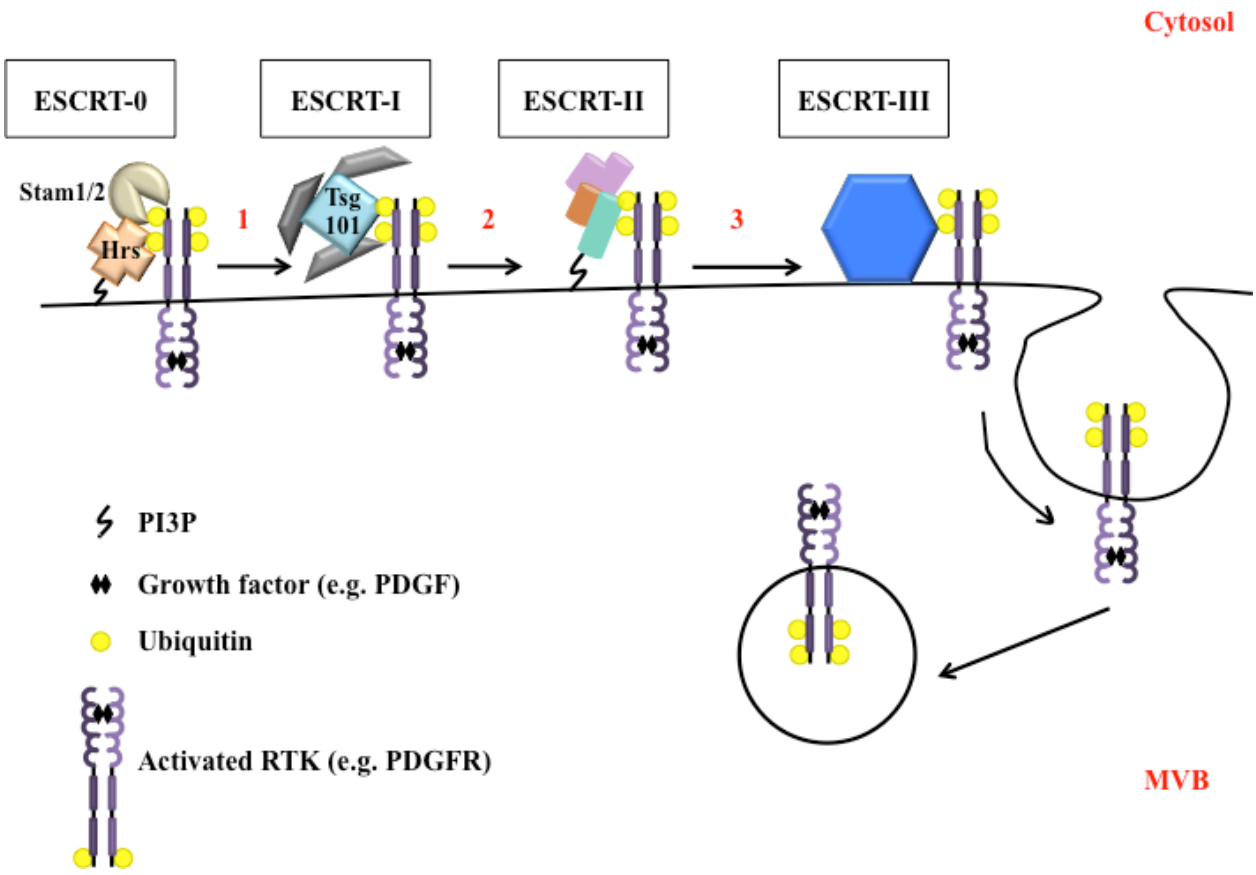

Figure 1.12: Late endosomal sorting of ubiquitnated RTK into multivesicular bodies (MVB) *via* the ESCRT machinery. 1) ESCRT 0 recognizes and binds to ubiquitin (Ub) on activated RTK complexes. ESCRT 0 interacts with ESCRT I, recruiting it to the site of ubiquitinated protein. 2) ESCRT I binds to Ub and recruits ESCRT II. ESCRT I and II function as cargo sorters. 3) ESCRT II recruits ESCRT III. ESCRT III deubiquitinates the RTK and initiates the invagination and scission of vesicles. ESCRT III is also responsible for disassembly of ESCRT complexes.

1.4.1.5 Rab Proteins and their regulation

Rab GTPase proteins belong to the Ras superfamily of GTPases (Pereira-Leal and Seabra, 2000). They regulate intracellular vesicle trafficking events from the plasma membrane to target organelles such as endosomes, lysosome, trans-Golgi network or endoplasmic reticulum (Brighouse *et al*., 2010; Hutagalung and Novick, 2011; Mitra *et al*., 2011; Stenmark, 2009; Zerial and McBride, 2001). Over 60 Rab proteins have been identified (Brighouse *et al*., 2010). Some Rab proteins, such as Rab4 and Rab5, are ubiquitously expressed while others, such as Rab3 (neurons) and Rab27 (platelets), are tissue specific (Stenmark and Olkkonen, 2001). Rab proteins are targeted to specific membrane locations possibly through the hypervariable region in the C-terminus (Pfeffer and Aivazian, 2004).

Rab proteins are regulated by their subcellular localization and their guanine nucleotide bound state, through interactions with a number of different proteins (Nottingham and Pfeffer, 2009). These proteins are GDP-bound and cytosolic when inactive, and GTP-bound with an insertion into a membrane when activated by their GEF (Bos *et al*., 2007; Nottingham and Pfeffer, 2009). The inactive Rab proteins are characteristically bound to a GDI protein that masks a C-terminal lipid prenylation site (Pfeffer *et al*., 1995). Rab proteins contain two geranylgeranyl motifs (20 carbon polyisoprenoid groups on two cysteine residues: C-C, C-X-C, C-C-X-X, C-C-X-X-X OR X-X-C-C; where X is any amino acid) at the C-terminus that can be prenylated by Rab geranylgeranyl transferases (Leung *et al*., 2006; Pfeffer and Aivazian, 2004; Seabra *et al*., 1992; Tuvim *et al*., 2001). In order to activate Rab proteins the GDI is removed, upon growth factor stimulation and receptor endocytosis, by a GDF (Figure 1.13) (Pfeffer and Aivazian, 2004; Seabra and Wasmeier, 2004; Stenmark and Olkkonen, 2001).

Displacement of the GDI exposes the lipid prenylation site, which can be inserted into the target membrane. GDP bound to the Rab protein is then exchanged for the more abundant GTP by the action of a GEF (Bos *et al*., 2007; Nottingham and Pfeffer, 2009; Stenmark and Olkkonen, 2001; Vetter and Wittinghofer, 2001). This leads to full activation of the Rab protein. Rab proteins contain two flexible switch regions, switch I and switch II, which are important for interaction with GEFs and GTPase activating proteins (GAP) (Bos et al., 2007; Fidyk and Cerione, 2002; Nottingham and Pfeffer, 2009; Scheffzek et al., 1998b; Tuvim et al., 2001). Binding of GTP to the Rab protein induces a conformational change within these two regions.

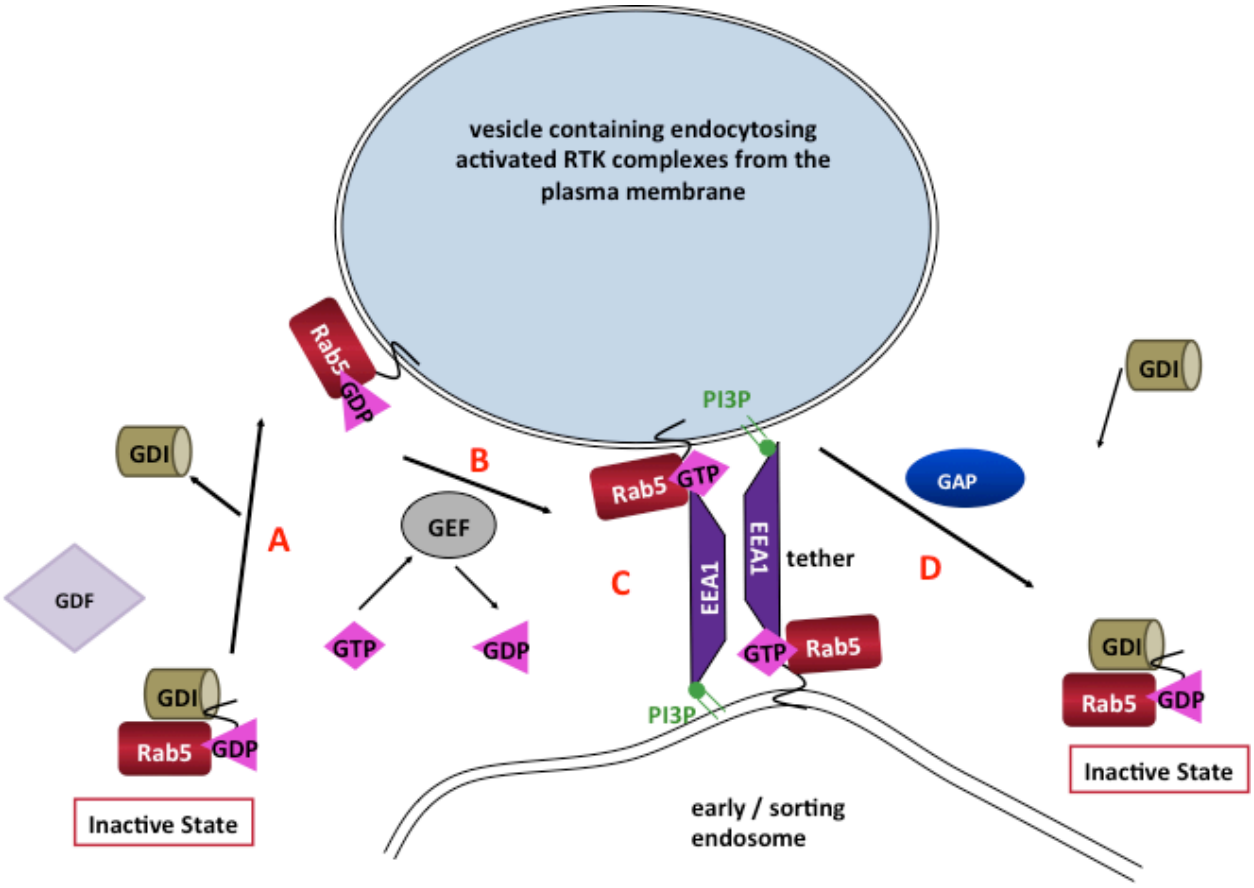

Figure 1.13: Regulation of Rab5 at the interface of endosome membrane fusion. A) The C-terminal prenylation site masked by a GDI (guanine nucleotide dissociation inhibitor) protein is removed by a GDF (GDI displacement factor) protein exposing the lipid site and allowing for membrane insertion. B) The GEF (guanine nucleotide exchange factor) protein stimulates the exchange of GDP for GTP, thus activating Rab5. C) Rab5-GTP recruits EEA1 (early endosomal auto-antigen 1) to the membrane that forms a tether between the two endosomes by binding to Rab5-GTP and PI3P. D) The GTPase activating protein (GAP) stimulates the weak intrinsic GTPase activity of Rab5. This returns Rab5 to an inactive state.

The conformational change creates a pocket that allows effector molecules to bind to active Rab proteins.

Rab proteins have a weak intrinsic ability to hydrolyse the bound GTP to GDP that is enhanced 100-1000 fold by GAPs (Bos et al., 2007; Scheffzek et al., 1998b; Vetter and Wittinghofer, 2001). The inactivated Rab protein is dislodged from the membrane, allowing for interaction between the lipid prenylyation site and the GDI to return Rab to its cytosolic GDP-bound state (Figure 1.13) (Pfeffer and Aivazian, 2004). GAPs serve two functions: (i) They provide a catalytic arginine residue (the arginine finger) that is involved in hydrogen bonding with a glutamic acid residue in the switch II region of the Rab protein, thereby positioning it within the nucleotide binding pocket and facilitating a hydrogen bond formation with the attacking nucleophilic water molecule (Clabecq et al., 2000; Li and Zhang, 2004; Vetter and Wittinghofer, 2001). The arginine finger is also involved in hydrogen bonding with the phosphate of GTP to stabilize the transition state (Li and Zhang, 2004; Sprang, 1997a). This is achieved by hydrogen bonding between the guanidine functional group of arginine and the α and γ phosphate oxygen atoms of GTP. The presence of Mg^{2+} within the nucleotide binding pocket is also important for stabilizing the switch region by interacting with oxygen atoms. These steps come together to promote the nucleophilic attack of water upon the γ phosphate of GTP to produce GDP and inorganic phosphate (Li and Zhang, 2004). Also, insertion of the arginine finger into the nucleotide binding pocket displaces water molecules and reduces the entropy of the reaction (Kotting *et al*., 2007); and (ii) They stabilize the switch regions of the Rab protein (Bos et al., 2007; Fidyk and Cerione, 2002; Li and Zhang, 2004; Scheffzek et al., 1998b; Seewald et al., 2002; Sprang, 1997b; Vetter and Wittinghofer, 2001). Interactions between the GAP and the Rab protein within the switch II region maintain the switch regions in an ordered state during the hydrolysis reaction.

A single protein can typically perform both functions (i.e. provide an arginine finger and stabilize the switch region), however, there also have been reports indicating that two proteins can work in concert to provide both functions (Szafer et al., 2000b). The ADP-ribosylation factor (Arf) GAP requires a coatomer to boost its GAP activity (Goldberg, 1999; Szafer et al., 2000b). A single Rab protein can be regulated by more than one GAP. Rab5 GAPs include p85, Tuberin, RN-Tre and TBC1D3/PRC17 (Chamberlain *et al*., 2004; Lanzetti *et al*., 2000; Pei *et al*., 2002; Xiao *et al*., 1997). Studies in our laboratory have demonstrated that arginine 274 in the BH

domain of p85α is the critical residue that serves as the arginine finger to enhance the GTPase activity of Rab5 (Chamberlain *et al*., 2004). The choice of Rab GAP may be dependent on differential GAP expression, subcellular localization of Rab protein or differential Rab protein target in response to specific stimuli (Frasa *et al*., 2012).

2.0 RATIONALE AND OBJECTIVES

The rationale for this thesis project stems from several inter-related experimental observations as follows. It has been documented in the literature that GAP proteins perform their function by stabilising the switch regions (which are crucial for interactions with GAPs and GEFs) in GTP-bound forms of Rab proteins. GAP proteins also provide a catalytic arginine required for the hydrolysis. A single GAP protein may perform both functions or two different proteins perform the separate functions (Bos et al., 2007; Fidyk and Cerione, 2002; Goldberg, 1999; Seewald et al., 2002; Seewald et al., 2003; Szafer et al., 2000a; Szafer et al., 2001). Our laboratory has shown, through mutational analysis, that arginine 274 in the BH domain of p85α serves as the arginine finger (Chamberlain *et al*., 2004). It was observed that p85 has a relatively low RabGAP activity and is able to bind both Rab5-GDP and Rab5-GTP, unlike most GAP proteins that bind selectively to the GTP forms (Chamberlain *et al*., 2004). One report found that p110β can bind selectively to Rab5-GTP (Christoforidis *et al*., 1999b) suggesting that it binds the conformation-specific switch regions and, thus, may work together with p85 to provide GAP functions. There are other reports indicating that PI3K has functions independent of its catalytic activity. Experiments performed with PI3K inhibitors show decreased PI3K activity and enlarged early endosomes that could not be restored by the addition of PI3K signalling products (Chen and Wang, 2001a, b). This observation is similar to observations made in cells expressing a constitutively active Rab5, Rab5-Q79L (Houle and Marceau, 2003). Expression of a constitutively inactive Rab5, Rab5-S34N, partially inhibited the effects of the PI3K inhibitor (Houle and Marceau, 2003). These results suggest that the PI3K complex may need to exist as a heterodimer of p85 and p110 in order to regulate Rab5. Furthermore, knock-out of p110β results in altered intracellular trafficking of EGFR and transferrin that can be restored by expression of a kinase dead p110β mutant (Ciraolo *et al*., 2008; Jia *et al*., 2008). This finding recapitulates the importance of both p85 and p110 in the regulation of endocytic events.

Further, since p85 binds to Rab5-GDP as well as Rab5-GTP, it may help recruit Rab5 to vesicle membranes containing activated PDGFR signalling complexes and thereby regulate PDGFR signalling events (Chamberlain *et al*., 2004). Taken together these results suggest the involvement of p110, and possibly the PDGFR, in enhancing (p110) or localizing (PDGFR) the RabGAP activity of p85.

The p85 protein regulates PI3K function and downstream signalling cascades, and also has a role in regulating Rab function (Chagpar *et al*., 2010; Chamberlain *et al*., 2004; Rabinovsky *et al*., 2009; Yu *et al*., 1998a; Yu *et al*., 1998b; Zhang *et al*., 2011). Defects in the ability of p85 to regulate Rab function are oncogenic (Chamberlain *et al*., 2008). As such, this thesis project was designed to examine how directed mutations in p85 known to affect its GAP activity as well as its ability to interact with p110 or PDGFR could influence the phenotype of NIH 3T3 mouse fibroblasts, an established model cell system for evaluating oncogenic transformation.

2.1 Hypothesis

We postulate that p85 acts in concert with p110 and/or PDGFR in order to enhance and/or localize its GAP activity near Rab proteins.

2.2 Objectives

1) To determine the effects of expressing a p85 mutant unable to bind to p110 or a RabGAP defective p85 mutant, unable to bind p110 on the
    a. Activation of PDGFR in response to PDGF
    b. Degradation of PDGFR in response to PDGF
    c. Activation of downstream effector molecules in response to PDGF
    d. Tumorigenicity of cells
2) To determine the effects of expressing a p85 mutant unable to bind to PDGFR or a RabGAP defective p85 mutant, unable to bind PDGFR on the
    a. Activation of PDGFR in response to PDGF
    b. Degradation of PDGFR in response to PDGF
    c. Activation of downstream effector molecules in response to PDGF
    d. Tumorigenicity of cells

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Reagents

All chemicals and reagents used were of molecular biological or analytical grade or higher. The majority of the chemicals and reagents used were procured from Sigma-Aldrich, VWR or BD Biosciences, unless otherwise specified.

3.1.2 Mammalian cells

NIH 3T3 mouse embryonic fibroblast cells and COS-1 African green monkey SV40 transformed kidney fibroblast-like cells were obtained from American Type Culture Collection (ATCC CRL1658 and ATCC CRL1650, respectively). Cell lines were maintained at 37 ºC in a 5% CO_2 humidified air environment.

3.1.3 Bacterial Strain

Replication of plasmid DNA was performed in the following *Escherichia coli* (*E. coli*) strain: TOP10 chemically competent *E. coli* [Genotype: F- mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 Δ lacX74 deoR recA1 araΔ139 Δ(ara, leu)7697 galU galK λ- rpsL (StrR) endA1 nupG] (Invitrogen, C4040-03).

3.1.4 Vectors and Plasmids

3.1.4.1 Vectors

Vectors used in this study include pFLAG3 and pMyc3, both of which carry both the ampicillin and geneticin resistance genes and allow for selection in bacterial transformation and stable expression of protein of interest in mammalian cell lines, respectively.

3.1.4.1.1 pFLAG3 vector

The pFLAG3 vector was modified from the pHA3 vector as described previously (Chamberlain *et al*., 2004). It encodes three copies of the FLAG-epitope (DYKDDDDK). The triple HA tag flanked by the HindIII and the BglII restriction sites and upstream of the multiple cloning site in the pHA3 vector (Figure 3.1) was replaced by the following sequence to generate the new pFLAG3 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′ (Figure 3.2) (Chamberlain *et al*., 2004). The pFLAG3 vector was used to express p85 wild type and p85 mutant fusion proteins with the FLAG-epitope at the N-terminus of the protein.

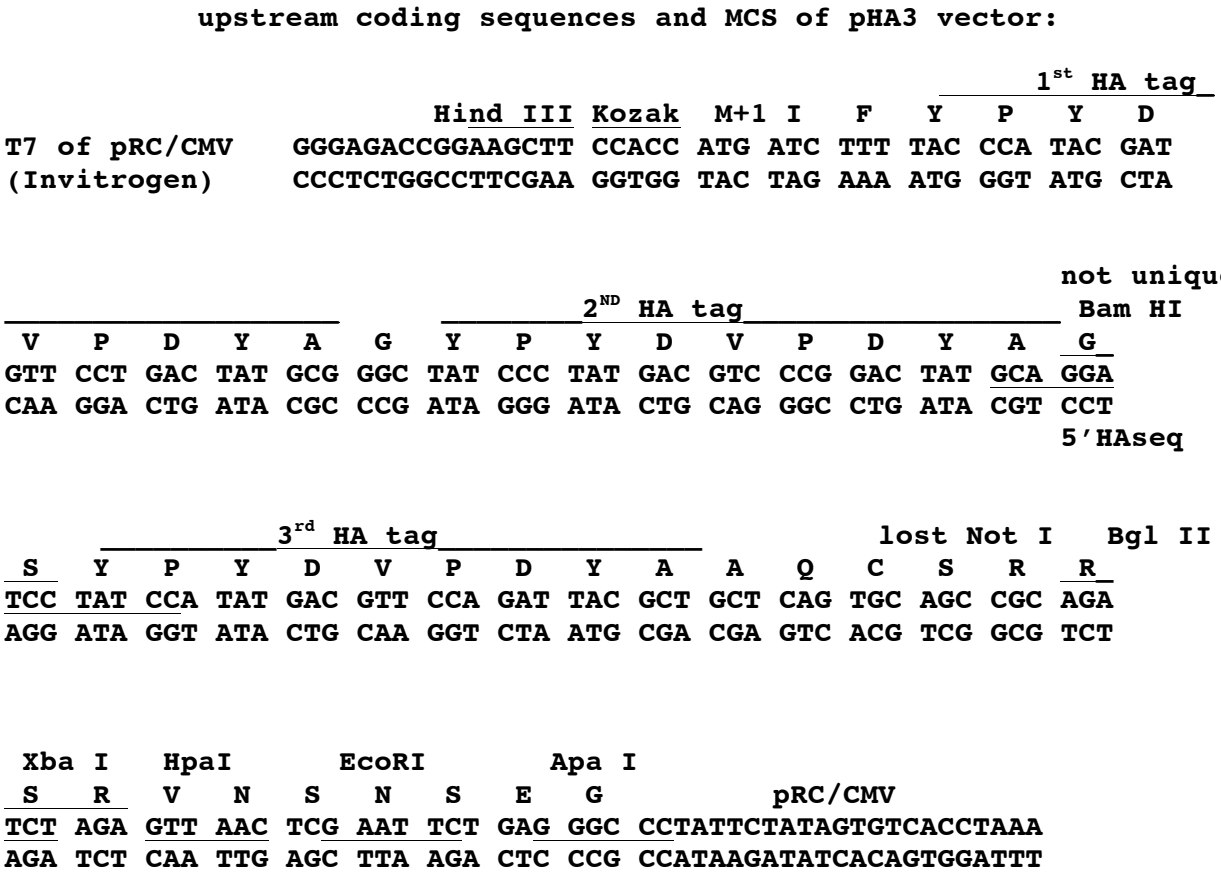

Figure 3.1: Multiple cloning site of pHA3 vector showing the location of the HA epitopes.

upstream coding sequences and MCS of pFLAG3 vector:

```
                                                          ________ 1st FLAG tag__
                          Hind III Kozak  M+1 D   Y   K   D   D   D
T7 of pRC/CMV    GGGAGACCGGAAGCTT CCACC ATG GAC TAC AAG GAC GAC GAT
(Invitrogen)     CCCTCTGGCCTTCGAA GGTGG TAC CTG ATG TTC CTG CTG CTA
                    5'seqFLAGeuk

                                                                unique
_______          __________2nd FLAG tag __________      Not I     ___
 D   K (no NheI) D   Y   K   D   D   D   D   K   A   A   A   D
GAC AAG GCT AGT GAC TAC AAG GAC GAC GAC GAT AAA GCG GCC GCT GAT
CTG TTC CGA TCA CTG ATG TTC CTG CTG CTG CTA TTT CGC CGG CGA CTA
  5'FLAG3seq

_____3rd FLAG tag___________ Nhe I   Bgl II Xba I Hpa I   Eco RI
 Y   K   D   D   D   D   K   A   S   R   S   R   V   N   S   N
TAC AAG GAC GAC GAC GAT AAA GCT AGC AGA TCT AGA GTT AAC TCG AAT
ATG TTC CTG CTG CTG CTA TTT CGA TCG TCT AGA TCT CAA TTG AGC TTA

        Apa I
 S   E   G   P   I       pRC/CMV
TCT GAG GGC CCT ATT CTATAGTGTCACCTAAA
AGA TCT CCG GGA TAA GATATCACAGTGGATTT
```

Figure 3.2: Multiple cloning site of pFLAG3 vector showing the location of the FLAG epitopes.

3.1.4.1.2 pMyc3 vector

The pMyc3 vector was generated by modifying the pFLAG3 vector. It encodes three copies of the Myc-epitope (EQKLISEEDL) upstream of the multiple cloning site (MCS). The fragment flanked by the HindIII and the NheI restriction sites encoding the three copies of FLAG-epitope (Figure 3.2) was replaced by the following sequence in the new pMyc3 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). This FLAG-modified vector was then digested with EcoRI and ApaI. A pair of oligonucleotides (5′-AAT TCG CGG CCG CGG GCC-3’ and 5’-CGC GGC CGC G-3′) were ligated into the EcoRI-ApaI-restriction enzyme digested FLAG-modified vector, so as to introduce a unique NotI restriction

site within the multiple cloning site and between EcoRI and ApaI restriction sites (Figure 3.3) (Chamberlain *et al*., 2004). The pMyc3 vector was used to express p110α fusion protein with the triple Myc-epitope tag at the N-terminus of the protein.

upstream coding sequences and MCS of pMyc3 vector:

```
                                                    .              1st Myc tag
                          Hind III Kozak  M+1 E    Q    K    L    I    S
T7 of pRC/CMV    GGGAGACCGGAAAGCTT CCACC ATG GAA CAG AAA CTG ATC AGC
(Invitrogen)     CCCTCTGGCCTTTCGAA GGTGG TAC CTT GTC TTT GAC TAG TCG

Unique
_____________.          .              2nd Myc tag              .  SacII
 E   E   D   L   L   S   E   Q   K   L   I   S   E   E   E   L   A
GAA GAG GAT CTG CTG AGC GAG CAG AAA CTG ATC AGC GAG GAA GAA CTG GCC
CTT CTC CTA GAC GAC TCG CAC GTC TTT GAC TAG TCG CTC CTT CTT GAC CGG

    .              3RD Myc tag                .  Nhe I        Bgl II
A   E   Q   K   L   I   S   E   E   D   L   A   S   R   S   R   V
GCG GAA CAG AAA CTG ATC AGC GAA GAG GAT CTG GCT AGC AGA TCT AGA GTT
CGC CTT GTC TTT GAC TAG TCG CTT CTC CTA GAC CGA TCG TCT AGA TCT CAA

                Unique
       EcoRI           Not I
 N   S   N   S   R     P   R   A   L   F   pRC/CMV
AAC TCG AAT TCG CGG CCG CGG GCC CTA TTC TATAGTGTCACCTAAA
TTG AGC TTA AGC GCC GGC GCC CGG GAT AAG ATATCACAGTGGATTT
```

Figure 3.3: Multiple cloning site of pMyc3 vector showing the location of the Myc epitopes.

3.1.4.2 Plasmids

Table 3.1 gives a summary of plasmids used for different investigations.

3.1.4.2.1 pFLAG3-p85 wild type and mutants

In order to generate plasmids encoding wild type p85, cDNA encoding full-length wild type p85 was subcloned into BglII and EcoRI sites of the MCS of the pFLAG3 vector (Chamberlain *et al*., 2004). Plasmids encoding pFLAG3-p85R274A, pFLAG3-p85Δ110 and pFLAG3-p85ΔR were generated by Quikchange mutagenesis method (Stratagene) (Chamberlain *et al*., 2004).

Table 3.1: List of Plasmids used in this thesis.

Protein	Species	Amino Acids	Vector backbone	Expression system	Selection
p85	Bovine	1 – 724, full-length	pFLAG3	Mammalian	Geneticin
p85R274A	Bovine	1 – 724 (R274 mutated to A)	pFLAG3	Mammalian	Geneticin
p85Δ110	Bovine	1 – 477 & 514 – 724 (deletion of amino acids 478-513, prevents p85 from binding to p110)	pFLAG3	Mammalian	Geneticin
p85Δ110+R274A	Bovine	1 – 477 & 514 – 724 (deletion of amino acids 478-513 plus R274 mutated to A)	pFLAG3	Mammalian	Geneticin
p85ΔR	Bovine	1 – 724 (2 amino acid substitutions: R358 to A and R649 to A, prevents binding to tyrosine phosphorylated PDGFR)	pFLAG3	Mammalian	Geneticin
p85ΔR+R274A	Bovine	1 – 724 (3 amino acid substitutions of R to A, at positions 358, 649 and 274)	pFLAG3	Mammalian	Geneticin
p110α	Mouse	1 – 1068, full-length	pMyc3	Mammalian	Geneticin

3.1.4.2.2 pMyc3-p110α

cDNA encoding full-length wild type mouse p110α was amplified by PCR from the pCMV-p110myc plasmid (a gift from Dr. L.T. Williams, University of California San Francisco, CA). The pCMV-p110myc plasmid was digested with BamHI and NotI. The pMyc3 vector was digested with BglII and NotI. The p110α insert DNA was subcloned into the BglII-NotI-digested pMyc3 vector to generate a plasmid encoding pMyc3-p110α (Chamberlain *et al*., 2004). Restriction enzyme digestion with BamHI or BglII results in sequences that have compatible overhanging cleavage sites that can be ligated. However, once ligated both the BamHI and BglII restriction recognition sites are lost.

3.1.5 Primers

All primers were ordered from Invitrogen, Gibco BRL, or the University of Alberta. Table 3.2 lists the primers used for site-directed mutagenesis and sequencing reactions.

Table 3.2: List of Primers used for Site-directed Mutagenesis and Sequencing

Primer Name	Sequence (5' to 3' notation)
Mutagenesis Primers	
5' p85R274A	GCC CTC TGC TTT TCG CAT TCC CAG CAG C
3' p85R274A	GCT GCT GGG AAT GCG AAA AGC AGA GGG C
Sequencing Primers	
3' 85 SEQ 157 (DAN31)	GTA ACT CGG CTG GGT
3' 85 SEQ 226	GGG CGA CCT AAT GAG
3' 85 SEQ 352	CAA AAA GGT CCC GTC
3' 85 SEQ 549	CCG CCT GCT TCT TCA
3' 85 SEQ 666 (DAN27)	GCA GTG CTT GAC CTC
5' p85R649A (DAN26)	TTC TTG TCG CGG AAA GC
SEQ 3' HA3	CAG CGA GCT CTA GCA

3.1.6 Antibodies

Antibodies were purchased from Santa Cruz Biotechnology (Dallas, Texas, USA), Sigma-Aldrich (St. Louis, Missouri, USA), Cell Signalling Technology (Danvers, Massachusetts, USA), EMD Millipore (Billerica, Massachusetts, USA), Ambion® Life Technologies Invitrogen (Carlsbad, California, USA), or BD Biosciences (Franklin Lakes, New Jersey, USA). Antibodies used for immunoprecipitation and Western blotting experiments are described in Table 3.3.

3.2 Methods

3.2.1 Generation of pFLAG3-p85Δ110+R274A and pFLAG3-p85ΔR+R274A encoding plasmids

3.2.1.1 Site-directed mutagenesis

Plasmids encoding pFLAG3-p85Δ110+R274A (deletion of amino acids 478-513 plus amino acid substitution of alanine for arginine at position 274) or pFLAG3-p85ΔR+R274A (three amino acid substitutions of alanine for arginine, at positions 358, 649 and 274) were generated using the Quikchange Site-Directed Mutagenesis method by Stratagene. The mutagenic PCR reaction mixture contained 1x Pfu buffer with $MgSO_4$, template DNA (30 ng for pFLAG3-p85Δ110 and 60 ng for pFLAG3-p85ΔR), 125 ng of each sense/antisense mutagenic primer (5' p85R274A and 3' p85R274A), 0.05 mM dNTPs and 2.5 units of Pfu DNA polymerase

Table 3.3: List of Antibodies and the Dilution or Concentration used for Western Blotting (WB) and Immunoprecipitation (IP) Analysis.

Antibody	**Company, Catalogue Number**	**Host Species**	**Dilution or Concentration used**	**Application**	**Figure (s)**
Akt	Cell Signalling Technology, 9272	Rabbit	1:500	WB	4.8, 4.20
Phospho-Akt (Ser473) (pAkt)	Cell Signalling Technology, 9271	Rabbit	1:500	WB	4.8, 4.20
Erk1 (MAPK)	BD Biosciences, 610030	Mouse	1:500	WB	4.7, 4.19
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (pMAPK)	Cell Signalling Technology, 9101	Rabbit	1:500	WB	4.7, 4.19
FLAG-M2	Sigma-Aldrich, F3165	Mouse	4.9 µg/mL	WB	4.2, 4.3, 4.4, 4.5, 4.14, 4.15, 4.16, 4.17
			4 µg	IP	4.2
GAPDH	Ambion, AM4300	Mouse	0.5 µg/mL	WB	4.3, 4.4, 4.6, 4.16, 4.18
c-Myc (A-14)	Santa Cruz Biotechnology, sc-789	Rabbit	1 µg/mL	WB	4.2
Normal mouse IgG-AC	Santa Cruz Biotechnology, sc-2343	Mouse	4, 5 or 10 µg	IP	4.2
Normal rabbit IgG-AC	Santa Cruz Biotechnology, sc-2345	Rabbit	4, 5 or 10 µg	IP	4.3
p85 (BH)	Anderson, affinity purified (amino acids: 78-332)	Rabbit	1 µg/mL	WB	4.4
PDGFR-β (958)	Santa Cruz Biotechnology, sc432	Rabbit	1 µg/mL	WB	4.3, 4.6, 4.15, 4.16, 4.18
			5 µg	IP	4.3, 4.15
Phospho-Tyr (pY20)	Santa Cruz Biotechnology, sc-508	Mouse	2 µg/mL	WB	4.6, 4.18
PI3K p85 N-SH2	EMD Millipore, 05-217	Mouse	1:100	WB	4.3, 4.15, 4.16
PI3K p85 N-SH3	EMD Millipore, 05-212	Mouse	1:100	WB	4.4

Table 3.4: List of Secondary Antibodies and the Concentration used for Western Blotting (WB) Analysis.

Antibody	**Company, Catalogue Number**	**Host Species**	**Concentration used**
IRDye 680 Goat anti-Mouse IgG [now IRDye® 680RD Goat anti-Mouse IgG, 926-68070]	LI-COR, 926-32220	Goat	0.067 or 0.2 or 0.5 ng/mL
IRDye 680 Goat anti-Rabbit IgG [now IRDye® 680RD Goat anti-Rabbit IgG, 926-68071]	LI-COR, 926-32221	Goat	0.067 or 0.2 or 0.5 ng/mL
IRDye 800CW Goat anti-Mouse IgG	LI-COR, 926-32210	Goat	0.067 or 0.2 or 0.5 ng/mL
IRDye 800CW Goat anti-Rabbit IgG	LI-COR, 926-32211	Goat	0.067 or 0.2 or 0.5 ng/mL

(Thermo Scientific/Fermentas, EP0502) in a 50 µL reaction volume. The thermocycler (Applied Biosystems, 2720 or Veriti Thermal Cycler) was programmed as follows: (i) initial melting: 95 ºC for 1 minute, (ii) 16 cycles of 95 ºC for 30 seconds (melting), 55 ºC for 1 minute (annealing) and 68 ºC for 16 minutes (extension), with (iii) a final extension at 68 ºC for 7 minutes. The amplification reaction products were digested with 20 units of DpnI (New England Biolabs, R0176) for 1 hour at 37 ºC. The entire DpnI digested mutagenesis amplification reaction was transformed into TOP10 chemically competent *E. coli* as described in Section 3.2.1.3.

3.2.1.2 Preparation of competent cells

Five mL of Luria-Bertani Broth Miller (LB, Novagen, 71753-6) [1% (w/v) bacto-tryptone, 0.5% (w/v) bacto-yeast extract and 1% (w/v) sodium chloride (NaCl) pH 7.0 per litre] was inoculated with TOP10 chemically competent *E. coli* cells from a glycerol stock. Cells were incubated overnight at 37 °C with agitation. The entire 5 mL overnight culture was used to inoculate 100 mL of LB and was incubated at 37 °C with agitation until an optical density (O.D.) at 550 nm of 0.4 was attained. The culture was transferred to pre-chilled sterile 50 mL falcon centrifuge tubes and incubated on ice for 15 minutes. Cells were pelleted at 805×*g* for 15 minutes at 4 °C. The supernatant was removed and the bacterial cell pellets were resuspended in 30 mL of ice cold RF1 buffer [100 mM RbCl, 50 mM $MnCl_2$, 30 mM KOAc pH 7.5, 10 mM

$CaCl_2$, 15% (w/v) glycerol]. The cell suspension was incubated for 15 minutes on ice. Cells were pelleted at 805×*g* for 15 minutes at 4 °C. The supernatant was discarded and the pellet was resuspended in 7.2 mL of ice cold RF2 buffer [10 mM MOPS pH 6.8, 10 mM RbCl, 75 mM $CaCl_2$, 15% (w/v) glycerol]. Cells were incubated on ice for 15 minutes. Cells were distributed into 200 µL aliquots, flash frozen in liquid nitrogen and stored at -80 °C.

3.2.1.3 Transformation of competent cells with DNA

After site-directed mutagenesis, TOP10 chemically competent *E. coli* cells (described in Section 3.2.1.2) were transformed with DNA. The reaction mixture was transferred to 200 µL of competent TOP10 cells and incubated on ice for 30 minutes. Cells were heat shocked at 42 ºC for 90 seconds and then placed on ice for 5 minutes after which 800 µL of liquid LB was added to each tube. Cells were incubated at 37 ºC for 30 minutes to allow for expression of the ampicillin resistant gene product. Cells were pelleted at 2,320×*g* and 4 °C for 1 minute. The supernatant was discarded and the cells were resuspended in 50 µL of LB. The cell suspension was spread on the surface of LB-agar plates [1% (w/v) bacto-tryptone, 0.5% (w/v) bacto-yeast extract and 1% (w/v) sodium chloride (NaCl) pH 7.0, 1.5% (w/v) agar (Sigma-Aldrich, A7002) per litre] supplemented with 100 µg/mL ampicillin (LBA agar plates) and incubated at 37 °C overnight. Ten distinct colonies were picked into 5 mL of LB supplemented with 100 µg/mL ampicillin and grown overnight at 37 °C with shaking (250 rpm). Plasmid DNA was isolated with a QIAprep Spin Miniprep Kit (Qiagen, 27106) according to the manufacturer's instructions. Samples were sequenced by the DNA Technologies Laboratory, National Research Council of Canada Plant Biotechnology Institute, University of Saskatchewan to confirm the mutation and verify the integrity of the entire inserted cDNA sequence.

3.2.2 Protein Analysis

3.2.2.1 Cell Lysate Preparation

Cells are harvested in either sodium dodecyl sulfate (SDS) sample buffer [62.5 mM Tris-HCl pH 6.8, 10% (v/v) Glycerol, 5% (v/v) β–mercaptoethanol, 2.3% (w/v) SDS, 0.1% (w/v) bromophenol blue] or phospholipase C (PLC) lysis buffer [50 mM HEPES pH 7.5, 150 mM NaCl, 10% (v/v) Glycerol, 1% (v/v) Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 10 mM NaPPi, 100 mM NaF] depending on the study. SDS sample buffer was used when the sole purpose of the

lysate was for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis. Cell lysates used for immunoprecipitation experiments were prepared with PLC lysis buffer plus protease and phosphatase inhibitors [10 µg/mL aprotinin, 10 µg/mL leupeptin, 1 mM AESBF, 1 mM sodium orthovanadate (Na_3VO_4)].

For lysates prepared with SDS sample buffer, cells were washed with cold phosphate buffered saline (PBS) and SDS sample buffer heated to 100 ºC was added directly to the tissue culture dish. Cells were scraped off the dish and collected into a tube. The cell lysates were sonicated for 10 to 20 seconds on the output setting of 1.5 with a Sonifier (Branson Sonifier 450 UltraSonic Processor Homogenizer Disruptor Sonicator) in order to shear the DNA (reduces the viscosity due to genomic DNA). Cell lysates were sometimes passed through a 25 G needle several times in order to shear the DNA. Lysates were then boiled at 100 ºC for 5 minutes. The protein concentration was determined using a Lowry assay (see below), and samples were loaded on an SDS-PAGE gel immediately or stored at -80 ºC for further analysis.

In order to harvest cells for immunoprecipitation experiments, tissue culture dishes were placed on ice, cells were washed with cold PBS and then lysed with cold PLC lysis buffer plus protease and phosphatase inhibitors. Cells were scraped off the dish and collected into a tube. Cell lysates were centrifuged at 16,100×*g* for 10 minutes at 4 ºC to pellet the insoluble cellular material. The supernatant was transferred to another tube and these pre-cleared lysates were used for immunoprecipitation experiments immediately or stored at -80 ºC. A protein concentration determination was performed by Lowry protein assay using the Total Protein Kit, Micro Lowry, Peterson's Modification (Sigma-Aldrich, TP0300) according to the manufacturer's instructions before cell lysates were used for different assays.

3.2.2.2 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Lysates were prepared as described in Section 3.2.2.1 and proteins were resolved by SDS-PAGE for visualization by Western blot analysis. This discontinuous gel system consists of a lower resolving (or separating gel) and an upper stacking gel sandwiched between two glass plates separated by 1 mm or 1.5 mm using precision spacers. SDS-PAGE experiments were performed using ether the Mini-PROTEAN II Electrophoresis Cell or the Mini-PROTEAN Tetra Cell electrophoresis system (Bio-Rad). Depending on the molecular weight of the proteins to be resolved, the resolving gels contained between 7.5% to 12% acrylamide solution [29.2:0.8

acrylamide: bisacrylamide], 375 mM Tris-HCl pH 8.8 and 0.1% (w/v) SDS. The lower percentage of acrylamide allowed for resolution of larger molecular weight proteins and *vice versa*. Polymerization was initiated by the addition of 0.16% (w/v) ammonium persulfate (APS, Sigma-Aldrich, 248614) and 0.1% (v/v) N,N,N',N'-Tetra-methylethylenediamine (TEMED, Sigma-Aldrich, T22500). The stacking gel contained 4.5% acrylamide solution, 125 mM Tris-HCl pH 6.8 and 0.1% (w/v) SDS. Polymerization of the stacking gel was initiated by the addition of 0.24% (w/v) APS and 0.2% (v/v) TEMED. Lysates containing equal amounts of total protein (determined by the Lowry assay above) were prepared in SDS sample buffer and the protein content denatured by heating to 100 °C for 5 minutes. Samples and prestained molecular weight markers (Fermentas, SM0671 or Fisher Scientific, BP3603-500) were loaded onto the gel and proteins were resolved at a constant voltage of 180 V in running buffer [25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS] until the bromophenol blue tracking dye ran off the bottom of the gel (Laemmli, 1970). The resolved proteins were visualized by Western blot analysis as described in Section 3.2.2.3. The size of each individual resolved proteins was compared to prestained molecular weight markers.

3.2.2.3 Western blot analysis

For Western blot analysis, proteins resolved by SDS-PAGE were transferred to a nitrocellulose membrane using a Panther semi-dry Electroblotter Owl Separation System (VWR, 27372-374 or Fisher Scientific, FB-SDB-2020) or a Trans-Blot Turbo Transfer System (Bio-Rad, 170-4155). Following protein separation by electrophoresis (Section 3.2.2.2), the SDS-PAGE gel was equilibrated in transfer buffer [48 mM Tris, 39 mM glycine, 0.038% (w/v) SDS, 20% (v/v) methanol] for 10 minutes. Components of the transfer system were prepared as follows for each gel: six pieces of 3MM filter paper (Whatman, 3030 700) were soaked in transfer buffer and one piece of nitrocellulose membrane (Whatman, 10401196) was rehydrated in distilled water. In order to transfer the proteins from the gel to the nitrocellulose membrane, a blotting sandwich consisting of three pieces of 3MM filter paper, the SDS-PAGE gel, the nitrocellulose membrane and three more pieces of 3MM filter paper was assembled and placed in the transfer apparatus with the membrane side closer to the anode ⊕. Air bubbles were removed from the layers by rolling a flat tube over the stack. Protein transfer was facilitated by applying a constant current of 400 mA (15 minutes per gel being transferred) to the Panther semi-dry

Electroblotter Owl Separation System or by applying a constant current of 1 A and a voltage of 25 V to the Trans-Blot Turbo Transfer System for 15 – 30 minutes.

After the transfer step, the membrane was incubated in blocking buffer. Fat-free milk is usually used as a blocking agent; however, certain antibodies can bind to the casein in milk-based blocking buffers. In those instances, BSA-based blocking buffers are used. Therefore, two different blocking buffers were used depending on the antibody: (i) 5% (w/v) Carnation fat-free instant skim milk powder in PBS [137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4(7H_2O)$ or Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.3], or (ii) 1% (w/v) BSA fraction V (Sigma-Aldrich, A9418) in PBS. Membranes were blocked for 1 hour at room temperature with gentle rocking or overnight at 4 °C. The membrane was then incubated with primary antibody made up in blocking buffer, plus 0.05% (v/v) Tween-20, overnight at 4 °C with nutation (see Table 3.3 for primary antibodies and their working concentrations). Following primary antibody incubation, the membrane was washed three times in PBST [PBS plus 0.05% (v/v) Tween-20] for 10 minutes per wash. The membrane was then incubated with a secondary antibody conjugated to an infrared-labelled dye (IRDye680 or IRDye800 for LI-COR Odyssey infrared imaging) made up in 5% (w/v) Carnation fat free instant skim milk powder in PBST for 1 hour at room temperature with nutation (see Table 3.4 for secondary antibodies and their working concentrations) and washed as described above.

For detection of proteins using infrared-labeled dyes, the membrane was washed three times in PBST and twice in PBS to remove Tween-20 (the presence of Tween-20 on the infrared scanner increases the amount of background on the membrane). The membrane was scanned using the Mandel LI-COR Odyssey Classic Infrared Imager (LI-COR, ODY-1079) and analyzed with the Odyssey V3.0 software.

It should be noted that, for Western blot analysis of samples that were compared with each other, multiple gels containing the samples were resolved together, transferred, probed and scanned side-by-side to ensure that they were treated as similar as possible.

3.2.3 Cell Culture Techniques

3.2.3.1 Mammalian Cell lines

NIH 3T3 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, Gibco, 12100-061) supplemented with 10% fetal bovine serum (FBS, Gibco, 16250-078) and grown at

37 °C in a 5% CO_2 humidified air environment. NIH 3T3 cells stably transfected with pFLAG3 vector as well as pFLAG3 plasmids containing p85wt, p85R274A, p85Δ110, p85Δ110+R274A, p85ΔR or p85ΔR+R274A cDNA were grown in selection medium containing DMEM supplemented with 10% FBS and 400 µg/mL Geneticin (G418 Sulfate, Gibco 11811-031) at 37 °C in a 5% CO_2 humidified air environment. Geneticin disrupts protein synthesis by inhibiting the elongation step (Bar-Nun et al., 1983). COS-1 cells were transiently transfected with pFLAG3 plasmids containing p85wt, p85R274A, p85Δ110, p85Δ110+R274A, p85ΔR or p85ΔR+R274A cDNA and/or pMyc3-p110 and grown in DMEM supplemented with 10% FBS at 37 °C in a 5% CO_2 humidified air environment. Cells were fed every 3 to 4 days. Cells were passaged at 80 to 90% confluency by detaching with 0.05% Trypsin-EDTA (Gibco, 15400-054).

3.2.3.2 Transient Transfection

COS-1 cells as well as NIH 3T3 cells were transiently transfected with the empty pFLAG3 plasmid vector or pFLAG3 plasmids containing p85wt, p85R274A (substitution mutation that abolishes p85 RabGAP activity), p85Δ110 (deletion mutation that prevents p85 from binding to p110), p85Δ110+R274A (deletion and substitution mutations that prevents p85 from binding to p110 and abolishes p85 RabGAP activity), p85ΔR (substitution mutations that prevent binding to tyrosine phosphorylated PDGFR) or p85ΔR+R274A (substitution mutations that prevent binding to tyrosine phosphorylated PDGFR and abolishes p85 RabGAP activity) cDNA (Chamberlain *et al*., 2004; King *et al*., 2000). One million (1×10^6) cells were seeded onto a 10 cm tissue culture dish and cultured at 37 °C in a 5% CO_2 humidified air environment for 24 hours. For each 10 cm dish to be transfected the following solutions were prepared: solution A [6 µg plasmid DNA, 600 µL Opti-MEM reduced serum medium (Gibco, 31985-070)] and solution B [600 µL Opti-MEM reduced serum medium, 18 µL Lipofectamine Transfection Reagent (Invitrogen, 18324-020)]. Solutions A and B were combined and incubated for 15 minutes at room temperature after which 4.8 mL serum-free DMEM was added to the mixture. The transfection mixture was added to cells that had been previously washed with serum-free DMEM. The cells were incubated for 5 hours at 37 °C. Six (6) mL of DMEM supplemented with 20% FBS was added to the transfection medium and the cells were incubated overnight at 37 °C. The medium was then changed to DMEM supplemented with 10% FBS and cells were left to grow for 24 to 72 hours. Cells were used for growth factor stimulation studies (Section 3.2.3.4),

adhesion assay (Section 3.2.3.8), or harvested for immunoprecipitation experiments (Section 3.2.3.5) or Western blot analysis (Section 3.2.2.3).

3.2.3.3 Stable transfection

Three hundred and fifty thousand (3.5×10^5) NIH 3T3 cells were seeded onto a 6 cm tissue culture dish and cultured at 37 °C for 24 hours. Plasmids encoding the pFLAG3 vector or pFLAG3-p85Δ110+R274A or pFLAG3-p85ΔR+R274A were transfected into NIH 3T3 cells. For each 6 cm dish to be transfected the following solutions were prepared: solution A [0.6 µg plasmid DNA, 300 µL Opti-MEM reduced serum medium] and solution B [300 µL Opti-MEM reduced serum medium, 10 µL Lipofectamine Transfection Reagent]. Solutions A and B were combined and incubated for 15 minutes at room temperature after which 2.4 mL serum-free DMEM was added to the mixture. The transfection mixture was added to cells that had been previously washed with serum-free DMEM. The cells were incubated for 5 hours at 37 °C. Three (3) mL of DMEM supplemented with 20% FBS was added to the transfection medium and the cells were incubated overnight at 37 °C. The medium was then changed to DMEM supplemented with 10% FBS and cells were left to grow until 90% confluency was observed.

Cells were trypsinized and resuspended in selection medium containing DMEM supplemented with 10% FBS and 400 µg/mL Geneticin. Cells were seeded onto 10 × 10 cm tissue culture dishes per 6 cm dish transfected and cultured in selection medium for up to 10 days until distinct colonies of drug resistant cells were seen. Twenty four (24) distinct colonies were picked from the 10 cm tissue culture dishes and transferred to 2 × 12-well tissue culture dishes. The rest of the colonies left on the 10 × 10 cm tissue culture dishes were pooled together, cultured on 10 cm dishes and referred to as the mixed population. Cells were cultured in selection medium until they were about 80% confluent. Each clonal isolate was trypsinized, resuspended in selection medium and seeded onto a 6-well tissue culture dish (to be lysed and analyzed for protein expression using a Western blot analysis) and a 12-well tissue culture dish (maintained as a stock plate).

Cells in the 6-well dishes were cultured in selection medium until they were about 90% confluent. Cells were lysed in sodium dodecyl sulfate (SDS) sample buffer as described in Section 3.2.2.1. Protein concentration was determined using the Total Protein Kit, Micro Lowry, Peterson's Modification (Sigma-Aldrich, TP0300) according to the manufacturer's instructions.

Proteins were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis as described in Section 3.2.2.3.

Clonal isolates expressing different amounts of the overexpressed protein were transferred from the 12-well stock dish onto a 6 cm dish and maintained in selection medium until they were about 90% confluent. Cells were then trypsinized, resuspended in selection medium and seeded onto 2 × 10 cm tissue culture dishes. Cells were further expanded, frozen down as stocks and used for the desired experiments.

3.2.3.4 Growth Factor Stimulation

In order to measure the rate of degradation of the PDGFR and activation of downstream signalling pathways events in response to PDGF treatment, a time-course experiment involving growth factor stimulation was performed using NIH 3T3 cells either transiently expressing p85 wild type or p85 mutants or NIH 3T3 cells stably expressing p85 wild type or p85 mutants. Cells were grown as described in Section 3.2.3.1 to 80% confluency. The cells were serum deprived for 12 to 18 hours in DMEM containing 0.5% FBS. The cells were then treated with 50 ng/mL of rhPDGF-BB (R&D Systems, 220-BB) for 0, 10, 30, 60, 120 or 240 minutes at 37 °C. Cells were harvested by placing tissue culture dishes on ice, washing with cold phosphate buffered saline (PBS) and lysing with SDS sample buffer or PLC lysis buffer as described in Section 3.2.2.1.

3.2.3.5 Immunoprecipitation

3.2.3.5.1 Anti-FLAG immunoprecipitation

COS-1 cells were transiently co-transfected with plasmids expressing the different p85 mutants and Myc-p110 as described in Section 3.2.3.2. Lysates were collected three days after transfection in cold PLC lysis buffer containing protease and phosphatase inhibitors. The protein concentrations of lysates were determined by the Lowry assay. An anti-FLAG immunoprecipitation was carried out using 125 µg of total cell protein. The volume of lysate equivalent to 125 µg of total protein was pre-cleared with 4 µg of normal mouse IgG-agarose conjugate (Santa Cruz Biotechnology, sc-2343) and 30 µL of a 50% slurry of protein G-agarose beads (Sigma-Aldrich, P7700) for 1 hour at 4 °C with nutation. This removed any proteins that interacted non-specifically with the mouse antibody. The agarose beads were pelleted at 18,188×*g* and 4 °C for 10 minutes. The pre-cleared supernatant was transferred to a new tube

and the pre-clear step was repeated as before. The resulting pre-cleared supernatant was transferred to a new tube and proteins of interest were immunoprecipitated with 4 µg of anti-FLAG M2 antibody (Sigma-Aldrich, F3165) and 30 µL of a 50% slurry of protein G-agarose beads. Another set of samples were incubated with 4 µg of normal mouse IgG-agarose conjugate and 30 µL of a 50% slurry of protein G-agarose beads. These samples served to demonstrate that any non-specific immunoprecipitation/interaction with antibody or agarose beads had been mitigated. All samples were incubated for 90 minutes at 4 °C with nutation. Beads were then pelleted at 2,320×*g* and 4 °C for 2 minutes. The supernatant was discarded and samples were washed in 1 mL HNTG buffer (20 mM HEPES pH 7.5, 150, mM NaCl, 0.1% Triton X-100, 10% glycerol and 1 mM Na_3VO_4) and centrifuged at 2320×*g* and 4 °C for 2 minutes. The washes and centrifugation were repeated three times to remove residual supernatant. The samples were either assayed for PI3K activity (Section 3.2.5.3) or used for Western blot analysis (Section 3.2.4.3). For Western blot analysis, the supernatant was completely removed from the beads and 20 µL of 2× SDS sample buffer was added to the pelleted beads. The samples were boiled for 5 minutes (to denature and dissociate the proteins from the beads) and centrifuged at 18,188×*g* and 4 °C for 1 minute (to pellet the dissociated agarose beads). The supernatant was loaded immediately on SDS-PAGE gels or stored at -20 °C for later analysis.

3.2.3.5.2 Anti-PDGFR immunoprecipitation

To determine whether p85 wild type or p85 mutants associate with the PDGFR and to measure the duration of any such association an anti-PDGFR immunoprecipitation was performed. NIH 3T3 cells stably expressing either p85 wild type or the individual p85 mutants were treated with growth factor (PDGF) for 0, 10, 30, 60, 120 or 240 minutes (Section 3.2.3.4) and lysed in PLC lysis buffer containing protease and phosphatase inhibitors. The protein concentrations of lysates were determined by the Lowry assay. An anti-PDGFR immunoprecipitation was carried out using 125 µg of protein. The volume of lysate equivalent to 125 µg of total protein was pre-cleared with 5 µg of normal rabbit IgG-agarose conjugate (Santa Cruz Biotechnology, sc-2345) and 30 µL of a 50% slurry of protein G-Sepharose 4B Fast Flow beads (Sigma-Aldrich, P3296) for 30 minutes at 4 °C with nutation. The beads were pelleted at 18,188×*g* and 4 °C for 10 minutes. The supernatant was transferred to a new tube and the pre-clear step was repeated as before. The supernatant was transferred to a new tube and proteins

were immunoprecipitated with 5 µg of anti-PDGFR-β (958) antibody (Santa Cruz Biotechnology, sc-432) and 30 µL of a 50% slurry of protein G-Sepharose 4B Fast Flow beads. A parallel set of samples was incubated with 5 µg of normal rabbit IgG-agarose conjugate and 30 µL of a 50% slurry of protein G-Sepharose 4B Fast Flow beads. These samples served as a control used to demonstrate specificity of the immunoprecipitation in the anti-PDFGR set of immunoprecipitated samples. All samples were incubated for 90 minutes at 4 °C with nutation. Beads were then pelleted at 2,320×*g* and 4 °C for 2 minutes. The supernatant was discarded and samples were washed in 1 mL HNTG buffer and centrifuged at 2,320×*g* and 4 °C for 2 minutes. The washes and centrifugation were repeated three times. The supernatant was completely removed from the beads and 20 µL of 2× SDS sample buffer was added. The samples were boiled for 5 minutes, centrifuged at 18,188×*g* (4 °C for 1 minute) and the supernatant loaded immediately on SDS-PAGE gels or stored at -20 °C until needed.

3.2.3.5.3 Anti-Rab5-GTP IP

To determine the levels of Rab5-GTP in NIH 3T3 cells stably expressing p85 wild type or the individual p85 mutants, an immunoprecipitation experiment was carried out using activation-specific Rab5 antibodies based on the manufacturer's protocol. The Rab5 activation assay from New East Biosciences required a preliminary optimization. For this, NIH 3T3 cells were treated with PDGF (Section 3.2.6.4) for 2 or 30 minutes and lysed in Rab5 activation assay Assay/Lysis buffer [50 mM Tris-HCl pH 8, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 1% Triton X-100]. The lysates collected were used immediately for the assay since the activation of Rab5 is a transient event. Either 1 mg total protein of NIH 3T3 cell lysate, or 1 or 5 µg of purified Rab5 wild type was used for the assay. Samples equivalent to the appropriate amount of protein were dispensed into tubes and Assay/Lysis buffer was added to a total volume of 500 µL. The negative and positive controls were set up as follows: the negative control tube had GDP (Sigma-Aldrich, G-7127) added to it while the positive control tube contained GTPγS (Sigma-Aldrich, G-8634).

In order to strip Rab5 of nucleotides, EDTA was added to the negative and positive control samples to a final concentration of 20 mM. The nucleotides were loaded onto Rab5 by adding GDP to the negative control tube to a final concentration of 1 mM and GTPγS to the positive control tube to a final concentration of 100 µM. Samples were incubated at 30 °C for 30

minutes with agitation. The nucleotide loading reaction was terminated by placing tubes on ice and adding $MgCl_2$ to a final concentration of 60 mM. From here on, the control samples as well as the lysates and purified proteins were subjected to the same procedure. The volume of the samples was adjusted to 1 mL with Assay/Lysis buffer. One (1) µg of anti-Active Rab5 mouse monoclonal antibody (New East Biosciences, 26911) and 20 µL of a 50% slurry of protein G-agarose beads was added to all samples. Samples were incubated at 4 °C for 1 hour with nutation. Beads were pelleted at 2,320×*g* and 4 °C for 2 minutes. The supernatant was discarded and the beads were washed three times with 1 mL of Assay/Lysis buffer. The supernatant was completely removed from the beads and 20 µL of 2× SDS sample buffer was added. The samples were boiled for 5 minutes, centrifuged at 18,188×*g* (4 °C for 1 minute) and the supernatant loaded immediately on SDS-PAGE gels or stored at -20 °C. It can be observed from the optimization results depicted in Figure 3.4 that the anti-Active Rab5 antibody that was supposed to specifically immunoprecipitate Rab5-GTP is also immunoprecipitating GDP loaded Rab5. Therefore, the Rab5 activation assay from New East Biosciences could not be used to measure the levels of Rab5-GTP in NIH 3T3 cells stably expressing p85 wild type or p85 mutants.

3.2.3.6 Foci formation assay

Proliferating normal cells are inhibited by contact with neighbouring cells, ensuring controlled regulation of cellular processes. However, transformed (oncogenic) cells are not inhibited and continue to grow, often piling on top of each other to form clumps or foci. To assess whether the stable expression of wild type p85 or the different p85 mutants could affect contact inhibition, a foci formation assay was carried out. The stable NIH 3T3 clones were seeded at a density of 1×10^5 cells/10 cm dish and maintained in normal medium for two weeks at 37°C. The medium was changed every three to four days. After two weeks, the medium was removed and the cells were fixed *in situ* in –20 °C methanol for at least 1 hour and allowed to air dry. Cells were stained with Giemsa stain (Sigma-Aldrich, GS1L) overnight with gentle rocking. Giemsa stains the nuclei blue/violet by binding to DNA (Horobin, 2011). Cells were washed with water until the excess unbound stain was completely removed. Photographs of each entire plate were taken with a Nikon D3100 digital camera (Nikon). The results shown were obtained from three independent experiments (i.e. three separate series of plated cells) with each independent experiment being performed in duplicate.

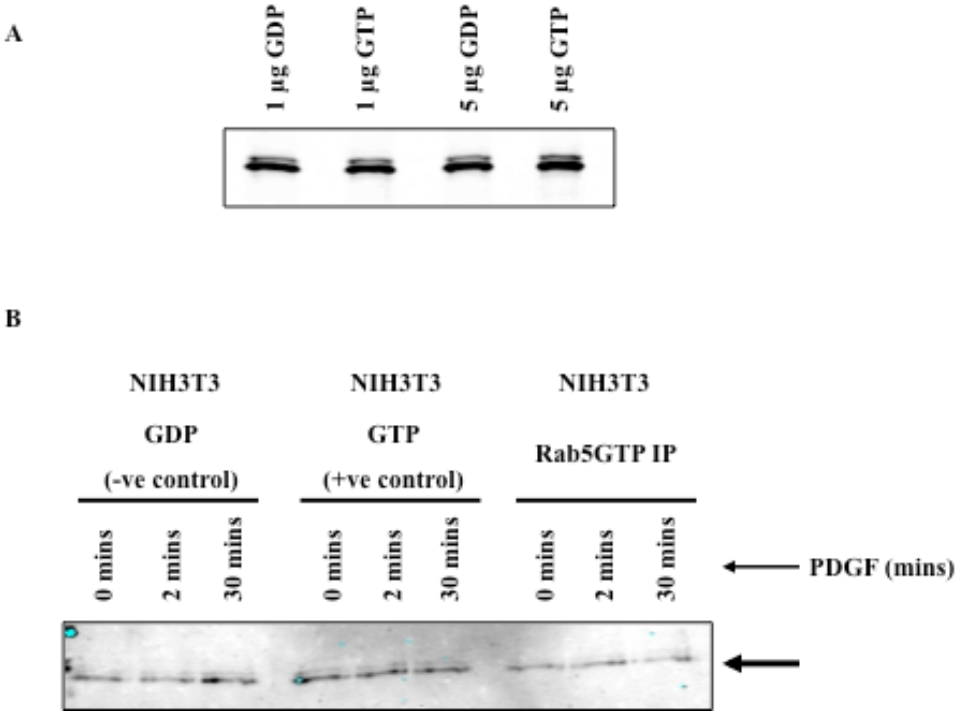

Figure 3.4: Optimization of Rab5GTP antibody. A) Different amounts of purified Rab5 wild type (1 µg, 5 µg) were loaded with either GDP or GTP. One (1) µg of RabGTP antibody was used to immunoprecipitate Rab5 for 1 hour at 4 °C. Samples were resolved and immunoblotted with an anti-Rab5 antibody. B) Cells were serum starved (DMEM supplemented with 0.5% FBS) for 48 hours and then left unstimulated or stimulated with 50 ng/mL PDGF-BB for 0, 2 and 30 minutes. One (1) µg of RabGTP antibody was used to immunoprecipitate Rab5 for 1 hour at 4 °C. Samples were resolved and immunoblotted with an anti-Rab5 antibody. This figure is a representation of data obtained from four independent experiments.

3.2.3.7 Colony formation in soft agar assay

The soft agar colony formation assay is a method routinely used to monitor anchorage-independent growth (a feature of transformed cells). To measure anchorage independent growth, a colony formation assay was performed in which cells stably expressing wild type p85 or the individual p85 mutants were grown in soft agar. Fifty thousand (5×10^4) cells/ 6 cm dish were cultured in a suspension of top agar (DMEM, 10% FBS, 3.75x MEM vitamin solution (Gibco Invitrogen, 11120-052), 0.36% agar (Sigma-Aldrich, A-7002)) above of a layer of bottom agar (DMEM, 10% FBS, 5x MEM vitamin solution, 0.61% agar). Cells were left to grow for a total of

20 days at 37°C. Cells were fed every four days by adding 2 mL of top agar to the plate. Colony formation was determined by the ability of individual cells to divide and ultimately form colony over time. Colonies often had uneven edges with smaller satellite colonies growing around their circumference. Photographs of colonies formed were captured either under phase contrast using a Nikon D3100 digital camera attached to a Nikon Eclipse TE300 microscope; or an Olympus IX51 microscope with the Infinity 3 software. Colonies that were greater than 17 μm in diameter, as measured using a grid and the Infinity 3 software, were considered true colonies and were counted. This value was determined by the size of colonies measured in control NIH 3T3 cells and cells expressing the FLAG empty vector plasmid. The results shown were obtained from two independent experiments with duplicate measurements per experiment.

3.2.3.8 Adhesion assay

Attachment to extracellular matrix is required for the survival and proliferation of normal cells. However, this can change in transformed cells and can often contribute to their ability to invade and grow in other tissues. Changes in adhesion often reflect changes in "anchorage independent" growth as tested in Section 3.2.3.7 above. An adhesion assay was performed to quantify the ability of cells to attach to different extracellular matrix proteins. Ninety-six (96)-well tissue-culture-treated microtiter plates were either left uncoated or coated with 50 ng/mL of either Fibronectin, human plasma (Calbiochem, 341635) or Collagen I, rat tail (Gibco Invitrogen, A10483-01) for 1 hour at 37°C. Each well was washed twice with 100 μL washing buffer (DMEM, 10% FBS, 0.1% BSA fraction V (Sigma-Aldrich, A9418)) and then blocked with 100 μL blocking buffer (DMEM, 10% FBS, 0.5% BSA fraction V) per well for 1 hour at 37°C. NIH 3T3 cells stably expressing wild type p85 or different p85 mutants were detached with 0.05% trypsin/EDTA/PBS (Gibco Invitrogen, 15400-054) for as short a time as possible to prevent digestion of proteins involved in the cell adhesion process and resuspended in DMEM supplemented with 10% FBS.

After the blocking step, wells were washed once with 100 μL washing buffer. Twenty thousand (2×10^4) cells were seeded per well and incubated for 30 minutes at 37°C to allow cells to attach. Wells were washed three times with 100 μL washing buffer to remove any cells that had not attached. Attached cells were then fixed with 100 μL 4% paraformaldehyde/PBS at room temperature for 15 minutes. Cells were washed once with 100 μL washing buffer and stained

with 100 µL crystal violet (5 mg/mL in 2% ethanol) (Sigma-Aldrich, C3886) for 10 minutes. Cells were washed 8 times with distilled water and the plate was left to air dry for at least 1 hour. Cells were lysed with 100 µL 2% SDS for 30 minutes with agitation to solubilize stain and mix evenly. Absorbance readings were measured at 550 nm on a microplate reader (Molecular Devices, SpectraMax M5 Multi-Mode Microplate Reader) as an indication of the cells' ability to adhere. The absorbance varied as a function of the amount of stain (i.e. the number of cells) that had remained attached to the surfaces coated with fibronectin or collagen. The results shown were obtained from two independent experiments with triplicate measurements per experiment.

3.2.3.9 Migration assay

In order to study the cells' ability to migrate towards a chemoattractant stimulus, a migration assay was carried out. The assay was based on a modification of the Boyden chamber, which consists of two chambers separated by a membrane with pores to allow for the passage of cells. The cells migrate from one chamber to another in response to a chemoattractant stimulus, e.g. along a concentration gradient (Boyden, 1962; Chen, 2005). The membrane was a transparent, polyethylene terephthalate (PET) membrane insert with a pore size of 8 µm (Falcon, 353097) supported by a 24-well cell culture insert companion plate (Falcon, 353504).

Cells were resuspended in DMEM supplemented with 0.1% FBS and 1×10^5 cells were added to the upper chamber of the membrane inserts. The lower compartment was filled with 1 mL of either DMEM supplemented with 0.1% FBS as control or DMEM supplemented with 10% FBS (the chemoattractant stimulus). Air bubbles were removed when the inserts were placed in the 24-well companion plate, as air bubbles at the interface between the upper and lower chambers can block the pores and prevent cell migration towards the chemoattractant stimulus. Cells were incubated at 37 °C for 24 hours. After cells had migrated for 24 hours, the media was removed from both chambers and the upper surface of the membrane was gently wiped with cotton swab dipped in serum-free DMEM to remove non-migratory cells. Cells that had migrated to the lower surface of the membrane were fixed in methanol at – 20 ºC overnight.

The membrane inserts were washed once in distilled water and the cells were stained with hematoxylin for 10 minutes. The membrane inserts were then immersed in tap water for 10 minutes. Membranes were dehydrated through increasing ethanol concentrations of 50%, 75%, 90% and 100% for 1 minute each. The membrane inserts were air dried for 1 hour. The

membrane was removed from the housing by cutting it out with a No.11 surgical scalpel blade. The membrane was immediately mounted on a microscope slide using a dibutylphthalate xylene (DPX) synthetic resin mountant. The number of cells that migrated to the lower chamber was quantified by counting eight random fields of view using the Stereo Investigator software (MBF Bioscience) at a magnification of 20X. The results shown were obtained from duplicate wells (each with an individual NIH 3T3 clone).

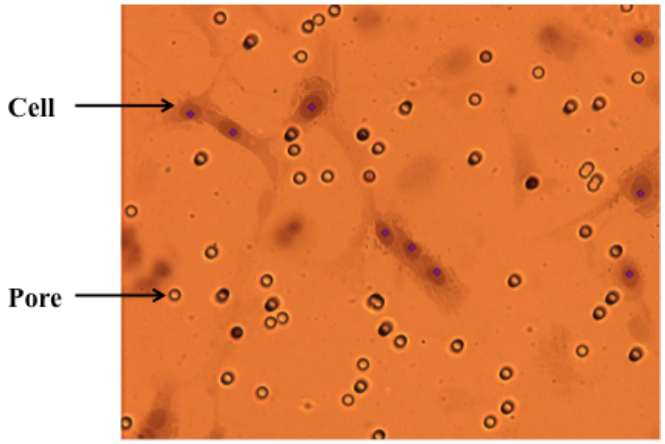

Figure 3.5: Picture of membrane insert with migrated cells viewed under an optical microscope. Cells that migrated to the lower chamber were fixed and stained. The Stereo Investigator software (MBF Bioscience) was used to quantify migrated cells. The photograph represents a typical field of view. Purple diamonds (a marker provided by the software) can be seen within the nuclei of cells that had fully migrated and identify those cells that were used for quantification.

3.2.4 PI3K activity assay

In order to determine whether the disruption of p85-p110 binding or p85-PDGFR interaction affected PI3K catalytic activity, a PI3K assay was carried out. COS-1 cells were transiently co-transfected with plasmids expressing the different FLAG-p85 mutants and Myc-p110 as described in Section 3.2.3.2. Lysates were collected three days after transfection in PLC

lysis buffer. The protein concentrations of lysates were determined by the Lowry assay. An anti-FLAG immunoprecipitation was carried out using 125 μg of protein as described in Section 3.2.3.5.1, after which samples were assayed for PI3K activity using 2.5 μg phosphatidylinositol (PI) as a lipid substrate and 10 μCi [γ-^{32}P]ATP (Perkin-Elmer, BLU502A). Reaction products were spotted onto a thin layer chromatography plate, developed in a chromatography chamber, and exposed to a phosphorimager screen as described below (Chamberlain *et al*., 2004). Anti-FLAG immunoprecipitates were washed twice with each of the following: wash 1 (phosphate buffered saline), 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 completely aspirated from the immunoprecipitate samples.

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 minutes. Each sample was incubated with lipid micelles (5 μg phosphatidylserine + 2.5 μg phosphatidylinositol) in PI3K assay buffer and 10 μCi [γ-^{32}P]ATP in a total volume of 50 μL for 15 minutes at 20°C with gentle agitation. The reaction was terminated by acidification with HCl (to a final concentration of 1.7 M). Lipids were extracted with chloroform:methanol (1:1) and washed with methanol:1 N HCl (1:1). The reaction products were dried down, resuspended in chloroform:methanol (1:1) and spotted onto a 20 x 20 cm Silica Gel 60Å TLC plate (VWR Canlab-BDH, Whatman 4865-821). Samples were developed in 1-propanol:water:acetic acid (17.4:7.9:1) in a chromatography chamber for four 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 Statistical Analysis

Data were analyzed and graphed with Prism software (GraphPad Software, Inc.). Unless otherwise noted, data was analyzed by Analysis of Variance (ANOVA) and significance was set at $p < 0.05$. *Post hoc* statistical analysis between experimental groups relied on Bonferroni's multiple comparison test. Data are presented as mean ± standard error of the mean (SEM).

4.0 RESULTS

4.1 Functional analysis of expression plasmids coding for p85 mutants tagged with a triple FLAG epitope

In order to examine how altering the RabGAP activity of p85, and/or its ability to bind either the p110 subunit or PDGFR, affects receptor signalling and trafficking events as well as cell behaviour (e.g. cell proliferation and survival), plasmids encoding different mutant forms of p85α were generated. The plasmid constructs were based on the pFLAG3 vector. The empty pFLAG3 was used as the vector control. The various p85α cDNA include (Figure 4.1):

- pFLAG3 (vector control)
- pFLAG3-p85 wild type
- pFLAG3-p85R274A (amino acid substitution of alanine for arginine, at position 274; lacks GAP activity towards Rab5 and Rab4 involved in receptor trafficking)
- pFLAG3-p85Δ110 (deletion of amino acids 478-513; prevents p85 from binding to p110)
- **pFLAG3-p85Δ110+R274A** (deletion of amino acids 478-513 plus amino acid substitution of alanine for arginine at position 274; lacks GAP activity and is unable to bind to p110)
- pFLAG3-p85ΔR (2 amino acid substitutions of alanine for arginine, at positions 358 and 649; prevents binding to tyrosine phosphorylated PDGFR)
- **pFLAG3-p85ΔR+R274A** (3 amino acid substitutions of alanine for arginine, at positions 358, 649 and 274; lacks GAP activity and is unable to bind to tyrosine phosphorylated PDGFR)

The p85α cDNA highlighted in bold above were generated during this project. All others were previously generated. In addition to the p85 constructs a triple Myc-tagged p110α plasmid was previously generated. Due to a lack of good quality p110 antibodies, the Myc tag was necessary for monitoring the interactions between the p85 proteins and p110. All plasmids were verified by sequencing and tested for protein expression by transient co-transfections into COS-1 cells and Western blot analysis using anti-Myc (exogenous Myc-p110) and anti-FLAG (exogenous FLAG-p85) antibodies (Figure 4.2A). COS-1 cells were used for these experiments since they are easy to transfect and are therefore an appropriate cell line to test if plasmids encoding proteins of interest are being expressed. Cells were co-transfected with plasmids

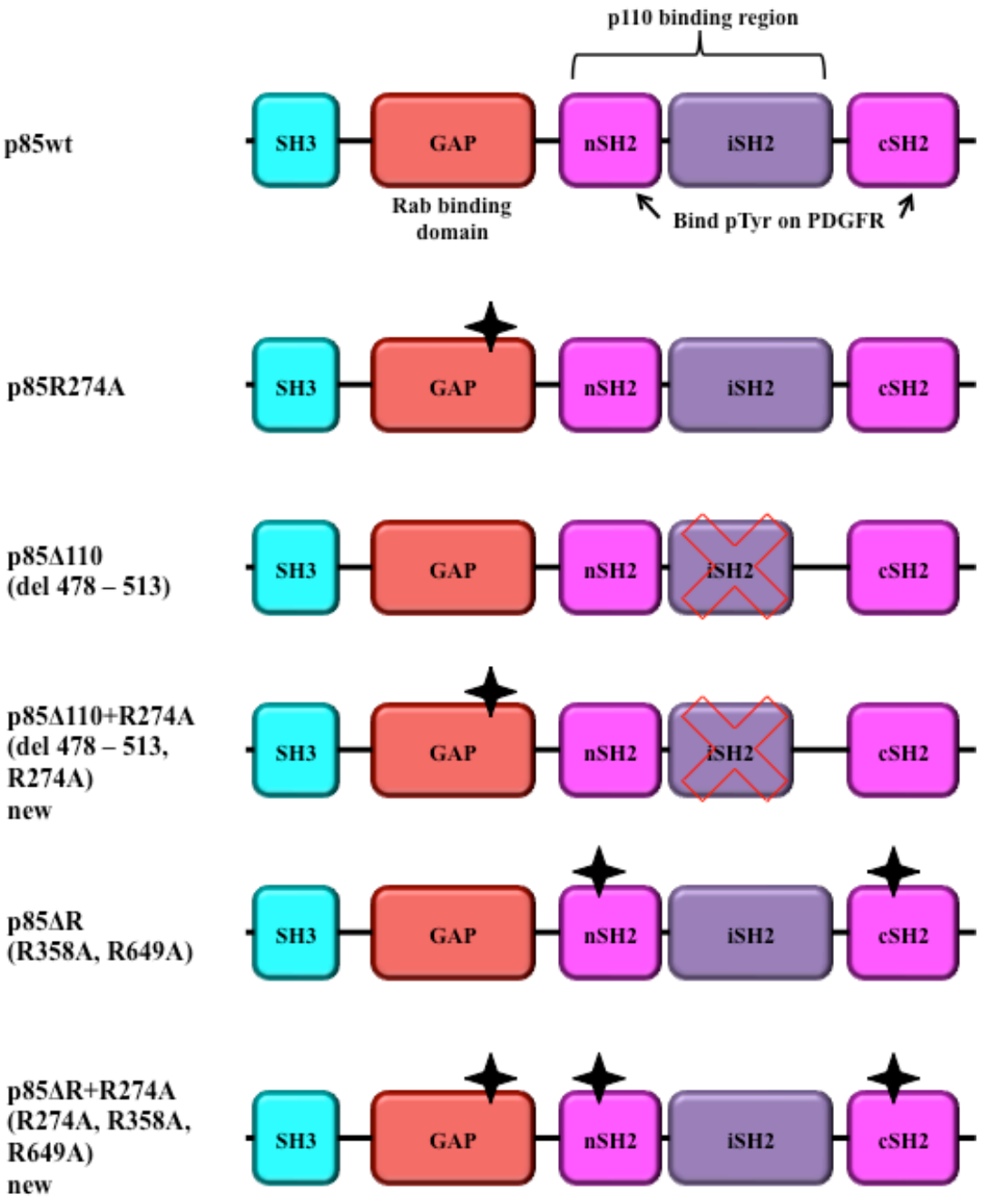

Figure 4.1: Schematic representation of the pFLAG3 plasmids expressing the different p85 mutants. The different domains of p85 are represented by different shapes and colours and labelled accordingly. p85 binds to phosphotyrosine (pTyr) residues on the PDGFR *via* the SH2 domains. The black stars indicate the site of the amino acid substitution of alanine (A) for arginine (R). The two new plasmids that were generated for this project are indicated. The shorter purple iSH2 domain represents the truncated iSH2 domain (i.e. deletion of aa478-513). The remaining plasmids and stable cell lines expressing them were previously described (Chagpar *et al*., 2010; Chamberlain *et al*., 2004).

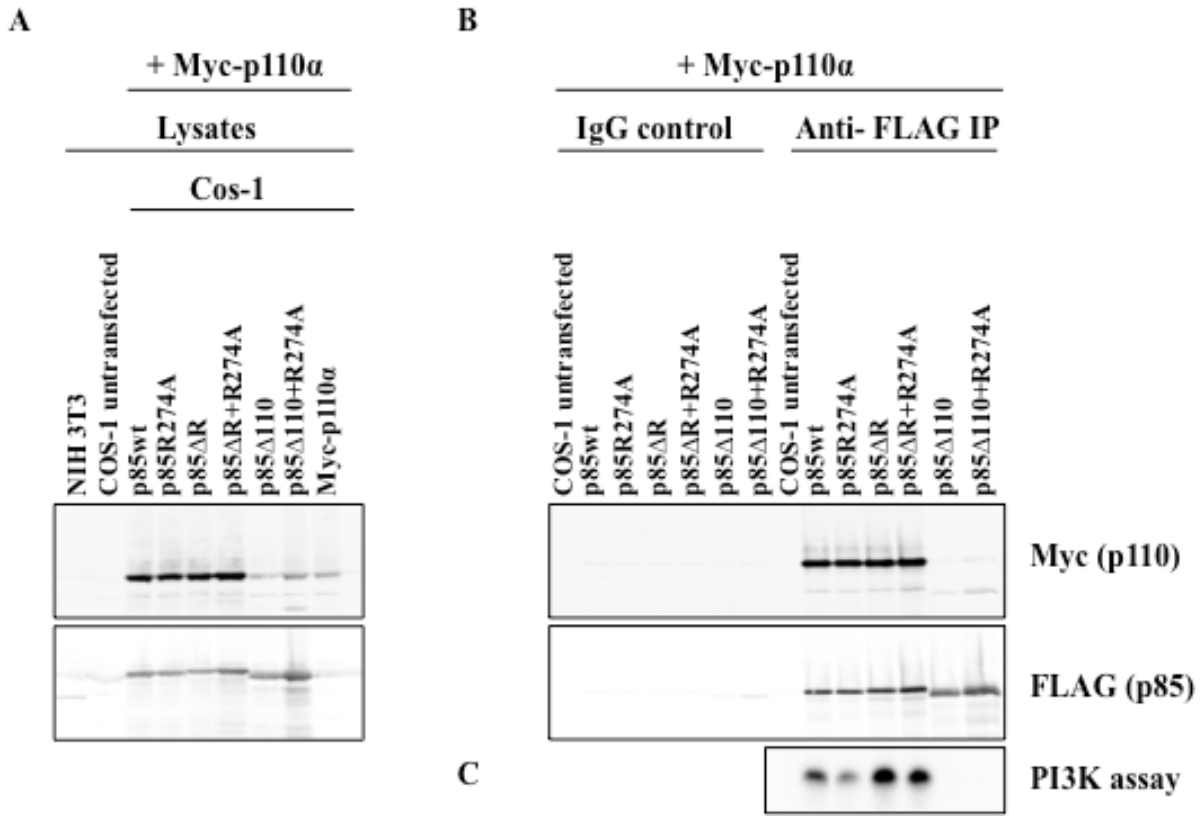

Figure 4.2: p85Δ110 and p85Δ110+R274A are unable to bind to p110 and when immunoprecipitated do not contain PI3K activity. A) COS-1 cells were transiently co-transfected with the indicated plasmids. Lysates were collected 3 days after transfection in SDS sample buffer for whole cell lysates. Proteins (25 μg) were resolved by SDS-PAGE. Untransfected COS-1 and NIH 3T3 whole cell lysates were used as negative controls. Proteins were resolved on an SDS-PAGE gel and probed with anti-Myc and anti-FLAG antibodies. B) Anti-FLAG immunoprecipitation (IP): COS-1 cells were transiently co-transfected with the indicated plasmids. Lysates were collected 3 days after transfection in PLC lysis buffer. An anti-FLAG immunoprecipitation was carried out using 125 μg of protein, after which proteins were resolved on an SDS-PAGE gel and probed with anti-Myc and anti-FLAG antibodies. A lack of co-immunoprecipitation with normal mouse IgG antibody confirms the specificity of the anti-FLAG IP. C) PI3K assay: Samples obtained after an anti-FLAG IP were assayed for PI3K activity using PI as a lipid substrate and [γ-^{32}P]ATP. The TLC plate was exposed to the phosphorimager screen for 20 min. Similar results were obtained in three independent experiments.

encoding either FLAG-p85 wild type (wt) or FLAG-p85 mutants and Myc-p110. Figure 4.2A indicates that the proteins are being expressed at the expected molecular weights and are immunoreactive. p85Δ110 and p85Δ110+R274A have a slightly lower molecular weight due to the deletion of 35 amino acids (478 to 513). In all replicates of the transient transfections performed, there was very low expression of Myc-p110 in cells expressing Myc-p110 alone or cells expressing Myc-p110 co-transfected with p85Δ110 or p85Δ110+R274A. This could be due to the fact that interaction between p85 and p110 is necessary for protecting p110 from degradation.

4.1.1 p85Δ110 and p85Δ110+R274A do not bind p110

The p85Δ110 proteins were tested for their ability to bind p110. COS-1 cells were transiently co-transfected with plasmids encoding either FLAG-p85wt or the different FLAG-p85 mutants and Myc-p110. Lysates were collected three days after transfection in PLC lysis buffer. The protein concentrations of lysates were determined after which an anti-FLAG immunoprecipitation was carried out using 125 μg of protein. The immunoprecipitates were resolved by SDS-PAGE followed by Western blot analysis using anti-Myc (exogenous p110) and anti-FLAG (exogenous p85) antibodies (Figure 4.2B). Consistent with the hypothesis, cells expressing p85Δ110 and p85Δ110+R274A were unable to bind p110, whereas the rest of the p85 mutants could bind p110 (Figure 4.2B). A parallel series of lysates were immunoprecipitated with normal mouse IgG antibody. The lack of any detectable p110 demonstrated the specificity of the anti-FLAG immunoprecipitation.

4.1.2 p85Δ110 and p85Δ110+R274A do not associate with PI3K activity

In order to determine whether disruption of p85-p110 binding or p85-PDGFR interaction affected PI3K activity, a PI3K assay was carried out. COS-1 cells were transiently co-transfected with plasmids encoding FLAG-p85wt or the different FLAG-p85 mutants and Myc-p110. An anti-FLAG IP was carried out as described in Section 4.1.1, after which samples were assayed for PI3K activity using 2.5 μg phosphatidylinositol (PI) as lipid substrate and 10 μCi [γ-^{32}P]ATP. Reaction products were resolved on a thin layer chromatography plate and exposed to a phosphorimager screen (Figure 4.2C). The results of the PI3K assay confirm that the p85Δ110 or p85Δ110+R274A proteins are devoid of any p85-associated PI3K activity (given that they are

unable to bind p110), whereas the other forms of p85 are associated with PI3K activity (given that their ability to co-immunoprecipitate with p110 is not affected) (Figure 4.2C).

4.1.3 p85ΔR and p85ΔR+R274A do not interact with PDGFR in response to growth factor treatment

To confirm that p85ΔR and p85ΔR+R274A are unable to bind activated receptor, NIH 3T3 cells were transiently transfected with FLAG-p85wt or the different FLAG-p85 mutants. NIH 3T3 cells were used in these experiments because they express PDGFR, in contrast with COS-1 cells that do not express endogenous PDGFR. Cells were serum starved (0.5% FBS) for 24 hours and then were stimulated with 50 ng/mL PDGF-BB for 10, 30, 60, 120 and 240 minutes. Control cells were left unstimulated. Proteins (125 μg) were immunoprecipitated using an anti-PDGFR antibody and a rabbit IgG as control. Immunoprecipitates were resolved by SDS-PAGE followed by Western blot analysis using anti-FLAG (exogenous FLAG-p85) and anti-p85 (both endogenous and exogenous p85) antibodies (Figure 4.3C). Figure 4.3A shows that similar amounts of the FLAG-tagged p85wt and mutants were expressed in NIH 3T3 cells. It can be observed from Figure 4.3B that there was an absence of non-specific immunoprecipitation of non-target proteins. Consistent with the hypothesis, no PDGFR-associated FLAG-tagged p85 was observed in cells expressing either p85ΔR or p85ΔR+R274A in response to growth factor treatment (Figure 4.3C). In cells expressing p85wt, both endogenous and exogenous p85 bound to the PDGFR after 10 minutes of growth factor treatment and stayed bound to the receptor for up to 2 hours when the receptor is normally dephosphorylated and inactivated. However, the amount of exogenous p85 that interacted with PDGFR at the 2 hour time point is higher than the endogenous p85. In cells expressing p85R274A, p85Δ110 or p85Δ110+R274A there appears to be a small amount of p85-PDGFR association in unstimulated cells (0 min time point) suggesting that there may be some abnormal binding of these p85 mutants to PDGFR in the absence of growth in these cells (Figure 4.3C). In these three cell types, the mutant form of p85 does not completely dissociate from the receptor even at 4 hours, though the levels start to decrease after the 2 hour treatment.

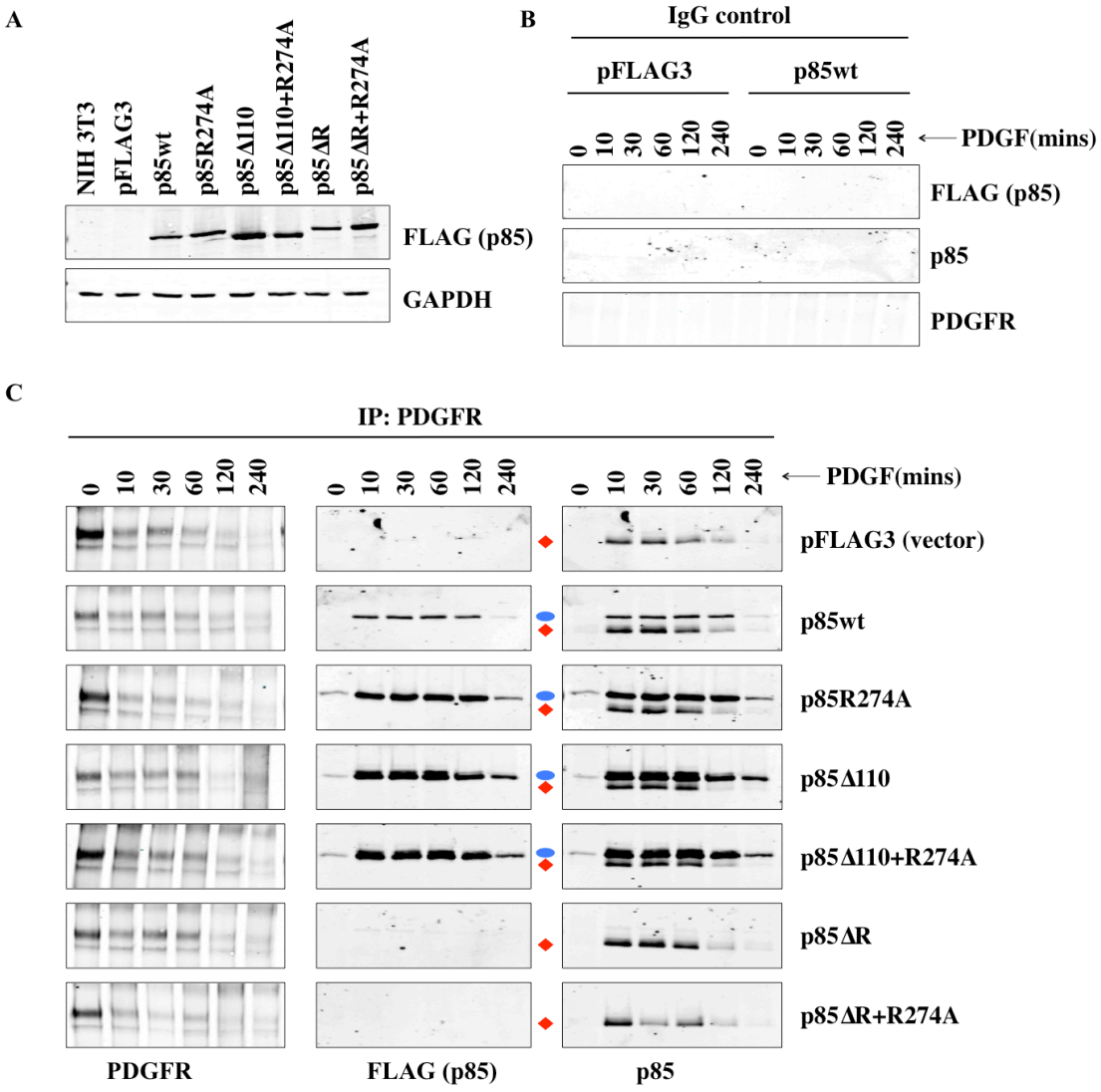

Figure 4.3: Association and dissociation pattern between p85 and PDGFR in response to growth factor stimulation. A) NIH 3T3 cells were transiently transfected with the indicated p85 wild type (wt) or the indicated mutants. Proteins (25 µg) were resolved by SDS-PAGE followed by Western blot analysis using anti-FLAG and anti-GAPDH antibodies. B) and C) NIH 3T3 cells were transiently transfected with the indicated p85 wt or mutants. Cells were serum starved (DMEM supplemented with 0.5% FBS) for 24 hours and then left unstimulated or stimulated with 50 ng/mL PDGF-BB for 10, 30, 60, 120 and 240 minutes. An anti-PDGFR immunoprecipitation (IP) was carried out using 125 µg of protein. IP products were resolved by SDS-PAGE and then probed with anti-PDGFR, anti-FLAG and anti-p85 antibodies. An IgG control experiment was performed to show the absence of non-specific interaction (B). Blue ovals for both FLAG and p85 blots identify exogenous pFLAG3-p85 (wt, R274A, Δ110 and Δ110+R274A). Red diamonds for p85 blots identify endogenous p85. This figure represents data obtained from one experiment.

4.2 Stable expression of pFLAG3-p85 wild type and p85 mutants

NIH 3T3 cells stably expressing pFLAG3-p85 wild type, pFLAG3-p85R274A, pFLAG3-p85Δ110 and pFLAG3-p85ΔR have been previously generated in the Anderson laboratory ((Chagpar *et al*., 2010; Chamberlain *et al*., 2004) and unpublished results). At least two clones per p85 construct were characterized in order to verify that effects observed were due to the p85 mutation and not an artefact of random integration into the genome (Figure 4.4 and Table 4.1). Clones expressing similar levels of FLAG-p85 were selected for the study and are indicated by the red asterisks in Figure 4.4. Clones selected from p85Δ110+R274A-expressing cells tend to lose FLAG-p85 expression after a few passages so three sets of stable transfections had to be performed to increase the probability of having enough clones for subsequent experiments. Cells were kept in culture for only a few passages and new batches of cells were thawed in order to continue the experiments with cells that still expressed FLAG-p85. Some clonal isolates lose gene expression with time possibly because the overexpression of gene of interest, or even the site of integration within the genome, could be toxic to the cells and they slowly die off leaving behind cells that have become resistant to the selective antibiotic but lack the gene of interest.

4.3 Properties of NIH 3T3 cells expressing pFLAG3-p85Δ110+R274A (a RabGAP defective p85 that is unable to bind the p110 subunit)

One of the main objectives of this thesis was to determine if the catalytic subunit of PI3K, i.e. p110, contributed a portion of the GAP function through its association with p85. The p110β subunit can bind selectively to Rab5-GTP (Christoforidis *et al*., 1999b) suggesting that it may bind the conformation-specific switch regions of Rab5 and thus work together with p85 to provide the switch stabilization GAP function. In this manner, p110β would enhance the GAP activity over and above that of p85 alone. In order to test this hypothesis, the rate of PDGFR degradation in cells expressing a RabGAP defective p85 that is unable to bind the p110 subunit was measured in comparison to cells expressing wild type p85, a RabGAP defective p85 or p85 unable to bind p110. The levels and duration of activated PDGFR as well as its effect on downstream signalling events were also measured in these cell lines.

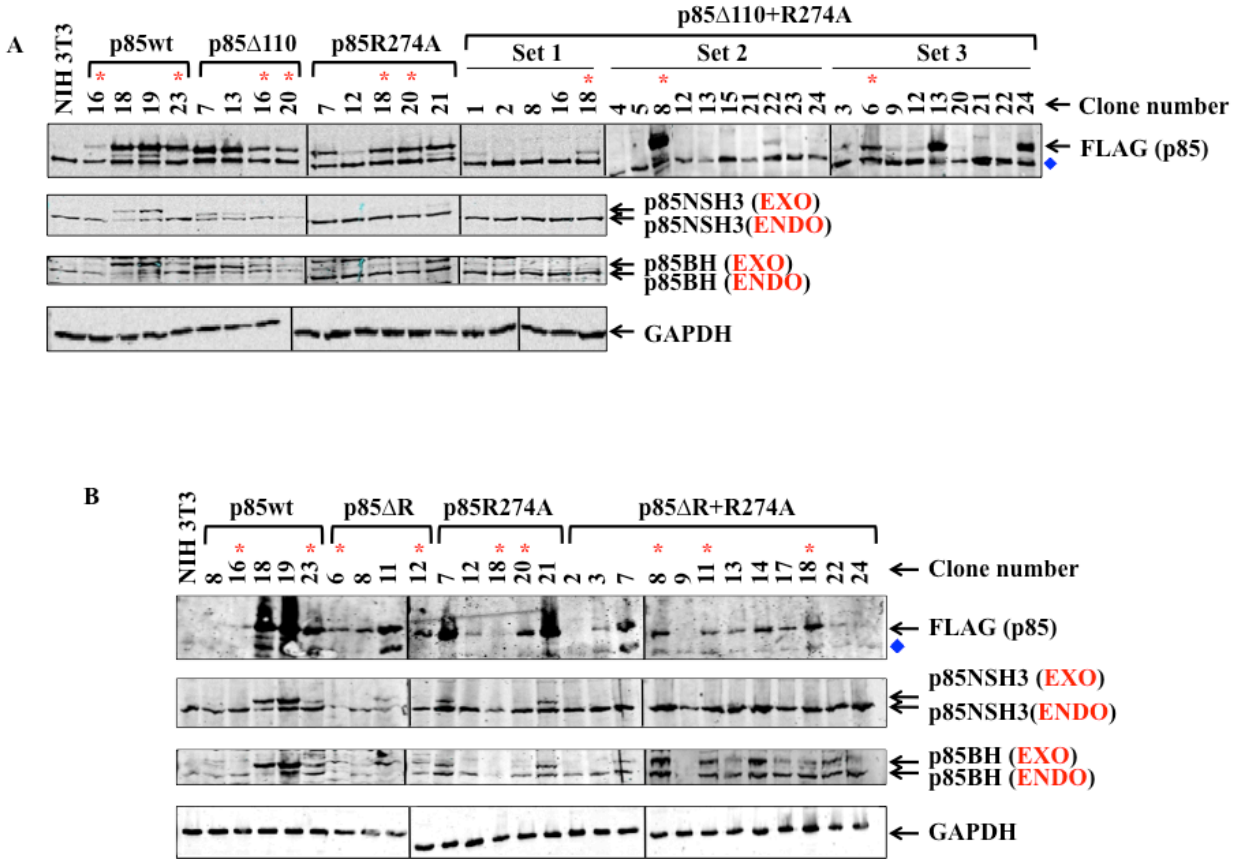

Figure 4.4: Relative expression of the different p85 mutants. NIH 3T3 cells were stably transfected with the indicated plasmids and selected in G418 (Geneticin). Individual clones from each FLAG-p85 wt or mutant were expanded and a fraction of each clone made into lysates in SDS sample buffer for analysis. Proteins (200 µg) were resolved by SDS-PAGE and probed with anti-FLAG, anti-p85 (p85NSH3 and p85BH antibodies recognise the N-SH3 and BH domains of p85 respectively) and anti-GAPDH antibodies. Western blots were visualised using the LICOR scanner. EXO: Transfected FLAG tagged p85. ENDO: Endogenously expressed p85. Blue diamond: non-specific band. Red asterisks: clones selected for subsequent experiments. A) p85Δ110 clones. B) p85ΔR clones.

Table 4.1: List of p85wt and mutant clonal isolates used for experiments in this thesis.

Cell type	Clonal isolate	Referred to as
pFLAG3-p85 wild type	Clone 16 Clone 23	C1 C2
pFLAG3-p85R274A	Clone 18 Clone 20	C1 C2
pFLAG3-p85Δ110	Clone 16 Clone 20	C1 C2
pFLAG3-p85Δ110+R274A	Set 1 Clone 18 Set 2 Clone 8 Set 3 Clone 6 Mixed population	C1 C2 C3 C4
pFLAG3-p85ΔR	Clone 6 Clone 12	C1 C2
pFLAG3-p85ΔR+R274A	Clone 8 Clone 11 Clone 18 Mixed population	C1 C2 C3 C4

4.3.1 PDGFR degradation rates and signalling kinetics of downstream effectors within stable cell lines expressing pFLAG3-p85Δ110+R274A compared to controls (pFLAG3-p85 wild type, pFLAG3-p85R274A and pFLAG3-p85Δ110)

In order to examine the response of p85Δ110+R274A to growth factor stimulation and activation of downstream signal transduction pathways, cells were serum starved for 24 hours and then were stimulated with 50 ng/mL PDGF-BB for 10, 30, 60, 120 and 240 minutes. Unstimulated cells served as controls. Proteins were resolved by SDS-PAGE followed by Western blot analysis using anti-PDGFR, anti-pTyr, anti-pMAPK/Erk, anti-MAPK/Erk, anti-pAkt and anti-Akt, antibodies to measure downstream signalling events in these cells. If p110

contributes to the GAP activity (possibly through switch stabilization), then in cells expressing p85Δ110+R274A, where p110 is not localized to Rab5, an even more sustained signalling (pMAPK/Erk), a slower rate of PDGFR degradation (i.e. sustained levels of PDGFR) and PDGFR that is highly activated (tyrosine phosphorylated) would be expected in comparison to cells expressing p85R274A (where p110 would still associate with p85 and be localized to Rab5).

4.3.1.1 Effect of Δ110 or Δ110+R274A mutation on PDGFR activation and degradation

In Figure 4.6, cells were stimulated with PDGF for increasing lengths of time and corresponding lysates were resolved and immunoblotted with anti-pTyr antibodies to determine the levels of tyrosine phosphorylated PDGFR, which is an indication of activated PDGFR. Figure 4.5 shows the levels of FLAG-tagged p85 wild type and mutants used in these experiments. The pTyr blots in Figure 4.6 reveal that in control cell lines (NIH 3T3, pFLAG3 and p85wt) the PDGFR is fully activated by 10 minutes of growth factor treatment and that the receptor begins to deactivate 30 minutes after treatment with growth factor. A similar pattern of receptor activation (i.e. change in pTyr status) is observed in p85Δ110-expressing cells (clone 2). On the other hand, clone 1 of p85Δ110-expressing cells shows a slight increase in the duration of receptor activation as seen by a marginally denser band in the 60 minute sample (Figure 4.6). As reported previously, cells expressing p85R274A have a more prolonged activation of PDGFR as shown by the high levels of tyrosine phosphorylated PDGFR at two hours of growth factor treatment (Figure 4.6 and (Chamberlain *et al*., 2004)). The pTyr blots also reveal that cells expressing p85Δ110+R274A have a more prolonged activation of PDGFR, indicated by higher levels of phosphorylated tyrosine residues, when compared to cells expressing p85R274A as well as control cell lines. It should be noted that clone 3 of p85Δ110+R274A-expressing cells does not show a dramatic increase in the duration of PDGFR activation, possibly due to the fact that these cells expressed lower amounts of FLAG-tagged p85 at the time the experiments were performed. As mentioned previously, the majority of clonal isolates expressing FLAG-tagged p85Δ110+R274A tend to lose FLAG expression over time. In all cell lines tested, the absence of a band in the 0 minute lane for the pTyr blots suggests that in these cells lines the PDGFR is only fully activated in response to growth factor stimulation.

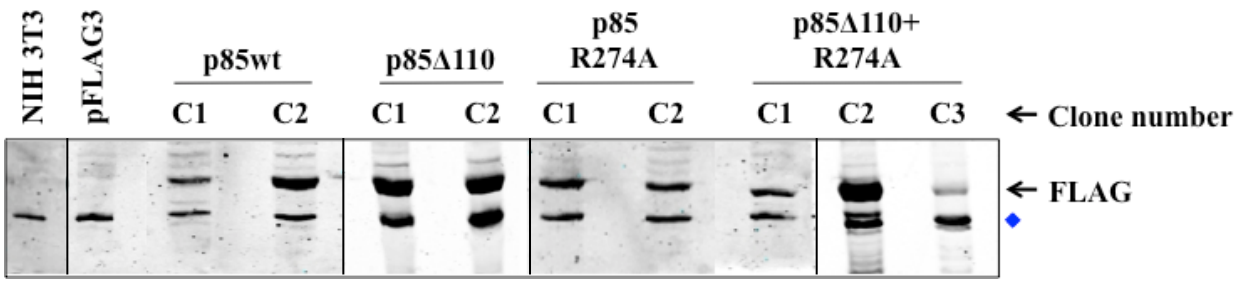

Figure 4.5: Relative expression of the different p85 Δ110 and R274A mutants. NIH 3T3 cells stably expressing the indicated plasmids were harvested in SDS sample buffer for whole cell lysates. Proteins (200 µg) were resolved by SDS-PAGE and probed with anti-FLAG antibodies. Western blots were visualised using the LICOR scanner. Blue diamond: non-specific band.

In order to measure the rate of receptor degradation, the same cell lysates obtained from the PDGF stimulation described above were Western blotted with anti-PDGFR antibodies (Figure 4.6). In control cell lines (NIH 3T3, pFLAG3 and p85wt) it can be seen that the total levels of PDGFR decrease after 30 minutes of growth factor stimulation. The PDGFR usually appears as two bands; a higher thicker band and a lower thinner band. The PDGFR undergoes glycosylation in order to yield a fully mature receptor that migrates at approximately 180 kDa in size (the higher band). The immature non-glycosylated receptor is about 170 kDa in size (the lower band). p85Δ110-expressing cells (clone 1) show a slight decrease in the rate of receptor degradation as can be observed by the presence of a stronger signal in the 60, 120 and 240 minute samples when compared to control cell lines. Cells expressing p85R274A show a significant decrease in the rate of receptor degradation as compared to control cell lines (Figure 4.6), thus confirming previous results (Chamberlain *et al*., 2004). However, p85Δ110+R274A-expressing cells have an even slower rate of receptor degradation when compared to cells expressing p85R274A. Since p85Δ110-expressing cells show intermediate effects between those of NIH 3T3/pFLAG3/p85wt and that of p85R274A/p85Δ110+R274A, it suggests that the p110 localized to receptors *via* p85 binding contributes to the altered PDGFR degradation kinetics in particular.

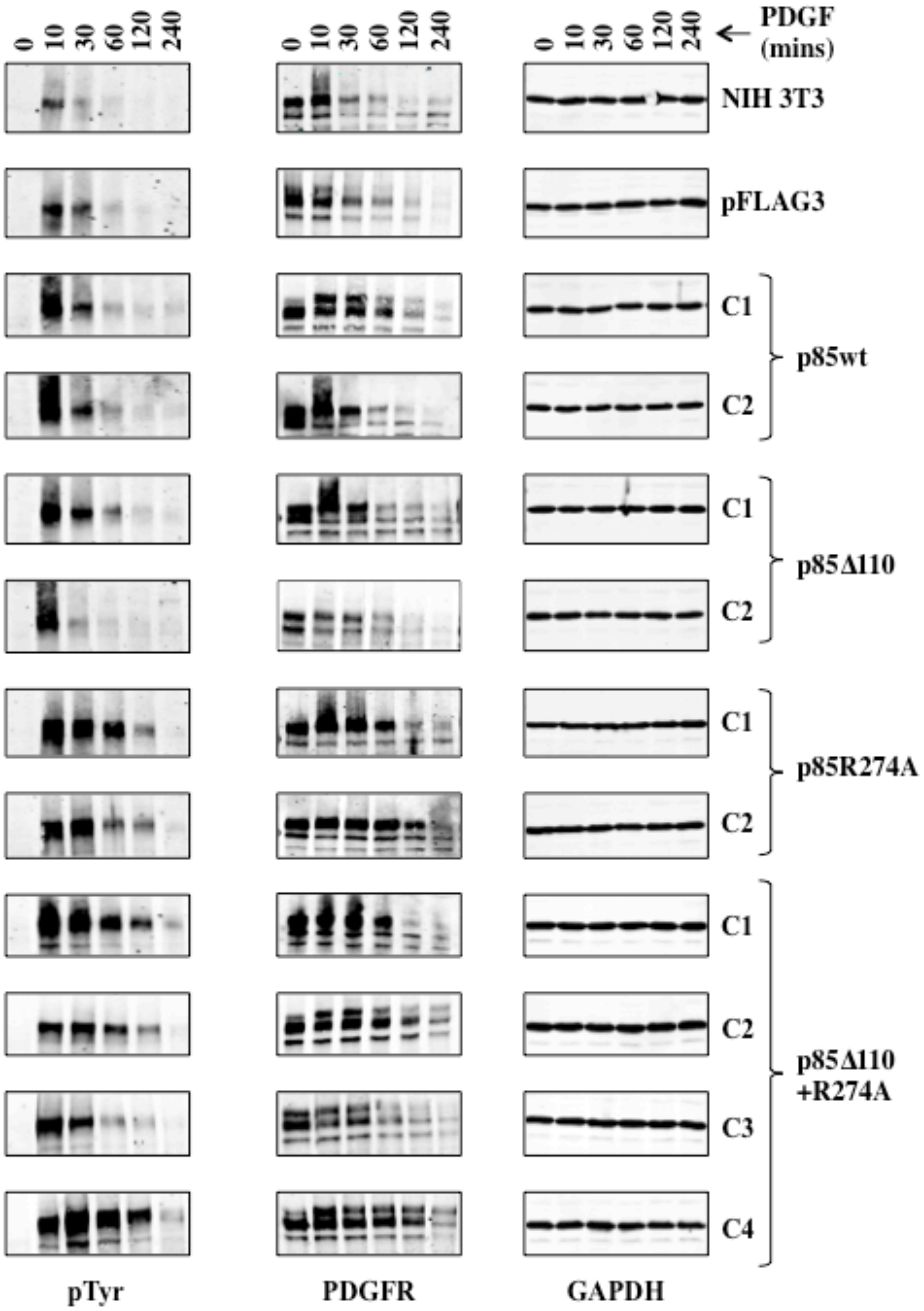

Figure 4.6: Prolonged PDGFR receptor activation and decreased receptor degradation in cells expressing p85R274A or p85Δ110+R274A. The cells indicated were serum starved (DMEM supplemented with 0.5% FBS) for 24 hours and then left unstimulated or stimulated with 50 ng/mL PDGF-BB for 10, 30, 60, 120 and 240 minutes. Proteins were resolved by SDS-PAGE followed by Western blot analysis using anti-pTyr, anti-PDGFR and anti-GAPDH antibodies. Similar results were obtained in three independent experiments.

4.3.1.2 The Δ110 or Δ110+R274A mutations in p85 affects downstream signalling in response to PDGF

The prolonged receptor activation and reduced rate of receptor degradation observed in p85Δ110+R274A-expressing cells (Figure 4.6) could alter signalling from the MAPK/Erk pathway or the PI3K/Akt pathway, both of which have been associated with the PDGFR signalling (Alvarez *et al*., 2006; Cross *et al*., 2000; Fruman *et al*., 1998; McKay and Morrison, 2007). Downstream effectors of the PDGFR signalling pathway were examined by Western blot analysis using the same lysates obtained from the growth factor stimulation experiments described in Section 4.3.1. Activation of the MAPK/Erk pathway was measured by Western blot analysis with antibodies targeting phospho-MAPK/Erk (pMAPK/Erk) (Figure 4.7). The total MAPK/Erk levels were also measured. MAPK/Erk can be seen as a doublet of 44 and 42 kDa. The 44 kDa protein represents the MAPK1/Erk1 isoform that is phosphorylated on both Thr202 and Tyr204 to activate it. On the other hand, the 42 kDa protein is the MAPK2/Erk2 isoform that is phosphorylated on Thr185 and Tyr187 for full activation. The pMAPK/Erk blots in Figure 4.7 show that in control cells (NIH 3T3, pFLAG3 and p85wt) phosphorylation of MAPK/Erk was induced from 10 minutes to 60 minutes, with minimal levels of pMAPK/Erk at the 2 hour and 4 hour time points. In cells expressing p85Δ110, there was slightly more phosphorylated MAPK/Erk present at the 2 hour and 4 hour time points. Expression of p85R274A leads to an enhanced activation of the MAPK/Erk pathway (Figure 4.7 and (Chamberlain *et al*., 2004)). In these cells, phosphorylation of MAPK/Erk can be observed even after 4 hours of growth factor treatment. Similar to p85R274A-expressing cells, phosphorylation of MAPK/Erk in p85Δ110+R274A-expressing cells is induced from 10 minutes to 4 hours. In both p85R274A- and p85Δ110+R274A-expressing cells there are very low amounts of pMAPK/Erk present before growth factor stimulation indicating that the presence of p85R274A and p85Δ110+R274A proteins in these cells in may interfere with the mechanism(s) that would inhibit MAPK/Erk signalling (e.g. any one of the 11 dual specificity MAPK/Erk phosphatases) (Boutros *et al*., 2008). The MAPK/Erk blots in Figure 4.7 show the total levels of the MAPK/Erk. All cell lines tested express similar levels of MAPK/Erk.

Activation of the PI3K/Akt pathway was also examined by Western blotting with phospho-specific antibodies for Akt (Figure 4.8). The total levels of Akt across the different clones show that the cells express similar levels of Akt. In all cell lines tested, Akt was

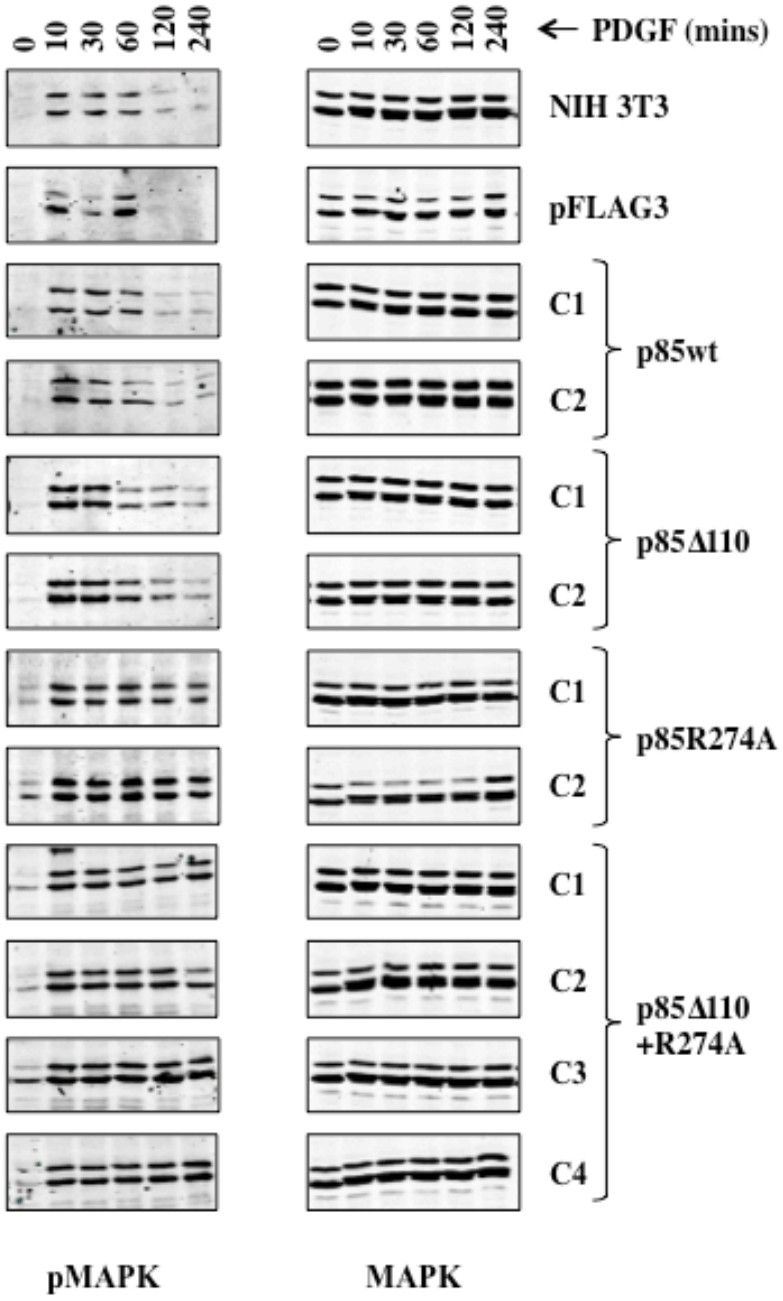

Figure 4.7: MAPK signalling following PDGFR stimulation is sustained by the expression of R274A. Cells were serum starved (DMEM supplemented with 0.5% FBS) for 24 hours and then left unstimulated or stimulated with 50 ng/mL PDGF-BB for 10, 30, 60, 120 and 240 minutes. Proteins were resolved by SDS-PAGE followed by Western blot analysis using anti-pMAPK/Erk and anti-MAPK/Erk antibodies. Similar results were obtained in three independent experiments.

phosphorylated on Ser473 within 10 minutes of beginning growth factor treatment and the phosphorylation was sustained over the time course. There was no difference in the activation of the Akt pathway between control cells and cells expressing p85Δ110, p85R274A or p85Δ110+R274A. Taken together, the data suggests that expression of p85R274A or p85Δ110+R274A results in sustained activation of the MAPK/Erk pathway (but not the PI3K/Akt pathway). The lack of any detectable effect in cells expressing p85Δ110 suggests that the sustained activation of the MAPK/Erk pathway is primarily due to the R274A substitution in p85.

4.3.2 Oncogenic properties of cells expressing pFLAG3-p85Δ110+R274A compared to controls

It has been reported by our laboratory that cells expressing the RabGAP defective p85R274A are transformed indicating that the RabGAP function of p85 plays an essential role in the regulation of cell survival and proliferation (Chamberlain *et al*., 2008). The hallmarks of some transformed cells include loss of cell-cell and cell-basement membrane contact inhibition, loss of anchorage dependence for cell survival and proliferation, and the ability to degrade and detach from the extracellular matrix (ECM) and migrate to secondary sites. Since the expression of p85Δ110+R274A resulted in enhanced PDGFR signalling much like p85R274A-expresssing cells, similar cell based assays were used to determine the oncogenicity of the p85Δ110+R274A mutation as had been used previously (Chamberlain *et al*., 2008).

4.3.2.1 Loss of contact inhibition (Foci formation assay)

Proliferation of normal cells in tissue culture is inhibited by contact with other cells ensuring that they grow in a monolayer. Transformed cells, however, continue to grow and pile on top of each other to form clumps, eventually forming foci. The foci formation assay is used to determine whether an adherent cell is transformed or not, however, it should be noted that not all cells that are transformed can form foci. Cells expressing p85wt or the different mutants of p85 were seeded at a density of 100 000 cells/10 cm dish and maintained in normal medium for two weeks, with routine medium changes, till they were well past 100% confluency. Cells were stained with Giemsa stain and the entire 10 cm dish was photographed to show macroscopic foci

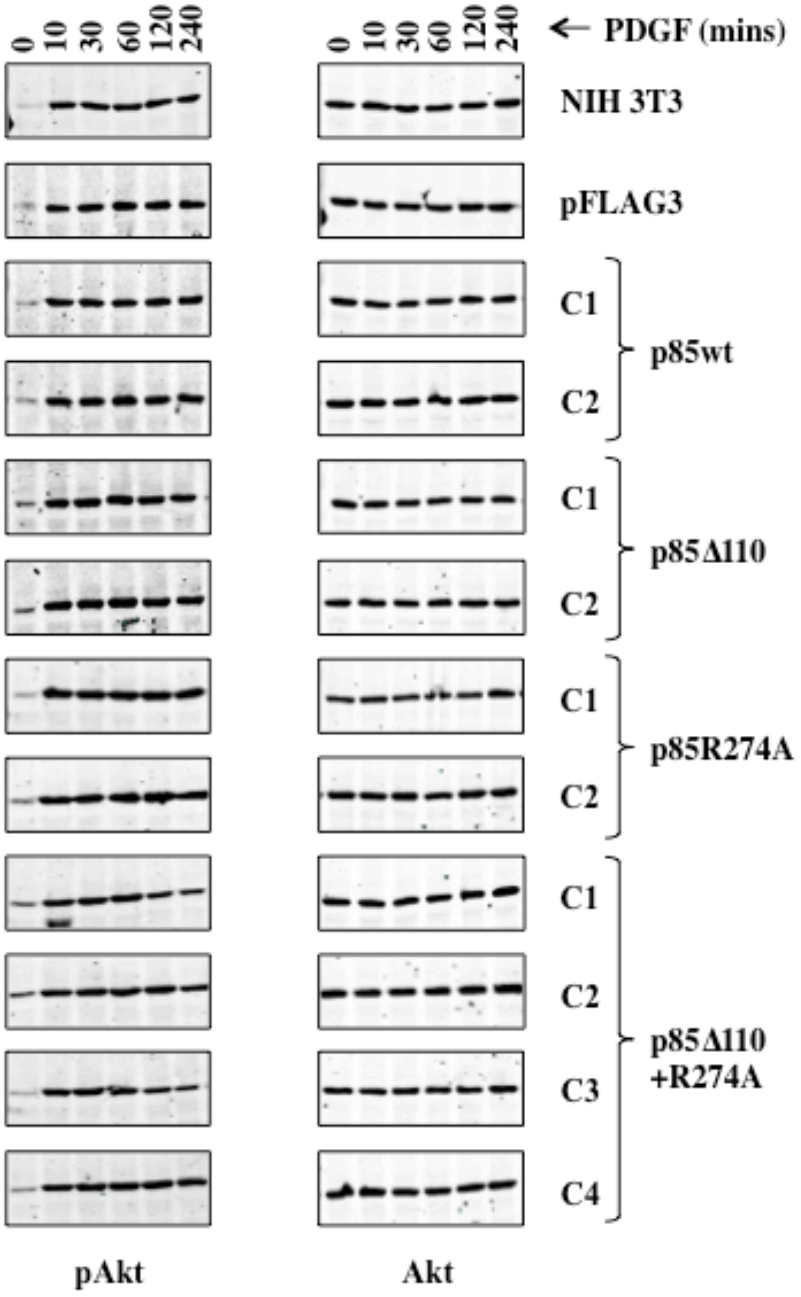

Figure 4.8: Akt signalling following PDGFR stimulation is not affected by the expression of either the Δ110 or the R274A variant of p85. Cells were serum starved (DMEM supplemented with 0.5% FBS) for 24 hours and then left unstimulated or stimulated with 50 ng/mL PDGF-BB for 10, 30, 60, 120 and 240 minutes. Proteins were resolved by SDS-PAGE followed by Western blot analysis using anti-pAkt and anti-Akt antibodies. Similar results were obtained in three independent experiments.

(Figure 4.9). Parental NIH 3T3 cells and cells stably expressing p85wt, p85Δ110 or p85Δ110+R274A remained as a monolayer for the duration of the experiment and started to lift off along the circumference of the 10 cm dish when the cells were just past confluency. In some experiments the cells lifted completely off the dish in an intact monolayer. No foci were observed with cells expressing p85wt, p85Δ110 or p85Δ110+R274A (Figure 4.9). Cells expressing p85R274A formed foci seen as black spots on the C1 and C2 dishes (Figure 4.9). Expression of p85Δ110 or p85Δ110+R274A does not lead to the formation of foci, which suggests that cells expressing these mutants are not transformed. This result is contrary to what was expected as cells expressing p85R274A or p85Δ110+R274A both had enhanced PDGFR signalling (pMAPK/Erk), which would contribute to cell proliferation and survival, and would usually result in transformation and oncogenic cell properties. The formation of foci in cells expressing p85R274A confirms a role for this RabGAP defective p85 in cellular transformation, as observed previously (Chamberlain *et al*., 2008). However, any condition that might interfere with the ability of a RabGAP defective p85 to bind to p110 would appear to inhibit this aspect of cellular transformation and strongly suggests that p85 needs to exist as the PI3K heterodimer for full transformation of cells to occur via the PI3K signalling pathway.

4.3.2.2 Anchorage independent growth (Colony formation in soft agar)

To further analyse the transformation ability of the p85Δ110 or p85Δ110+R274A mutation another transformation assay was used; the colony formation in soft agar assay (anchorage-independent growth assay). In normal tissue, cells adhere both to one another and to the protein mesh filling the intercellular space. The intricate network of proteins occupying the space is referred to as the extracellular matrix (ECM). In contrast with normal cells that require proper cell-cell and cell-matrix interactions for maintaining cellular functions, transformed cells can continue to survive and proliferate without being attached to a surface or communicating through cell-cell interactions. The colony formation in soft agar assay is used to demonstrate a cell's capacity for anchorage-dependent versus -independent growth. Cells were seeded in a suspension of top agar above of a layer of bottom agar and cultured for 20 days. Colonies formed were visualized under a microscope with a 10X objective lens and photographed (Figure 4.10). Colonies formed from cells expressing the pFLAG3 vector or those expressing p85wt had an average diameter 28 μm. Therefore, only colonies that were 28 μm or larger in diameter were

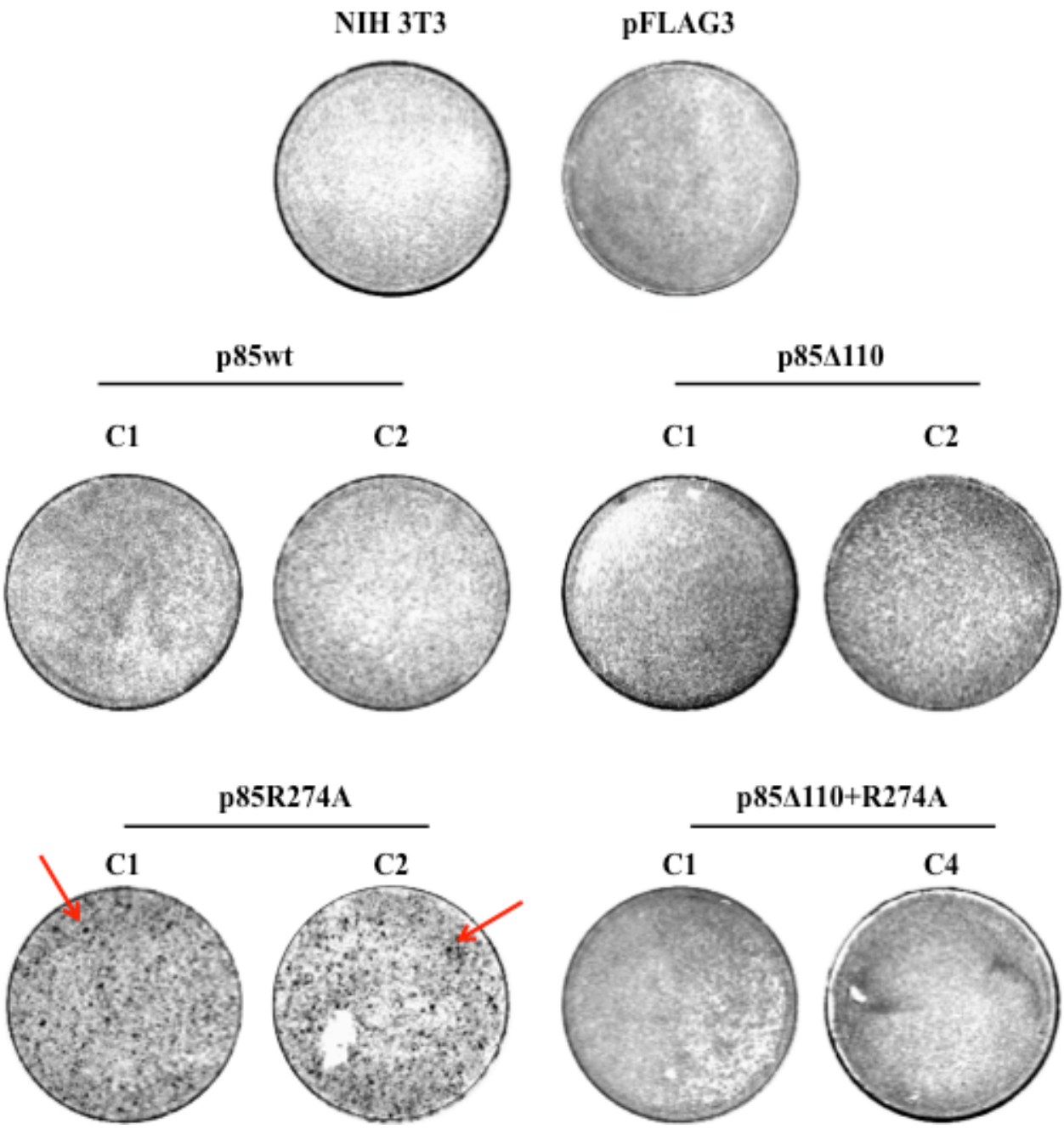

Figure 4.9: Cells expressing p85R274A form foci in a contact inhibition assay, but cells expressing p85Δ110 and p85Δ110+R274A do not. Cells were seeded at a density of 100 000 cells/10 cm dish and maintained in normal medium for two weeks. Cells were fixed in -20 °C methanol, stained with Giemsa stain and photographed. Images show the entire surface of a 10 cm dish and each image is representative of four different experiments (except for p85Δ110+R274A clone 4 where only two experiments were performed). C: clone number. Red arrows point to foci.

counted. p85Δ110-expressing cells developed colonies that were marginally wider than vector control and p85wt (Figure 4.10). These colonies ranged in diameter from 28 to 39 μm. Cells expressing p85R274A formed numerous colonies that ranged in diameter from 28 to 180 μm (Figure 4.10). The observations that these cells were able to grow on soft agar (anchorage-independent) (Figure 10) and also lost any contact inhibition (Figure 4.9) further supports the report that they are transformed (Chamberlain *et al*., 2008). p85Δ110+R274A clones 1 and the mixed population, referred to as clone 4, formed colonies that ranged in size from 28 to 39 μm in diameter. Both p85Δ110- and p85Δ110+R274A-expressing cells formed similar sized colonies and similar number of colonies (Figures 4.10 and 4.11). Taken together, the data suggests that expression of a p85 mutant unable to bind to the p110 subunit or a RabGAP defective p85 that is unable to bind p110 does not lead to cellular transformation.

4.3.2.3 Attachment to fibronectin and collagen (Adhesion assay)

Cell adhesion is important for the maintenance of cell-cell communication as well as for the maintenance of cellular structure *via* the connections made with the extracellular matrix (ECM) and the cytoskeleton. One of the early steps involved in cancer cell metastasis is the transformed cells' ability to degrade components of the ECM, thereby diminishing their adherence and increasing their potential for escaping from the primary tumour site. The ECM contains connective tissue proteins such as collagen and fibronectin. To examine the cells' ability to interact with the ECM, 96-well microtiter plates were coated with either of two common connective tissue proteins, e.g. fibronectin or collagen type I. Twenty thousand (20 000) cells were then seeded onto the coated plates and allowed to adhere to the plates for 30 minutes. They were then washed, fixed, stained with crystal violet and solubilised. Absorbance readings were taken at 550 nm, with the absorbance reflecting the cell's ability to adhere (Figure 4.12). Cells expressing p85wt or p85Δ110 adhered to collagen I to the same extent. It can be seen from Figure 4.12 that cells expressing p85R274A were less adherent to collagen I than cells expressing p85wt. Two cell lines expressing p85Δ110+R274A (clones 2 and 3) were less adherent to collagen I when compared to cells expressing p85wt, whereas clone 1 appeared to be more adherent. Adhesion to fibronectin, however, was similar for all FLAG-tagged p85 expressing cell lines tested. This suggests that expression of p85R274A or p85Δ110+R274A changes the adherent properties of cells by making them less adherent to collagen I.

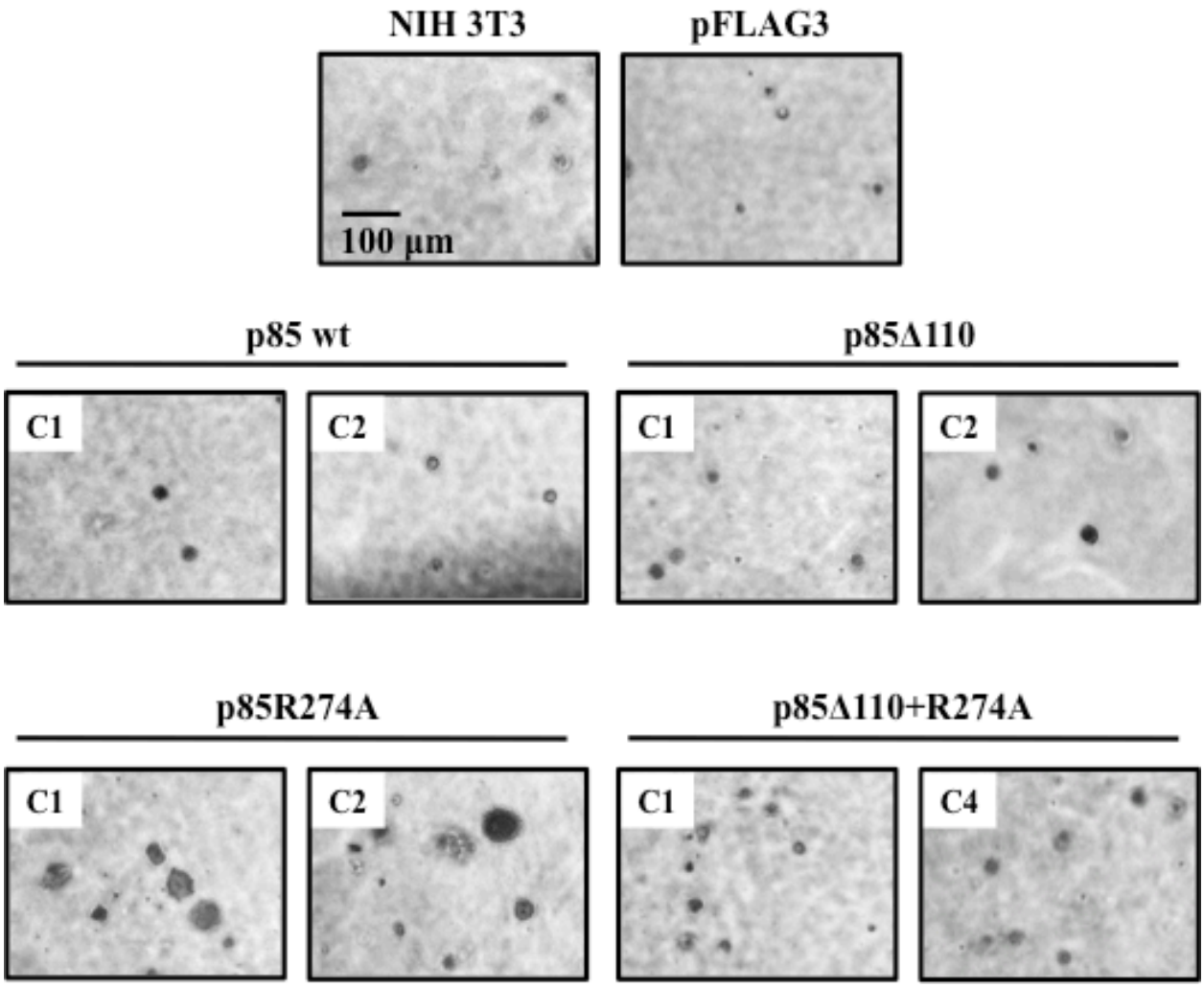

Figure 4.10: Cells expressing p85R274A form colonies in soft agar, but cells expressing p85Δ110 or p85Δ110+R274A do not. Fifty thousand cells/6 cm dish were cultured in a suspension of top agar (3.75X MEM vitamins, 0.36% agar) above of a layer of bottom agar (5X MEM vitamins, 0.61% agar). Cells were left to grow for a total of 20 days. Images are representative of the colonies formed by the individual cells expressing the different p85 mutants in four different experiments (except for p85Δ110+R274A clone 4 where two experiments were performed). C: clone number. Colonies are spots that are 28 µm or larger in diameter.

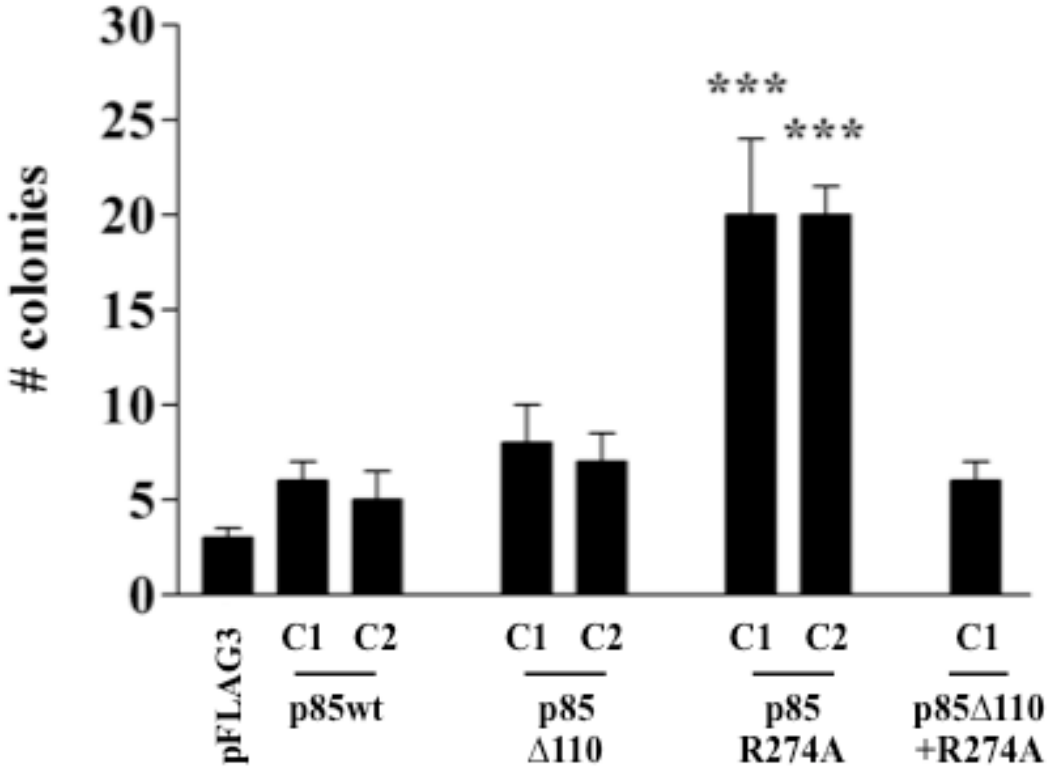

Figure 4.11: Cells expressing p85R274A form numerous colonies in soft agar. The graph depicts the number of colonies formed per 50 000 cells plated that are expressing different p85 mutants (see Figure 4.10). Each value represents the mean (± SD) of four independent experiments, each based on three separate viewing fields. Bonferroni's multiple comparison test indicates ***, $p<0.001$ as compared to the vector control or either of the p85wt clones. C: clone number.

4.3.2.4 Migratory properties (Boyden chamber assay)

Cell migration is used as a measure of cells' ability to detach from the primary location and move. Most migration assays are a modification of the Boyden chamber, which is simply two chambers separated by a membrane with pores to allow cells to migrate from one chamber to the other along a chemical gradient, e.g. towards a chemoattractant such as a growth factor or ECM substrates (Boyden, 1962; Chen, 2005). In order to study the cells' ability to migrate towards a stimulus, cells were resuspended in DMEM supplemented with 0.1% FBS and $1x10^5$ cells were added to the top chamber. The bottom chamber was filled with either DMEM supplemented with 0.1% FBS as control or DMEM supplemented with 10% FBS. Samples were incubated at 37 °C for 24 hours. The cells that migrated to the lower chamber were stained with hematoxylin. The results were expressed as the number of cells that migrated towards 10% FBS minus the number of cells that migrated towards 0.1% FBS (Figure 4.13). All cells migrated to the same extent towards 0.1% FBS except for p85Δ110+R274A clones 1 and 3 that migrated more and p85Δ110+R274A clone 2 that migrated much less. Cells expressing p85wt clone 2 migrated to a greater extent towards 10% FBS than cells expressing p85wt clone 1. Statistical analyses were therefore performed in relation to p85wt clones 1 and 2 separately. There was no difference between the number of cells expressing p85wt or p85Δ110 that migrated towards 10% FBS. Cells expressing p85R274A clone 2 migrated significantly more than both p85wt- and p85Δ110-expressing cells. Cells expressing p85Δ110+R274A clones 2 and 4 migrated more towards 10% FBS in comparison to both p85wt- and p85Δ110-expressing cells. Cells expressing p85Δ110+R274A clone 2 had an even higher rate of migration than p85R274A-expressing cells. The data indicate that expression of p85R274A or p85Δ110+R274A could result in an increase in the migratory properties of cells. However, this increase appears to be clone specific.

4.3.3 Analysis of PDGFR and p85 association in cells expressing pFLAG3-p85Δ110+R274A

It was previously observed in the Anderson laboratory that a significant amount of p85 is still bound to the PDGFR in cells expressing pFLAG3-p85Δ110 even after 2 hours of growth factor stimulation (when the PDGFR is usually dephosphorylated and inactivated) (Figure 4.14). This suggests that the p110 subunit facilitates dissociation of the PI3K complex from the PDGFR, and without it, p85 stays bound to PDGFR. In order to better understand this signalling

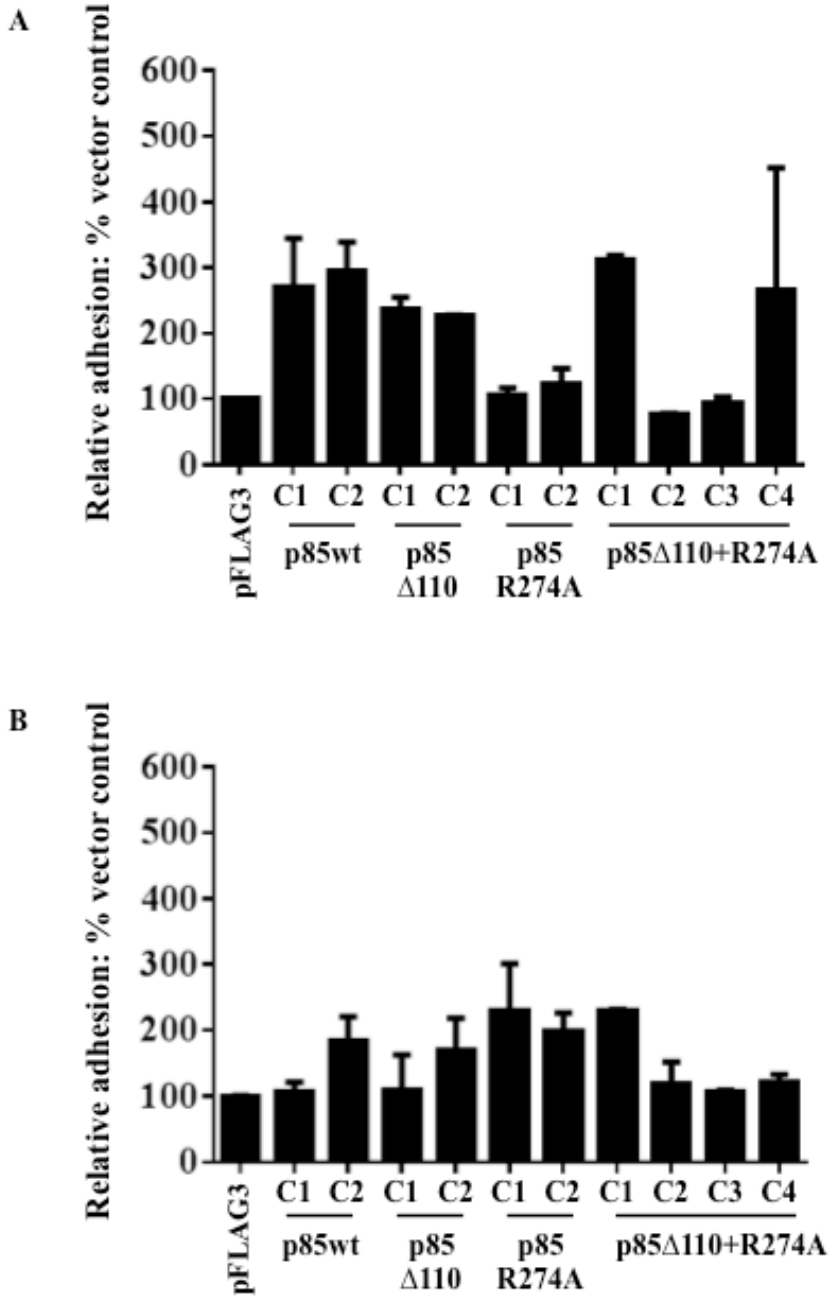

Figure 4.12: The inability of p85 to bind to p110 affects the adhesion properties of the R274A mutation in p85. Cells were seeded on 96-well plates coated with either collagen (A) or fibronectin (B) and incubated for 30 minutes. Cells were then fixed, stained with crystal violet and solubilised. Relative fluorescent units were quantified at 550 nm and represented relative to cells expressing the vector control (pFLAG3) from two independent experiments with triplicate measurements for each experiment. ANOVA for overall groups indicates p value = 0.014 for collagen (A) and p value = 0.015 for fibronectin (B). *Post hoc* analysis using the stringent Bonferroni's adjustment for multiple comparisons, however, showed no statistical significance.

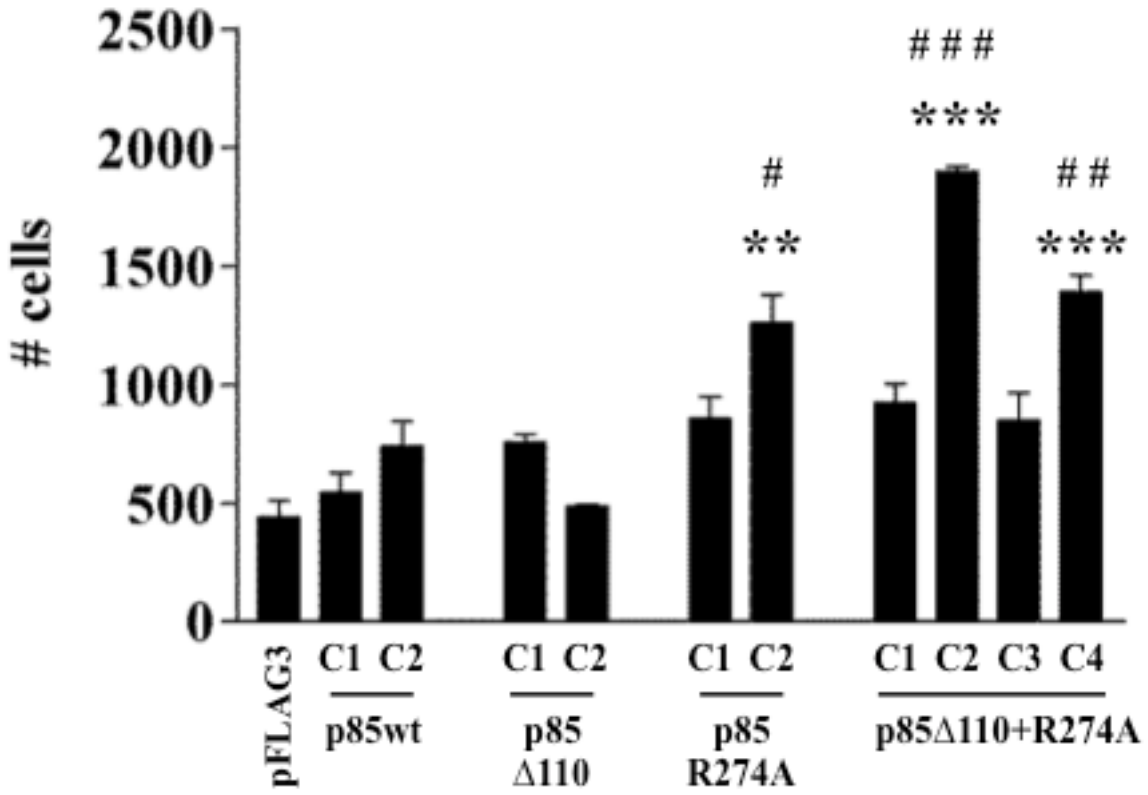

Figure 4.13: p85R274A and p85Δ110+R274A mutations induce more migration of cells in a clone specific manner. Cells were seeded into the top section of a Boyden chamber and left to migrate towards either 0.1% FBS or 10% FBS at 37 °C for 24 hours. Cells that had migrated to the bottom side of the Boyden chamber were then fixed and stained with hematoxylin. Cells observed in 8 fields of view at a magnification of 20X were quantified using the Stereo Investigator software (MBF Bioscience). The results represent cells that migrated towards 10% FBS minus cells that migrated towards 0.1% FBS from one experiment with duplicate measurements. Bonferroni's multiple comparison test indicates ** $p<0.01$, *** $p<0.001$ as compared to p85wt C1 and # $p<0.05$, ## $p<0.01$, ### $p<0.001$ as compared to p85wt C2.

event, the association and dissociation pattern between p85 and PDGFR in response to PDGF stimulation in cells expressing pFLAG3-p85Δ110 or pFLAG3-p85Δ110+R274A was studied.

Cells were first serum deprived (0.5% FBS) for 24 hours and then left unstimulated or stimulated with 50 ng/mL PDGF-BB for 10, 30, 60, 120 and 240 minutes. An anti-PDGFR immunoprecipitation was carried out and samples resolved by SDS-PAGE were probed using anti-FLAG (exogenous p85) and anti-p85 (both endogenous and exogenous p85) antibodies (Figure 4.15). Based on the results from the previous study shown in Figure 4.14, the levels of PDGFR-associated FLAG-tagged p85, in response to growth factor treatment, were expected to increase after the 0 minute time point and slowly decline to almost undetectable levels by the 60 minute time point in control cells (NIH 3T3, pFLAG3 and p85wt). It can be observed from Figure 4.15 that cells expressing p85wt (clone 2), p85Δ110 and p85Δ110+R274A (clone 2) have PDGFR-associated FLAG-tagged p85 up until the 240 minute time point. All the other cell lines tested did not show any co-immunoprecipitation of FLAG-p85 protein and activated PDGFR. This could be due to the fact that most of these cell lines had lost expression of the FLAG-p85 protein or had very low expression by the time this work was carried out (p85wt clone 1, both clones expressing p85R274A and p85Δ110+R274A clones 1, 3 and 4) (Figure 4.16). As can be seen from Figure 4.16, cells expressing p85wt (clone 2), p85Δ110 and p85Δ110+R274A (clone 2) expressed the highest level of FLAG-tagged p85 when compared to the other clones with that same mutation. This may suggest that the sustained levels of FLAG-tagged p85 observed at the 240 minute time point that was previously observed (Figure 4.14) may just be a consequence of over-expression; in that, clones with higher expression will stay associated to receptor longer since there is an abundance of p85 and those with lower expression will show the same association and dissociation pattern as endogenous p85.

The data also indicates that p85 does not require being part of a p85-p110 heterodimer to associate with the PDGFR.

4.4 Properties of NIH 3T3 cells expressing pFLAG3-p85ΔR+R274A (a RabGAP defective p85 that is unable to bind PDGFR)

The second major objective of this thesis was to determine whether p85 needed to associate with PDGFR in order to exert its GAP activity on Rab5 and Rab4. Since p85 binds to Rab5-GDP as well as Rab5-GTP, it may help recruit Rab5 to vesicle membranes containing

activated PDGFR signalling complexes and thereby regulate PDGFR signalling and/or trafficking events (Chamberlain *et al*., 2004). We postulate that PDGFR may help localize the RabGAP activity of p85.

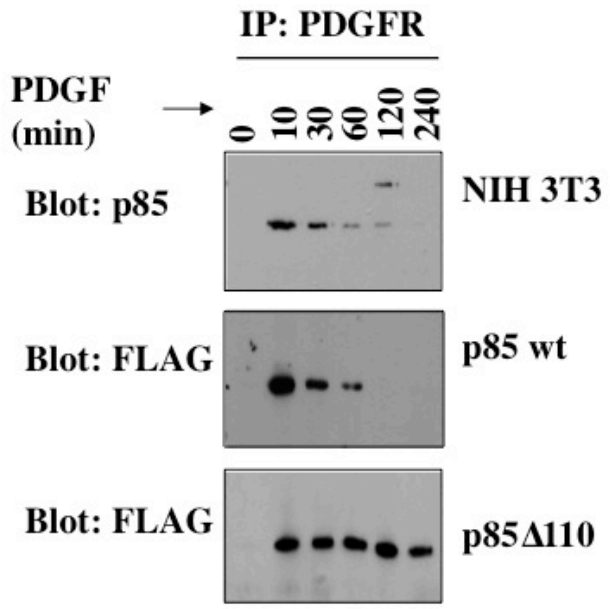

Figure 4.14: p85Δ110 stays bound to activated PDGFR. Cells were serum starved (DMEM supplemented with 0.5% FBS) for 24 hours and then left unstimulated or stimulated with 50 ng/mL PDGF-BB for 10, 30, 60, 120 and 240 minutes. An anti-PDGFR IP was carried out after which IP products were resolved by SDS-PAGE followed by Western blot analysis using anti-FLAG and anti-p85 antibodies. Experiments were performed by Jonathan Hanson.

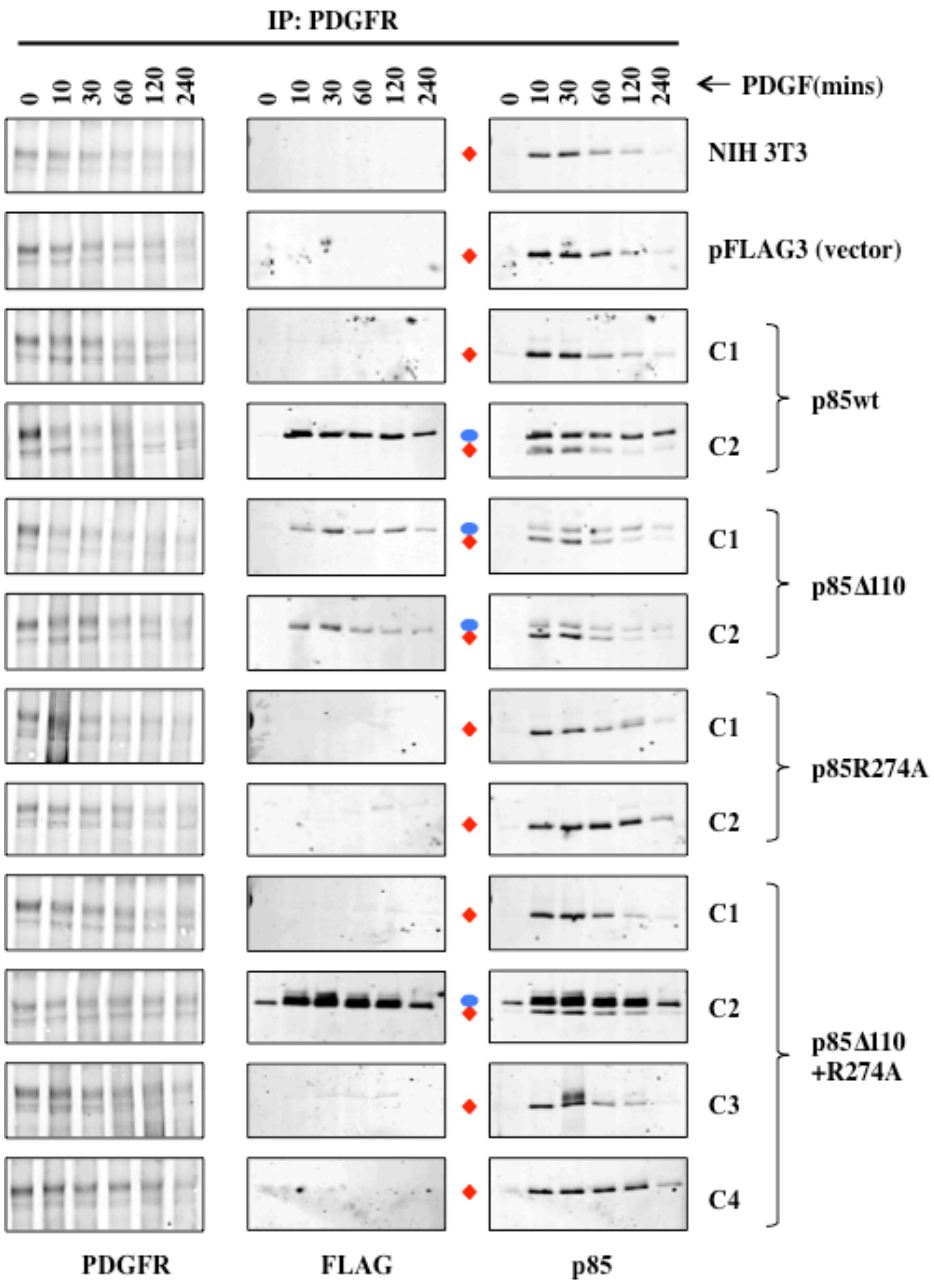

Figure 4.15: Association and dissociation pattern between p85Δ110 or p85Δ110+R274A and PDGFR in response to growth factor stimulation. Cells were serum starved (DMEM supplemented with 0.5% FBS) for 24 hours and then left unstimulated or stimulated with 50 ng/mL PDGF-BB for 10, 30, 60, 120 and 240 minutes. An anti-PDGFR IP was carried out using 125 µg of protein. IP products were resolved by SDS-PAGE followed by Western blot analysis using anti-PDGFR, anti-FLAG and anti-p85 antibodies. Blue ovals for both FLAG and p85 blots identify exogenous pFLAG3-p85 (wt, R274A, Δ110 and Δ110+R274A). Red diamonds for p85 blots identify endogenous p85. This figure represents data obtained from three independent experiments.

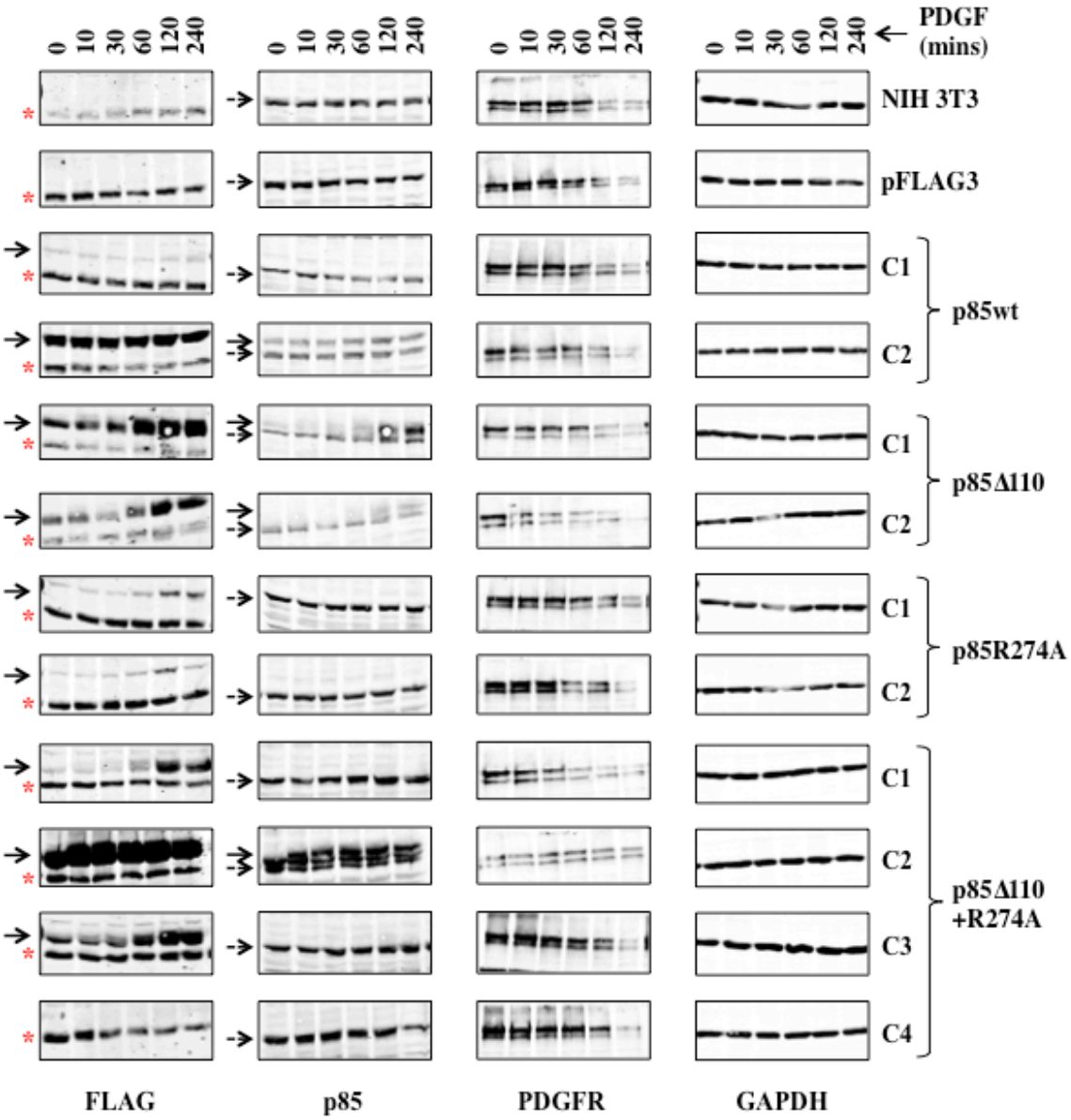

Figure 4.16: Input amount of pFLAG3-tagged p85 used for the anti-PDGFR IP. Cells stably expressing p85 wt or the different mutants were serum starved for 24 hours and then left unstimulated or stimulated with 50 ng/mL PDGF-BB for the indicated time course. Cells were lysed in PLC lysis and cleared of cellular debris by centrifugation. A fraction of the lysates were used for the anti-PDGFR IP described in Figure 4.15. Proteins (50 µg) were denatured with SDS sample buffer and resolved by SDS-PAGE followed by Western blot analysis using anti-FLAG, anti-p85, anti-PDGFR and anti-GAPDH antibodies. Similar results were obtained from three independent experiments. Solid arrows for both FLAG and p85 blots: exogenous pFLAG3-tagged p85. Broken arrow for p85 blots: endogenous p85. Red asterisks: non-specific bands.

4.4.1 PDGFR degradation rates and signalling kinetics of downstream effectors within stable cell lines expressing pFLAG3-p85ΔR+R274A compared to controls (pFLAG3-p85 wild type, pFLAG3-p85R274A and pFLAG3-p85ΔR)

p85 regulates PDGFR trafficking events and, therefore, signal transduction initiated by PDGFR (Chamberlain *et al*., 2008; Chamberlain *et al*., 2010). The consequence of disrupting p85-PDGFR interaction was examined by measuring receptor activation and degradation rates as well as the activation of downstream signalling molecules.

4.4.1.1 Effect of the ΔR or ΔR+R274A mutations in p85 on PDGFR activation and degradation

To test the impact of disrupting binding between p85 and PDGFR on signalling initiated by PDGF, NIH 3T3 cells stably expressing FLAG-p85wt or FLAG-p85 mutants (Figure 4.17) were stimulated with PDGF for increasing intervals of time. Cell lysates were probed for levels of activated receptor and the expression levels of PDGFR (Figure 4.18). As described earlier, in control cell lines (NIH 3T3, pFLAG3 and p85wt) the phosphorylation of PDGFR is induced within 10 minutes of growth factor stimulation and sustained for 30 minutes (Figure 4.18). The same pattern was observed in p85ΔR-expressing cells. Cells expressing p85R274A have a more sustained activation of PDGFR as shown by the high levels of tyrosine phosphorylated PDGFR at 2 hours of growth factor stimulation (Figure 4.18) and confirms a previous report (Chamberlain *et al*., 2004)). Cells expressing p85ΔR+R274A mimic receptor activation patterns of cells expressing p85R274A. In all clones expressing p85ΔR+R274A, it can be seen that there is an increase in the duration of receptor activation (pTyr blots) in comparison to control cells.

The rate of receptor degradation was measured by stimulating cells with PDGF for the indicated times and Western blotting with anti-PDGFR antibodies (Figure 4.18). Again, it can be seen that the total levels of PDGFR decrease after 30 to 60 minutes of growth factor stimulation in control cell lines (NIH 3T3, pFLAG3 and p85wt). Cells expressing p85ΔR are comparable to control cells with regards to the rate of PDGFR degradation. As previously reported, cells expressing p85R274A show a significant reduction in the rate of receptor degradation as compared to control cell lines (Figure 4.18 and (Chamberlain *et al*., 2004)). Similarly, a reduced rate of degradation of the receptor was also observed in cells expressing p85ΔR+R274A. These observations suggest that disrupting the interaction between p85 and PDGFR has no effect on the

p85 RabGAP-mediated signalling, since both p85R274A- and p85ΔR+R274A-expressing cells show the same response to PDGF stimulation. Regulation of PDGFR activation by the RabGAP function of p85 is independent of p85-PDGFR association.

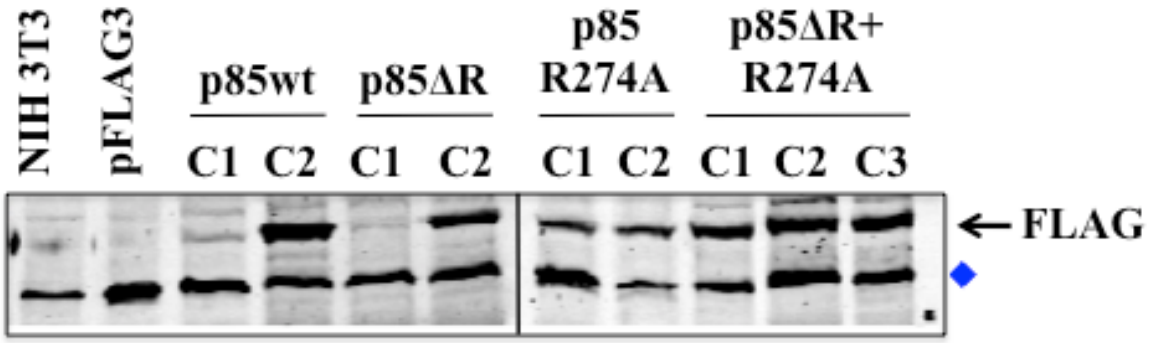

Figure 4.17: Relative expression of the different p85 mutants. NIH 3T3 cells stably expressing the indicated plasmids were harvested in SDS sample buffer for whole cell lysates. Proteins (200 μg) were resolved by SDS-PAGE and probed with anti-FLAG antibodies. Western blots were visualised using the LICOR scanner. Blue diamond: non-specific band.

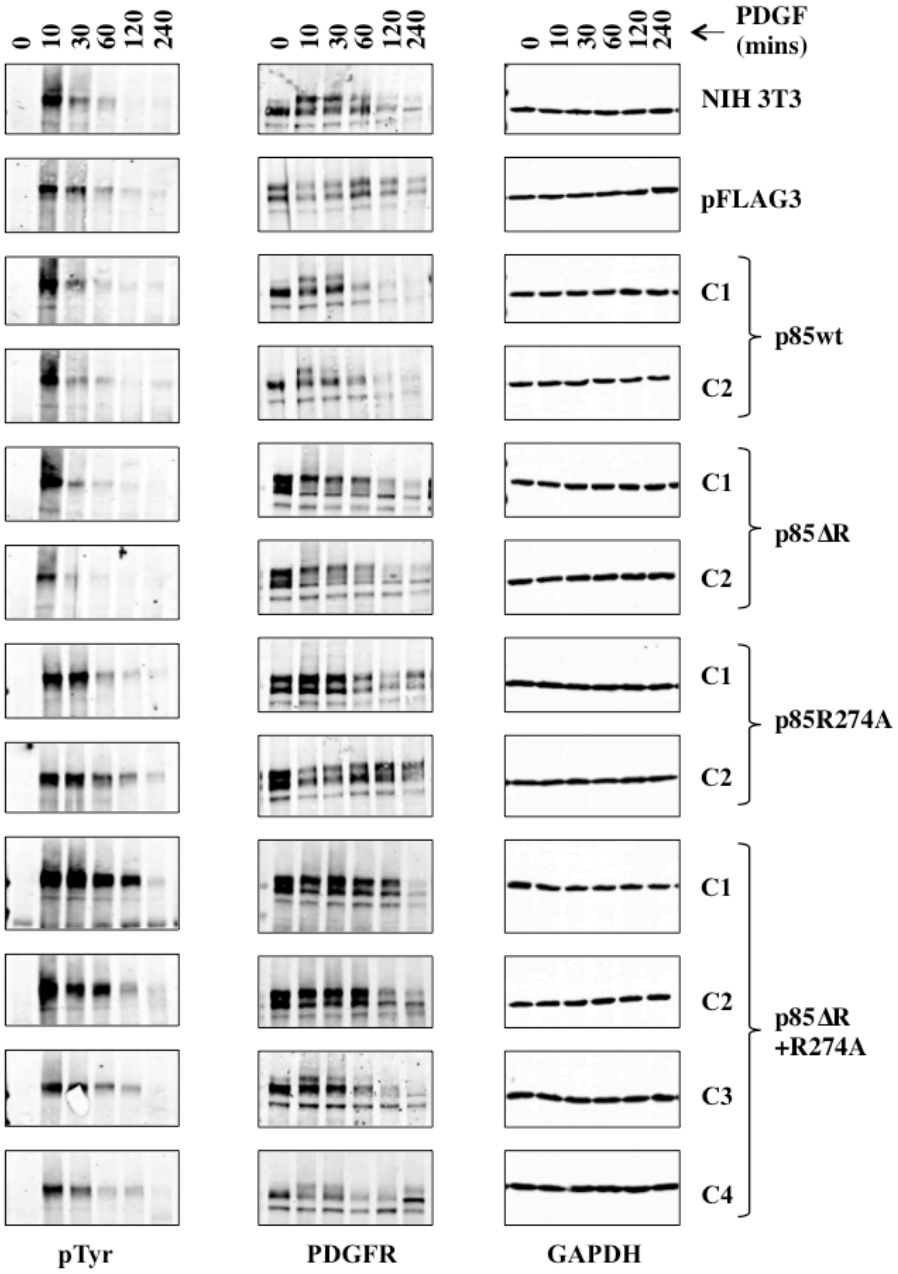

Figure 4.18: Prolonged PDGFR receptor activation and decreased receptor degradation in cells expressing p85R274A or p85ΔR+R274A. The cells indicated were serum starved (DMEM supplemented with 0.5% FBS) for 24 hours and then left unstimulated or stimulated with 50 ng/mL PDGF-BB for 10, 30, 60, 120 and 240 minutes. Proteins were resolved by SDS-PAGE followed by Western blot analysis using anti-pTyr, anti-PDGFR and anti-GAPDH antibodies. Similar results were obtained in three independent experiments.

4.4.1.2 Effect of ΔR or ΔR+R274A mutation on downstream signalling in response to PDGF

PDGFR signalling cascade induces downstream signalling through cascades such as the MAPK/Erk pathway or the PI3K/Akt pathway. Having observed that the PDGFR is highly activated in p85ΔR+R274A-expressing cells, the impact of this prolonged activation on downstream signalling was examined. In control cells (NIH 3T3, pFLAG3 and p85wt) as well as cells expressing p85ΔR, MAPK/Erk was activated 10 minutes after growth factor stimulation and levels of activated MAPK/Erk started to decline 60 to 120 minutes after growth factor stimulation. p85R274A-expressing cells showed an enhanced activation of the MAPK/Erk pathway (Figure 4.19 and (Chamberlain *et al*., 2004)). Expression of p85ΔR+R274A resulted in an induction of MAPK/Erk phosphorylation from 10 minutes to 4 hours after growth factor stimulation. In both p85R274A- and p85ΔR+R274A-expressing cells there are very low amounts of pMAPK/Erk present before growth factor stimulation indicating that in these cells the MAPK/Erk pathway may be constitutively (although only modestly) active.

The effect of the ΔR+R274A mutation on the PI3K/Akt pathway was also measured by Western blotting with phospho-specific antibodies for Akt (Figure 4.20). In all cell lines tested, Akt phosphorylation was induced from 10 minutes to 4 hours. There was no significant difference in the activation of the Akt pathway between control cells and cells expressing p85ΔR, p85R274A or p85ΔR+R274A.

We hypothesized that p85-PDGFR interaction is necessary to localize the RabGAP function of p85. We would therefore expect cells expressing p85ΔR and p85ΔR+R274A-expressing cells to have similar phenotypes. However, it was observed that cells expressing ΔR+R274A have similar responses to PDGF in comparison to p85R274A-expressing cells, with regards to activation of PDGFR and of its downstream effectors. The results obtained so far suggest that p85 does not need to be associated with activated PDGFR to exert its RabGAP regulatory effects on PDGFR activation and signalling.

4.4.2 Oncogenic properties of cells expressing pFLAG3-p85ΔR+R274A (a RabGAP defective p85 that is unable to bind PDGFR)

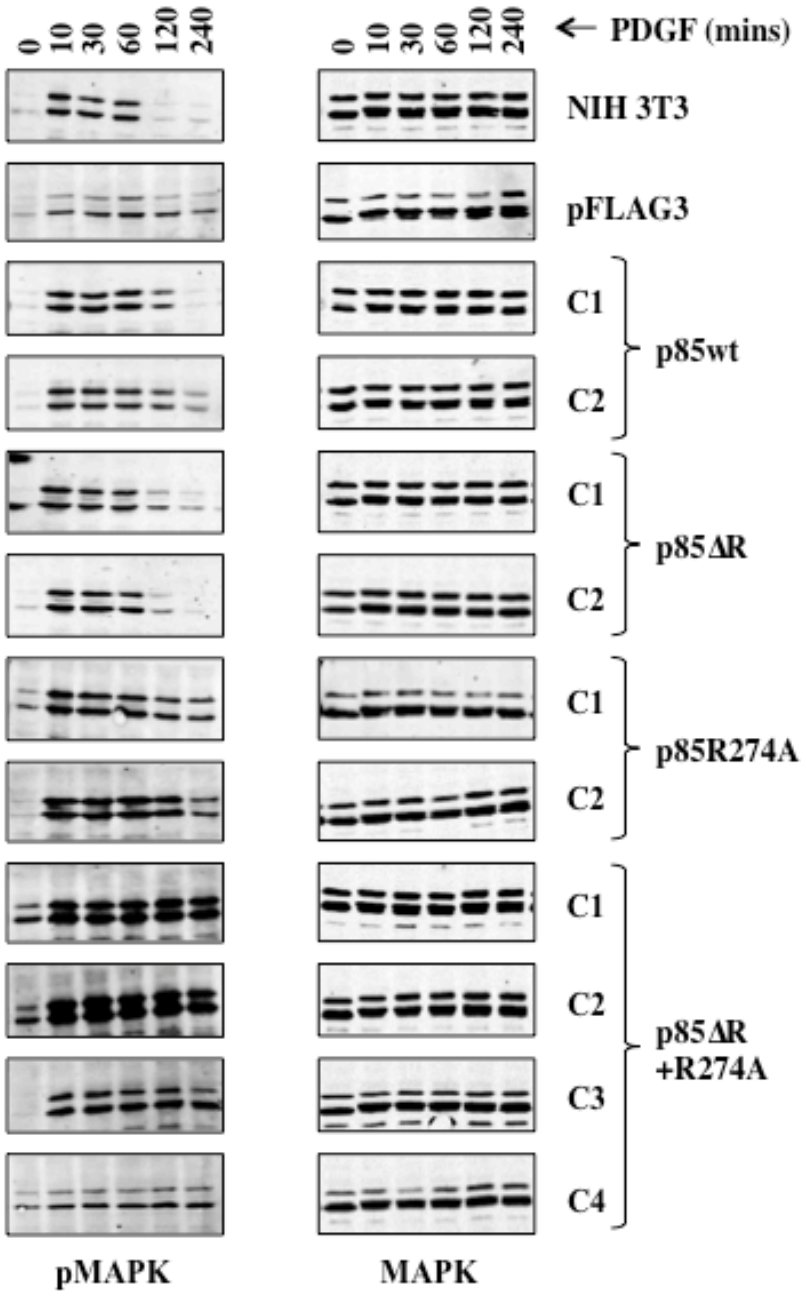

Figure 4.19: Sustained MAPK signalling downstream of PDGFR in cells expressing p85R274A or p85ΔR+R274A. Cells were serum starved (DMEM supplemented with 0.5% FBS) for 24 hours and then left unstimulated or stimulated with 50 ng/mL PDGF-BB for 10, 30, 60, 120 and 240 minutes. Proteins were resolved by SDS-PAGE followed by Western blot analysis using anti-pMAPK/Erk and anti-MAPK/Erk antibodies. Similar results were obtained in three independent experiments.

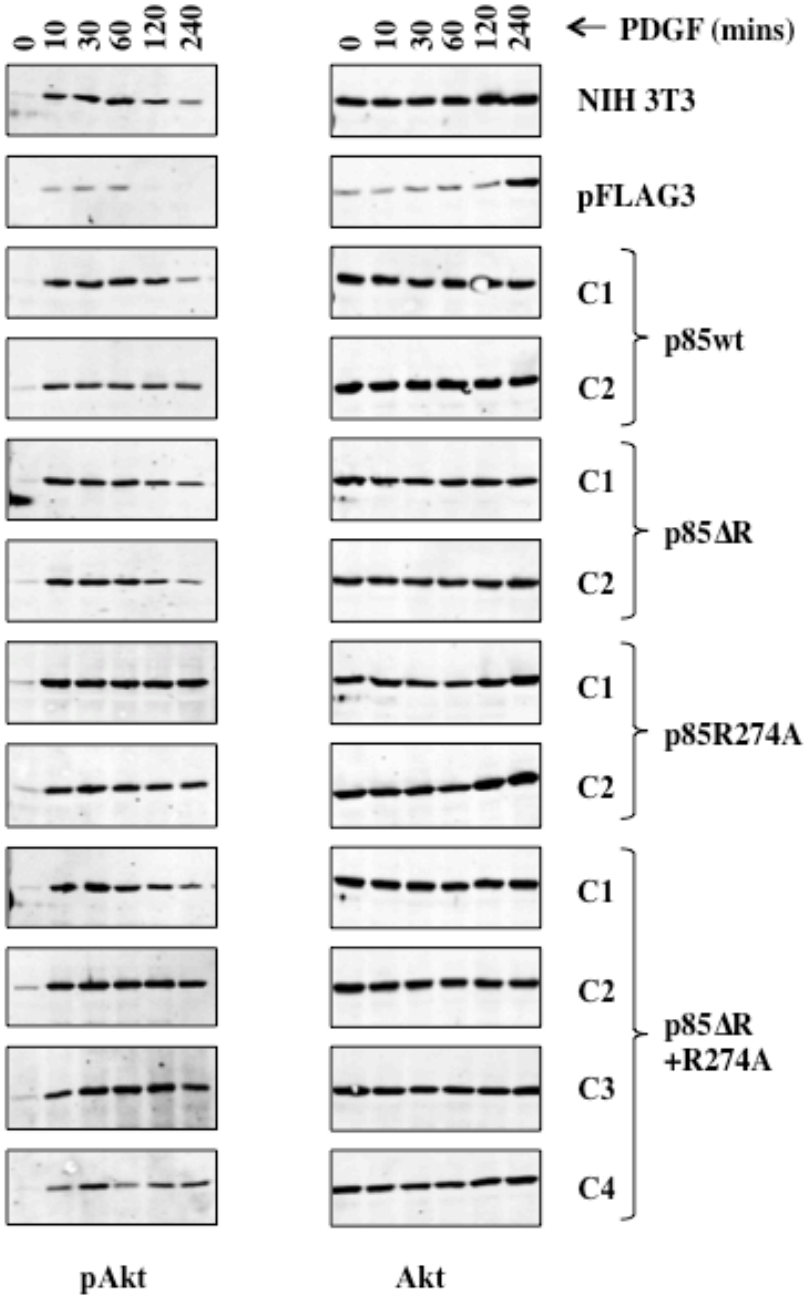

Figure 4.20: Akt signalling is unaffected by the expression of any p85 variant in cells stimulated with PDGF. Cells were serum starved (DMEM supplemented with 0.5% FBS) for 24 hours and then left unstimulated or stimulated with 50 ng/mL PDGF-BB for 10, 30, 60, 120 and 240 minutes. Proteins were resolved by SDS-PAGE followed by Western blot analysis using anti-pAkt and anti-Akt antibodies. Similar results were obtained in three independent experiments.

It was shown in the previous section that cells expressing p85ΔR+R274A have a sustained activation of signalling in response to PDGF. A disruption in the 'brakes' and 'checkpoints' required to halt cellular proliferation can lead to transformation of cells. The oncogenicity of these cell lines was therefore characterized.

4.4.2.1 Loss of contact inhibition (Foci formation assay)

The foci formation assay described in Section 4.3.2.1 was used to determine whether cells expressing p85ΔR+R274A were transformed. Cells expressing p85wt or p85ΔR did not form foci (Figure 4.21). Furthermore, cells expressing p85ΔR+R274A clone 1 failed to form any foci. However, p85R274A- and p85Δ110+R274A-expressing cells (clones 2, 4, and to some extent 3) formed foci indicating that these cells are transformed. Expression of p85ΔR+R274A results in the formation of foci and based on this assay, cells expressing this mutant could be considered transformed, just as are cells expressing p85R274A. These results further support the observations made with the response to PDGF experiments and suggest that p85-PDGFR association is not critical for the regulation of PDGFR signalling by the RabGAP function of p85.

4.4.2.2 Anchorage independent growth (Colony formation in soft agar)

The colony formation in soft agar assay described in Section 4.3.2.2 was also used to test whether p85ΔR+R274A-expressing cells are transformed. Colonies of cells expressing either pFLAG3 vector or p85wt had an average diameter of 17 μm. Colonies that were 17 μm and above in diameter were counted and reported (Figure 4.23). Cells expressing p85ΔR developed colonies that were at most twice the diameter of colonies produced by cells expressing the vector or p85wt (Figure 4.22). These colonies ranged in size from 17 to 33 μm in diameter. Cells expressing p85ΔR appeared to form more colonies per cells seeded than vector and p85wt control, however the apparent increase in number of colonies especially in clone number 2 was not statistically significant (Figure 4.23).

Cells expressing p85R274A or p85ΔR+R274A formed numerous colonies (Figure 4.22). These cells have lost their response to contact inhibition as well as their anchorage dependence and are therefore transformed. As shown before, p85R274A-expressing cells formed colonies in soft agar that typically ranged in size from 17 to 150 μm in diameter, with a few colonies getting

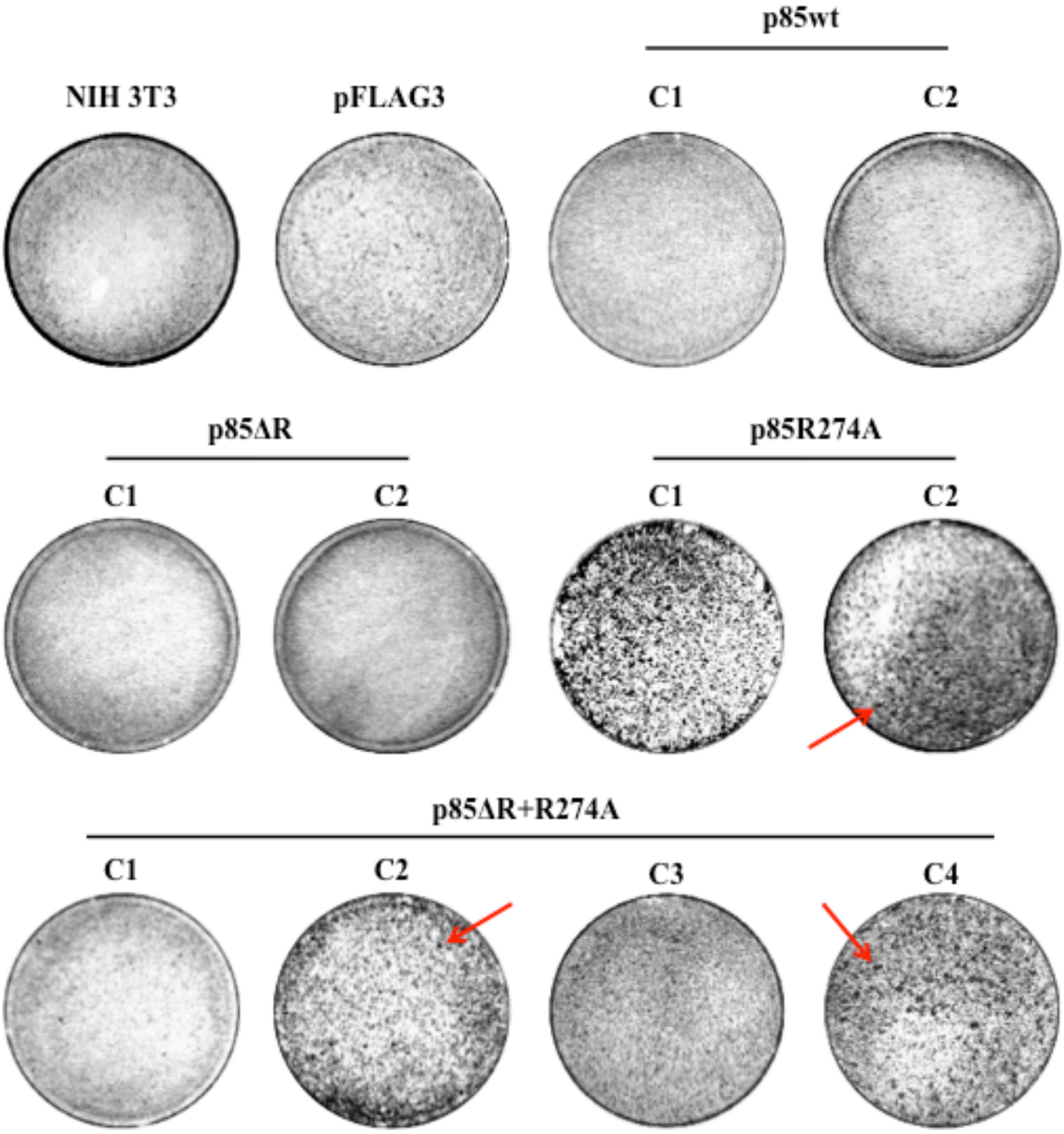

Figure 4.21: Cells expressing p85R274A and p85ΔR+R274A form foci in a contact inhibition assay. Cells were seeded at a density of 100 000 cells/10 cm dish and maintained in normal medium for 2 weeks. Cells were fixed in -20 °C methanol, stained with Giemsa stain and photographed. Images are representative of the entire 10 cm dish from four different experiments. C: clone number. Red arrows point to foci.

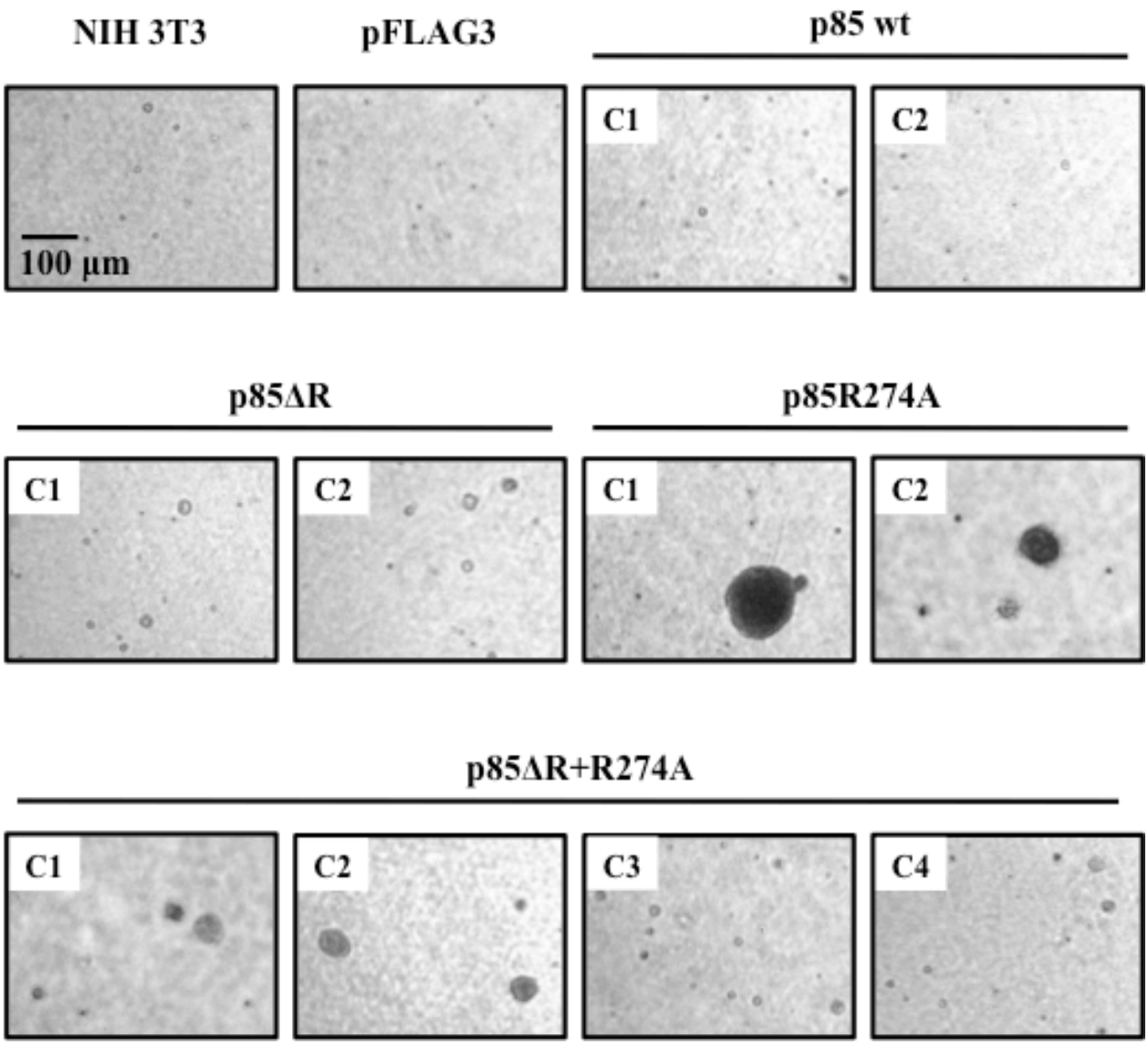

Figure 4.22: Cells expressing p85R274A and p85ΔR+R274A form colonies in soft agar. Fifty thousand cells/ 6 cm dish were cultured in a suspension of top agar (3.75X MEM vitamins, 0.36% agar) above of a layer of bottom agar (5X MEM vitamins, 0.61% agar). Cells were left to grow for a total of 20 days. Images are representative of the colonies formed by the individual cells expressing the different p85 mutants in four different experiments. Colonies are spots that are 17 µm or larger in diameter.

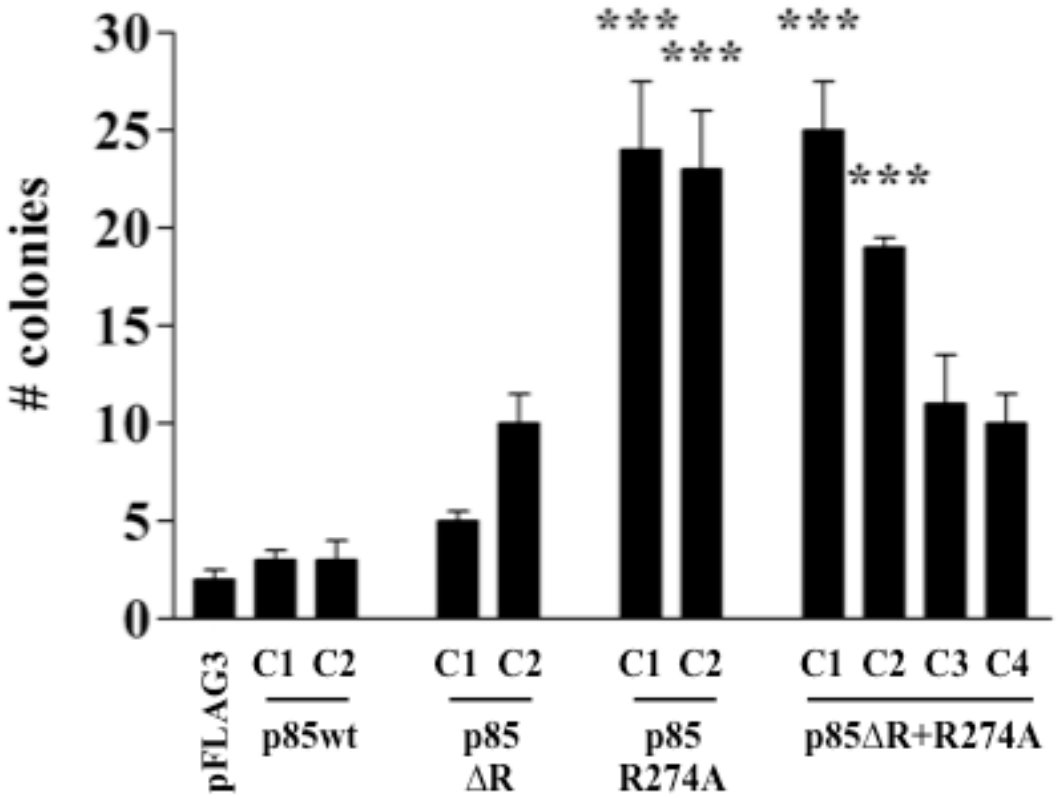

Figure 4.23: Cells expressing p85R274A and p85ΔR+R274A form numerous colonies in soft agar. The graph depicts the number of colonies formed per 50 000 cells plated that are expressing different p85 mutants (see Figure 4.22). Each value represents the mean (± SD) of four independent experiments, each based on three separate viewing fields. Bonferroni's multiple comparison test indicates ***, $p<0.001$ as compared to the vector control or either of the p85wt clones.

as big as 300 μm in diameter. Clones 1 and 2 of p85ΔR+R274A-expressing cells developed colonies that were between 17 to 73 μm in diameter. On the other hand, clone 3 and the mixed population referred to as clone 4 formed colonies that ranged in size from 17 to 39 μm in diameter. The data suggests that expression of a RabGAP defective p85 (p85R274A) results in a loss of anchorage dependence. Expression of a p85 mutant unable to bind to the receptor results in a marginal loss in anchorage dependence as shown in cells expressing p85ΔR or in two clones of cells expressing p85ΔR+R274A (clones 3 and 4), which is not statistically significant. The data also showed a statistically significant increase in the loss of anchorage dependence of cells expressing p85ΔR+R274A in clones 1 and 2.

4.4.2.3 Attachment to fibronectin and collagen (Adhesion assay)

As mentioned earlier, cell adhesion to ECM is vital to the normal function of the cell. The adherent properties of cells expressing p85ΔR and p85ΔR+R274A were investigated by examining the ability of these cells to attach to fibronectin and collagen I coated surfaces. Adherence to fibronectin was not altered by expression of p85ΔR, p85R274A or p85ΔR+R274A. When compared to cells expressing p85wt, three out of the four clonal isolates of p85ΔR+R274A are less adherent to collagen I, as are p85R274A-expressing cells. Clone 1 of p85ΔR+R274A-expressing cells shows a significant increase in attachment to collagen I. This suggests that expression of p85R274A or p85ΔR+R274A can result in cells adhering less to collagen I. Transformed cells can become more or less adherent to components of the ECM (Junker and Heine, 1987).

4.4.2.4 Migratory properties (Boyden chamber assay)

The Boyden chamber was again used to determine whether cells expressing p85ΔR or p85ΔR+R274A are able to migrate. All cells migrated to the same extent towards 0.1% FBS. Cells expressing p85wt clone 2 were migrated more towards the chemoattractant than cells expressing p85wt clone 1. Statistical analyses were performed in relation to p85wt clones 1 and 2 separately. Cells expressing p85ΔR migrated towards 10% FBS much less than p85wt control cells (Figure 4.25). Whereas cells expressing p85R274A clone 2 are significantly more migratory than both p85wt- and p85ΔR-expressing cells. Cells expressing p85ΔR+R274A clones 3 and 4 migrated more readily towards 10% FBS in comparison to both p85wt- and p85ΔR-expressing

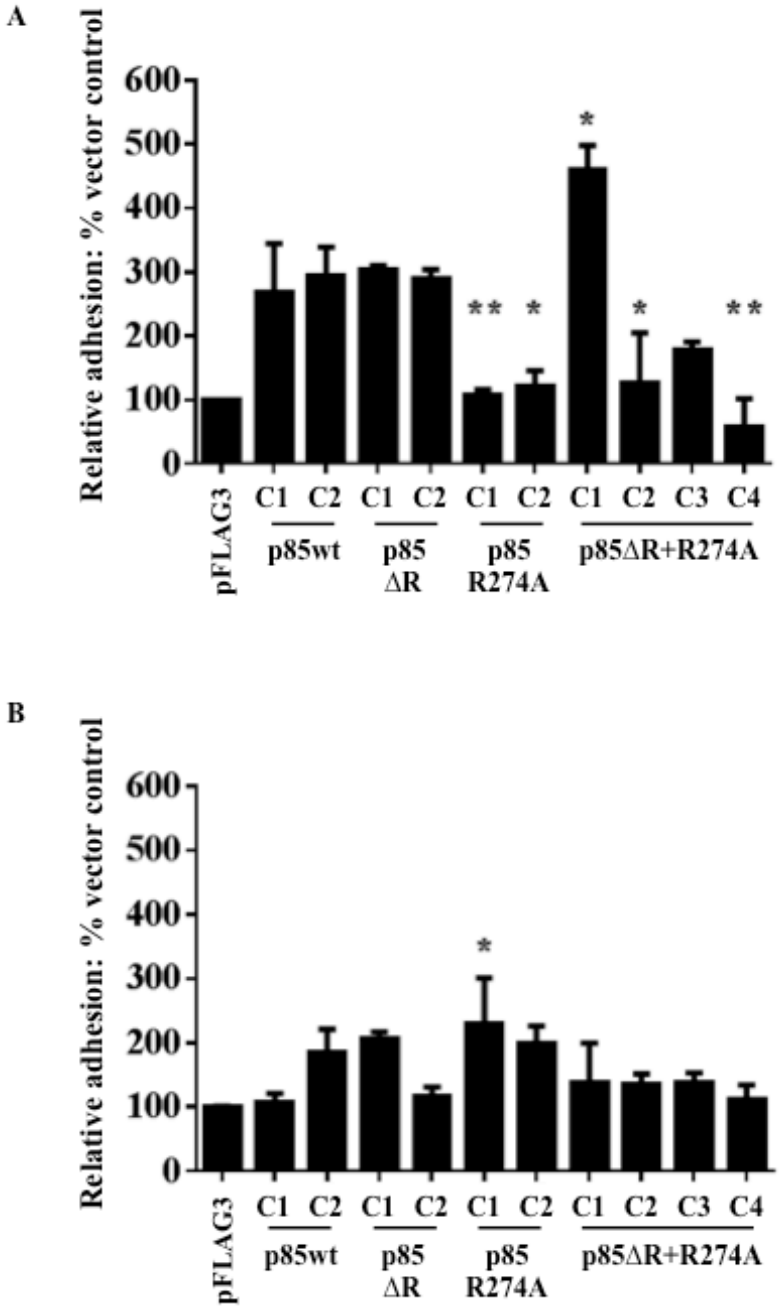

Figure 4.24: Cells expressing p85R274A and p85ΔR+R274A are less adherent to collagen coated surfaces. Cells were seeded on 96-well plates coated with either collagen (A) or fibronectin (B) and incubated for 30 minutes. Cells were then washed, fixed, stained with crystal violet and solubilised. Relative fluorescent units were quantified at 550 nm and represented relative to cells expressing the vector control (pFLAG3) from two independent experiments with triplicate measurements for each experiment. Bonferroni's multiple comparison test indicates * $p<0.05$, ** $p<0.01$ as compared to p85wt.

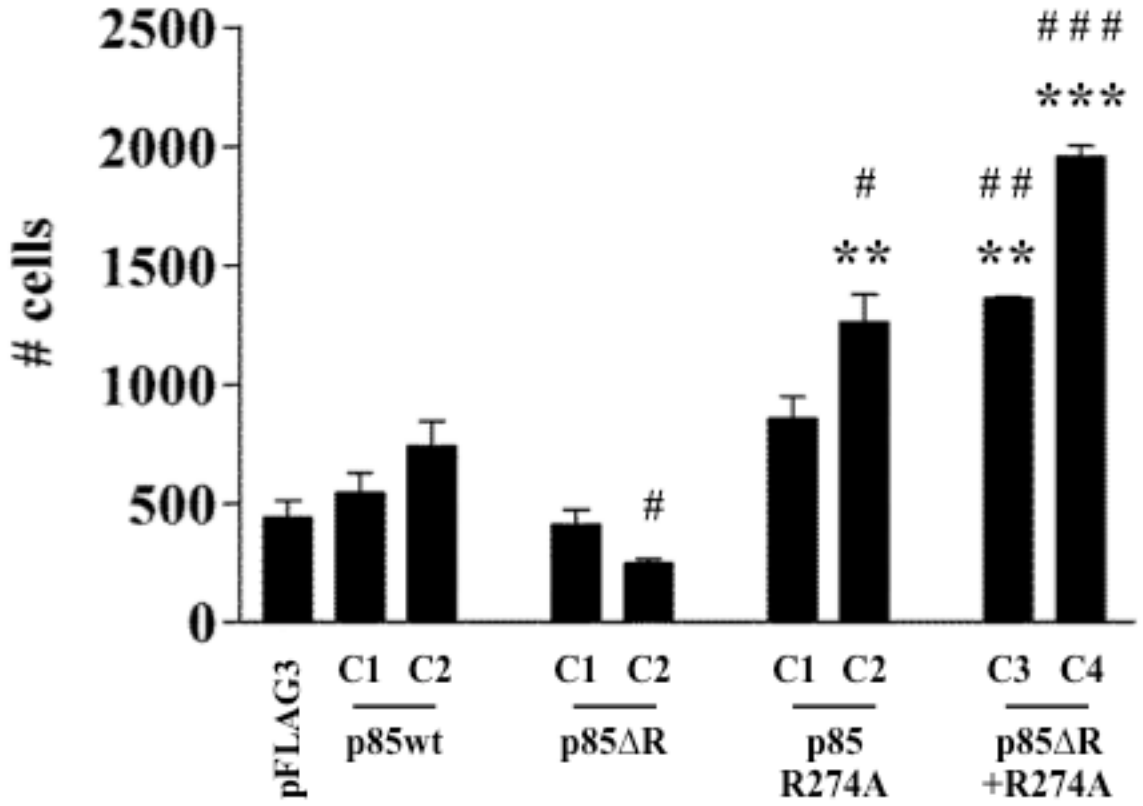

Figure 4.25: p85R274A and p85ΔR+R274A mutations induce more migration of cells, whereas the p85ΔR mutation induces less migration. Cells were seeded into the top section of a Boyden chamber and left to migrate towards either 0.1% FBS or 10% FBS at 37 °C for 24 hours. Cells that had migrated to the bottom side of the Boyden chamber were then fixed and stained with hematoxylin. Cells observed in 8 fields of view at a magnification of 20X were quantified using the Stereo Investigator software (MBF Bioscience). The results represent cells that migrated towards 10% FBS minus cells that migrated towards 0.1% FBS from one experiment with duplicate measurements. Bonferroni's multiple comparison test indicates ** $p<0.01$, *** $p<0.001$ as compared to p85wt C1 and # $p<0.05$, ## $p<0.01$, ### $p<0.001$ as compared to p85wt C2.

cells (Figure 4.25). Cells expressing p85ΔR+R274A clone 4 had an even higher rate of migration than p85R274A-expressing cells. The data indicate that expression of p85R274A or p85ΔR+R274A results in an increase in the migratory properties of cells.

5.0 DISCUSSION

The p85 protein plays several different roles in regulating signalling by, as well as the trafficking of, activated growth factor receptors such as the PDGFR (Mellor *et al*., 2012). Our laboratory previously reported on a role for p85 in regulating the Rab GTPases, Rab5 and Rab4, which in turn regulate PDGFR intracellular trafficking and degradation (Chamberlain *et al*., 2004; Chamberlain *et al*., 2010). Expression of a RabGAP defective mutant of p85, e.g. p85R274A, acts as a dominant negative mutant and blocks degradation of activated PDGFR, alters receptor trafficking and transforms cells (Chamberlain *et al*., 2004; Chamberlain *et al*., 2008; Chamberlain *et al*., 2010). These observations suggest that the RabGAP activity of p85 is associated with PDGFR down-regulation. The main objective of this research was to determine whether p85R274A needs to associate physically with either the p110β catalytic subunit of PI3K and/or with the PDGFR in order to elicit its effects on receptor signalling, trafficking and transformation of cells.

5.1 Role of p85-p110β binding in the RabGAP function of p85

5.1.1 p110β as the switch stabilization effector protein of the p85-p110β GAP protein complex

The selective interaction between p110β and Rab5-GTP (Christoforidis *et al*., 1999b) suggests binding within the conformation-specific switch regions. This interaction may be important for a number of different functions including switch region stabilization by p85-p110β during Rab5 effector functions that require Rab5GTP and the generation of lipid substrates required for tethering and fusion of endocytic vesicles. It should also be noted that p85 is unable to stabilize the switch regions of Rab5 and would therefore require a binding partner in order to perform the full function as a GAP protein (Scheffzek et al., 1998a; Terzyan et al., 2004).

It was hypothesized that if p110β contributes to the GAP activity (possibly through switch stabilization), then cells expressing p85Δ110+R274A would be expected to have phenotypes even more pronounced than p85R274A-expressing cells. Expression of p85Δ110+R274A led to an even more prolonged activation of PDGFR, reduced rates of degradation and a more sustained MAPK/Erk signalling than was previously described for p85R274A-expressing cells (Chamberlain *et al*., 2004). The enhanced signalling observed in p85R274A-expressing cells resulted in transformation of cells (Chamberlain *et al*., 2004;

Chamberlain *et al*., 2008). Yet, contrary to the cellular transformation seen for p85R274A-expressing cells, expression of p85Δ110+R274A did not lead to cellular transformation. This was clear from the lack of foci formation on over-confluent plates and colony formation in a semi-solid environment (soft agar). Even though the cells expressing p85Δ110+R274A neither formed foci nor colonies in soft agar, half of the clones tested (clones 2, 3) were less adherent to collagen I, similar to p85R274A-expressing cells. However, the other two clones expressing p85Δ110+R274A (clones 1, 4) adhered to collagen I to the same extent as cells expressing p85wt further suggesting that these cells are not transformed. The differences in adhesion to collagen I in clonal isolates of p85Δ110+R274A-expressing cells could be due to the levels of FLAG-tagged p85 expressed by the individual clones. Cells expressing p85Δ110+R274A clones 2 or 3 expressed similar levels of FLAG-tagged p85 in comparison to cells expressing p85R274A clone 2 and may account for the similar pattern of adhesion that was observed. It should be noted that clone 4 of p85Δ110+R274A-expressing cells also expressed similar levels of FLAG-tagged p85 when compared to cells expressing p85R274A clone 2.

Cell adhesion to the extracellular matrix (ECM) can be used as a marker of cellular transformation. Transformed cells may change the properties of adhesion to the different proteins found on other cells or found within the ECM. For example, collagen (type IV) serves as a scaffold for laminin as well as other components of the basement membrane (Yurchenco *et al*., 1986). A loss of collagen due to an increase in collagenase activity promotes invasion and metastasis (Junker and Heine, 1987). Fibronectin is important for mesenchymal cell attachment, cell proliferation and cell migration (Bitterman *et al*., 1983; Couchman *et al*., 1982; Hynes and Yamada, 1982). It is often reduced and/or absent from the surface of transformed cells (Junker and Heine, 1987). During the initial stages of metastasis, transformed cells can degrade and invade the ECM allowing these cells to migrate to secondary locations. The MAPK/Erk signalling pathway is a regulator of cell migration and invasion. In terms of the migratory properties of cells expressing p85Δ110+R274A, two of the clones tested (clones 2, 4) appeared to migrate better than p85wt-expressing cells, as had already been observed with p85R274A-expressing cells. The other two clones tested (clones 1, 3) migrated to the same extent as p85wt-expressing cells.

Even though 15 – 20% expression of p85R274A (compared to endogenous p85 levels) is sufficient to disrupt receptor signalling and trafficking events (Chamberlain *et al*., 2004;

Chamberlain *et al*., 2008), it is possible that this same level of expression is not enough to observe the RabGAP effects of p110β on receptor signalling and trafficking. It should be noted that all the cell lines used in this thesis express endogenous levels of p85 and the effects of p85 mutations may be reduced by normal signalling from endogenous p85. Alternatively, the different functions of the PI3K complex, i.e. p85-mediated GAP activity (arginine finger), p110β-mediated GAP activity (switch stabilization) and lipid generation, may yield different phenotypes, and the assays used to test cell transformation in this thesis simply indicated the sum of an individual change within the p85 protein on any one or all three of these functions. It has already been shown that Rab5-GTP, and not Rab5-GDP, Rab4 or Rab11, can bind to p110β (Christoforidis *et al*., 1999b). This implies that p110β exerts its effects only on Rab5 and therefore only at the internalization step, whereas p85 has effects on both internalization and recycling during endocytosis since it serves as a GAP for both Rab5 and Rab4 (Chamberlain *et al*., 2004). Phosphatidylinositol-3,4,5-trisphosphate ($PI3,4,5P_3$) generated by p110β can also supply Rab5-associated phosphatases with the substrate required to produce phosphatidylinositol-3-phosphate (PI3P), which is important for tether formation between the vesicle budded off from the plasma membrane and the early-sorting endosome (again, the internalization step). Indeed, it has recently been reported that in response to growth factor limitation, p110β can bind to Rab5-GTP and enhance the interaction between Rab5 and Vps34 (Dou *et al*., 2013). Vps34 is a class III PI3K catalytic subunit that converts phosphatidylinositol (PI) to PI3P. The study conducted by Dou and colleagues also revealed that p110β is important for Rab5 activation, although they also show that binding of p110β to Rab5 antagonizes the Rab5GAP activity of p85 (Dou *et al*., 2013). The non-canonical functions of p110β therefore include switch stabilization of Rab5, regulation of Rab5 activation as well as regulation of internalization of receptor complexes and fusion to the early sorting endosome.

5.1.2 Model for cells expressing p85Δ110 or p85Δ110+R274A

Based on the hypothesis that p110β provides the switch stabilization function, it was further hypothesized that p110β needed to be bound to p85 for full GAP activity. As mentioned above, the different functions of the PI3K complex (i.e. p85-mediated GAP activity (arginine finger), p110β-mediated GAP activity (switch stabilization) and p110β-mediated lipid

generation) may yield different phenotypes and may explain the varying phenotypes observed during the course of these studies.

The following model is proposed for cells expressing p85Δ110 or p85Δ110+R274A. In NIH3T3 cells, activated PDGFR complexes are internalized and loaded onto the early/sorting endosome *via* a Rab5-mediated pathway. From the early/sorting endosome PDGFRs are either deactivated and recycled back to the plasma membrane rapidly by a Rab4-dependent process, or slowly by a Rab4 and Rab11-dependent process, or ubiquitinated and sorted into a lysosomal degradation pathway. In this endocytic pathway, p85 functions as a GAP protein for Rab5 and Rab4 enhancing their intrinsic GTPase activity and maintaining the normal balance between activation and duration of signalling. p110β can bind to Rab5-GTP. Binding of p110β to Rab5 can serve 2 functions: 1) provide a switch stabilization function to enhance GAP activity, and/or 2) provide PI3,4,5P_3 which can serve as a substrate for phosphatases associated with Rab5 and EEA1 to generate PI3P required for the tether formation during vesicle fusion.

In p85Δ110-expressing cells, it is proposed that p85 is unable to bind p110β preventing the formation of the p85-p110β-Rab5 complex. A slight increase in the rate of internalization will be expected since p110β cannot bind to p85-Rab5 and enhance its GAP activity. GAP activity will remain low since p110β is unable to provide the switch stabilization function. Also, the tether formation could be slightly impaired (Note: there are other sources of PI3P for tether formation). The rate of recycling and degradation will not be affected since p110β only regulated the function of Rab5 at the internalization step. Cells expressing p85Δ110 were comparable to NIH 3T3 cells.

In cells expressing p85R274A, p85 is RabGAP defective and is unable to inactivate Rab5 and Rab4. This is expected to result in an increase in both receptor internalization (Rab5-dependent) and recycling (Rab4-dependent), with limited receptor degradation. In support of this model, p85R274A-expressing cells show increased rate of PDGFR internalization and recycling (Chamberlain *et al*., 2010) and reduced PDGFR degradation (Chamberlain *et al*., 2004; Chamberlain *et al*., 2010). These cells also showed prolonged PDGFR activation and enhanced signalling (MAPK/Erk) (Chamberlain *et al*., 2004). Cells expressing p85R274A were also shown to be transformed (Chamberlain *et al*., 2008).

It is postulated that in cells expressing p85Δ110+R274A, the RabGAP defective p85 is unable to bind p110β preventing the formation of the p85-p110β-Rab5 complex. This effect is

expected to result in an increase in receptor internalization (Rab5-dependent) and recycling (Rab4-dependent), with limited receptor degradation. That is, we expect these cells to have phenotypes comparable to cells expressing p85R274A. Even though p85Δ110+R274A-expressing cells have prolonged PDGFR activation, enhanced signalling (MAPK/Erk) and reduced PDGFR degradation, these cells are not transformed.

Based on the working model, it appears that a defect in GAP activity of p85 alone could be enough to lead to transformation of cells. When binding of p110β to the p85R274A RabGAP defective variant is abolished, cellular transformation seems to be reversed. Yet, receptor signalling remains enhanced, probably due to the fact that the receptor complexes are sequestered either at the plasma membrane or within the budding vesicle, and consequently are incapable of reaching the early/sorting endosome. These data support the involvement of p110β in the RabGAP function of p85.

5.2 Role of the binding of p85 to the PDGFR in the RabGAP function of p85

5.2.1 p85-PDGFR binding for full GAP function of p85

It was hypothesized that PDGFR may help localize the RabGAP function of p85 through the binding of p85 to phosphorylated tyrosine residues on activated PDGFR. During internalization and uptake of activated receptor complexes into endosomes, Rab5 binds to both the internalized vesicle and the early sorting endosome and is therefore appropriately positioned to interact with p85. If p85-PDGFR binding is necessary to localize the RabGAP function of p85, p85ΔR-expressing cells and cells expressing p85ΔR+R274A would be expected to have similar phenotypes, but different from other p85 variants that would retain their ability to bind PDGFR. The hypothesis was tested by examining the levels of activated receptor and total receptor levels as well as the levels of molecules in downstream signalling cascades, such as MAPK/Erk. Whereas cells expressing p85ΔR have normal signalling, e.g. similar to signalling in control NIH 3T3 cells and cells expressing p85wt, the cells expressing p85ΔR+R274A have a much more prolonged activation of PDGFR, a slower degradation rate of PDGFR, and a sustained activation of the MAPK/Erk pathway, just like cells expressing p85R274A. Defects in receptor activation, signalling and down-regulation have been implicated in a number of different cancers (Bache *et al*., 2004; Knowles *et al*., 2009; Platt *et al*., 2009; Sebastian *et al*., 2006). Cells expressing p85ΔR+R274A were transformed, just like p85R274A-expressing cells.

Both p85R274A- and p85ΔR+R274A-expressing cells had lost response to contact inhibition as demonstrated by the formation of foci in clones 2 and 4 of p85ΔR+R274A-expressing cells and both clones of p85R274A-expressing cells. The R274A mutation also affected the cells' ability for anchorage-dependent growth (e.g. clones 1 and 2 of p85ΔR+R274A-expressing cells and both clones of p85R274A-expressing cells easily formed colonies in a semi-solid environment (soft agar)).

As mentioned earlier, cell adhesion is important for cell-cell communication and for the maintenance of cellular structure. When adherent cells become transformed they can change their adherent properties by modifying how they interact with various components of the ECM. Cells expressing p85R274A or p85ΔR+R274A (clones 2, 3 and 4) were less adherent to collagen I when compared to p85wt control cells. Collagen is important for providing and maintaining the integrity of the ECM. Loss of adherence to collagen suggests that these cells are able to detach from the extracellular matrix. The migration assay utilized in this research confirmed that cells expressing p85R274A as well as cells expressing p85ΔR+R274A (clones 2, 3 and 4) were more migratory than cells expressing p85wt. This corroborates the observation that cells expressing p85R274A are invasive (Chamberlain *et al*., 2008).

In summary, expression of p85R274A or p85ΔR+R274A in cells leads to an increased PDGFR activation and sustained PDGFR-mediated signalling, and decreased PDGFR down-regulation, which eventually culminates in changes in cellular behaviour that support a tumorigenic phenotype. The effects observed are more pronounced in p85ΔR+R274A-expressing cells. Taken together, these results suggest that binding of p85 to PDGFR is not necessary for the localization of the RabGAP function of p85.

5.2.2 p85 as a GDI displacement factor (GDF)

Our laboratory has shown that p85 is able to bind both Rab5-GDP and Rab5-GTP (Chamberlain *et al*., 2004), unlike most GAP proteins that bind selectively to the GTP forms only. The inactive Rab5-GDP is cytosolic and exists in complex with a GDP-dissociation inhibitor (GDI) that obstructs the lipid geranylgeranylated site that is crucial for membrane insertion (Leung *et al*., 2007; Leung *et al*., 2006; Pfeffer and Aivazian, 2004; Wu *et al*., 2010). Rab5 is recruited to the vesicle that buds off from the plasma membrane and the early sorting endosome by the removal of the GDI and the subsequent exchange of GDP for GTP, which is

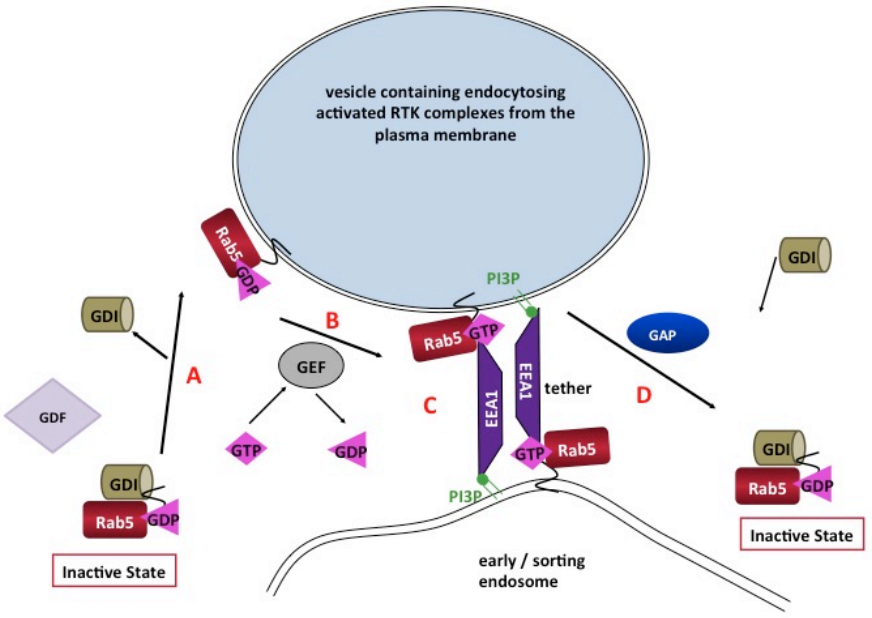

Figure 5.1: Mechanism of Rab5 regulation. A) Lipid prenylated Rab GTPases are cytosolic and inactive in their GDP-bound conformations bound to a cytosolic protein termed GDP-dissociation inhibitor (GDI) that masks the lipid prenylation site. The GDI is displaced by a GDI displacement factor (GDF) and Rab5-GDP is delivered to both the membrane of the vesicle containing activated receptor tyrosine kinases (RTKs) that have budded off from the plasma membrane and the early endosomal membrane. This step has been speculated to occur in conjunction with unidentified targeting factors that may aid in Rab5-GDP recruitment (for e.g. PDGFR/p85?). B) A guanine nucleotide exchange factor (GEF) promotes the exchange of GDP for GTP resulting in an active form of Rab5. C) Rab5-GTP recruits effector proteins such as EEA1 to the membrane and vesicle fusion events are initiated. Active Rab5-GTP binds effector proteins like EEA1, a protein important in tethering two membranes together by binding both Rab5-GTP and the lipid phosphatidylinositol 3-phosphate (PI3P). D) Rab5 has the intrinsic ability to hydrolyse the bound GTP to GDP with the aid of GTPase activating protein (GAP proteins), returning Rab5 to an inactive state and resulting in the re-extraction of Rab5 from the membrane by binding to GDI.

facilitated by a guanine nucleotide exchange factor (GEF) (Pfeffer and Aivazian, 2004; Wu *et al*., 2010). The removal of the GDI is facilitated by a GDI displacement factor (GDF) (Figure 5.1) (Pfeffer and Aivazian, 2004; Sivars *et al*., 2003; Wu *et al*., 2010).

Since p85 binds to Rab5-GDP as well as activated PDGFR it may help recruit Rab5 to vesicle membranes containing activated PDGFR signalling complexes. The formation of a p85/Rab5-GDP/PDGFR complex places Rab5 in proximity to a membrane. This allows the GDI to be displaced, possibly, by p85 and allows Rab5 to be inserted into the membrane and be fully activated by the exchange of GDP for GTP. This hypothesis is being tested in our laboratory as components of three graduate thesis projects. (i) The first examines the importance of p85-PDGFR binding for the normal functioning of the Rab5 pathway by measuring the levels of Rab5-GTP and its effects on signalling events initiated by PDGF (this thesis). (ii) The second project investigates p85 binding sites on Rab5 through mutational analysis (Dielle Detillieux, MSc thesis). (iii) The third project analyzes the effect of activated PDGFR on p85 Rab5GAP activity using GAP assays, and the effect of activated PDGFR on p85-Rab5 binding by using surface plasmon resonance (SPR) (Cody Bergman, MSc thesis). The last two theses are now complete. Specific binding sites on Rab5 that are important for interaction with p85 have not been identified. GAP assays performed by Cody Bergman to measure the effect of activated and inactivated PDGFR on p85 Rab5GAP activity suggested that PDGFR had no significant effect on p85 Rab5GAP activity. This finding supports my observation made in cells expressing p85ΔR+R274A, where it was shown that p85-PDGFR interaction was not required for RabGAP function of p85.

If the p85/Rab5-GDP/PDGFR complex is important for the GDF function of p85 then we expect that in cells expressing p85ΔR or p85ΔR+R274A Rab5-GDP cannot be recruited to the membrane and be activated. This will result in a sequestration of Rab-GDP in the cytosol and a reduction in the levels of Rab5-GTP. The levels of active Rab5-GTP were to be measured by immunoprecipitating Rab5-GTP with an anti-Rab5GTP antibody (an activation specific antibody that is supposed to recognize only Rab5 bound to GTP – the active form) followed by SDS-PAGE and Western blot analysis using anti-Rab5 antibodies. Optimization experiments were carried out to determine the conditions required for this Rab5GTP antibody from New East Biosciences, which is the only activation specific Rab5 antibody available on the market. Experiments were performed using both purified Rab5 protein and cell lysates collected from

NIH 3T3 cells treated with growth factor for different times. The activation specific Rab5GTP antibody pulled down both GDP and GTP bound Rab5 and therefore could not be used for further experiments.

5.2.3 A model for cells expressing p85ΔR or p85ΔR+R274A

We hypothesized that since p85 binds to Rab5-GDP as well as Rab5-GTP, it may help recruit Rab5 to vesicle membranes containing activated PDGFR signalling complexes and thereby regulate PDGFR signalling events (Chamberlain *et al*., 2004) (Figure 5.1). In order to test this hypothesis, cells expressing either p85 unable to bind to the PDGFR (p85ΔR) or a RabGAP defective p85 unable to bind to the PDGFR (p85ΔR+R274A) were generated and tested for changes in PDGFR signalling, trafficking and degradation. Based on our model illustrated in Figure 5.2, cells expressing p85ΔR have lower levels of Rab5-GTP presumably because Rab5-GDP cannot be recruited to the endosomal membrane. Sequestration of Rab5-GDP in the cytosol would be expected to result in a slower rate of internalization of activated receptor complexes. In p85R274A-expressing cells, PDGFR-associated p85 may aid in Rab5-GDP recruitment, however, p85 is RabGAP defective leading to an inability to inactive Rab5. The accumulation of Rab5-GTP leads to prolonged PDGFR-mediated signalling events. In p85ΔR+R274A-expressing cells, p85 is unable to bind to the PDGFR preventing the recruitment of Rab5-GDP to the endosomal membrane. Rab5-GDP is sequestered in the cytosol. Cells expressing p85ΔR+R274A were expected to have phenotypes comparable to p85ΔR-expressing cells. However, it was observed that p85R274A-expressing cells and p85ΔR-expressing cells were comparable to each other, which indicates that binding of p85 to PDGFR is not required for Rab5-GDP recruitment to endosomal membranes.

5.3 Future studies

The results obtained have paved the way to better understand the non-canonical functions of the PI3K complex. The results have provided the basis for several interesting models of events that could be transpiring in cells when interactions between p85 and some of its signalling interactors are altered. However, the models that have been proposed remain simply conjecture at this point and will certainly need to be tested further to demonstrate biological or pathological relevance. Some suggested experiments are outlined below.

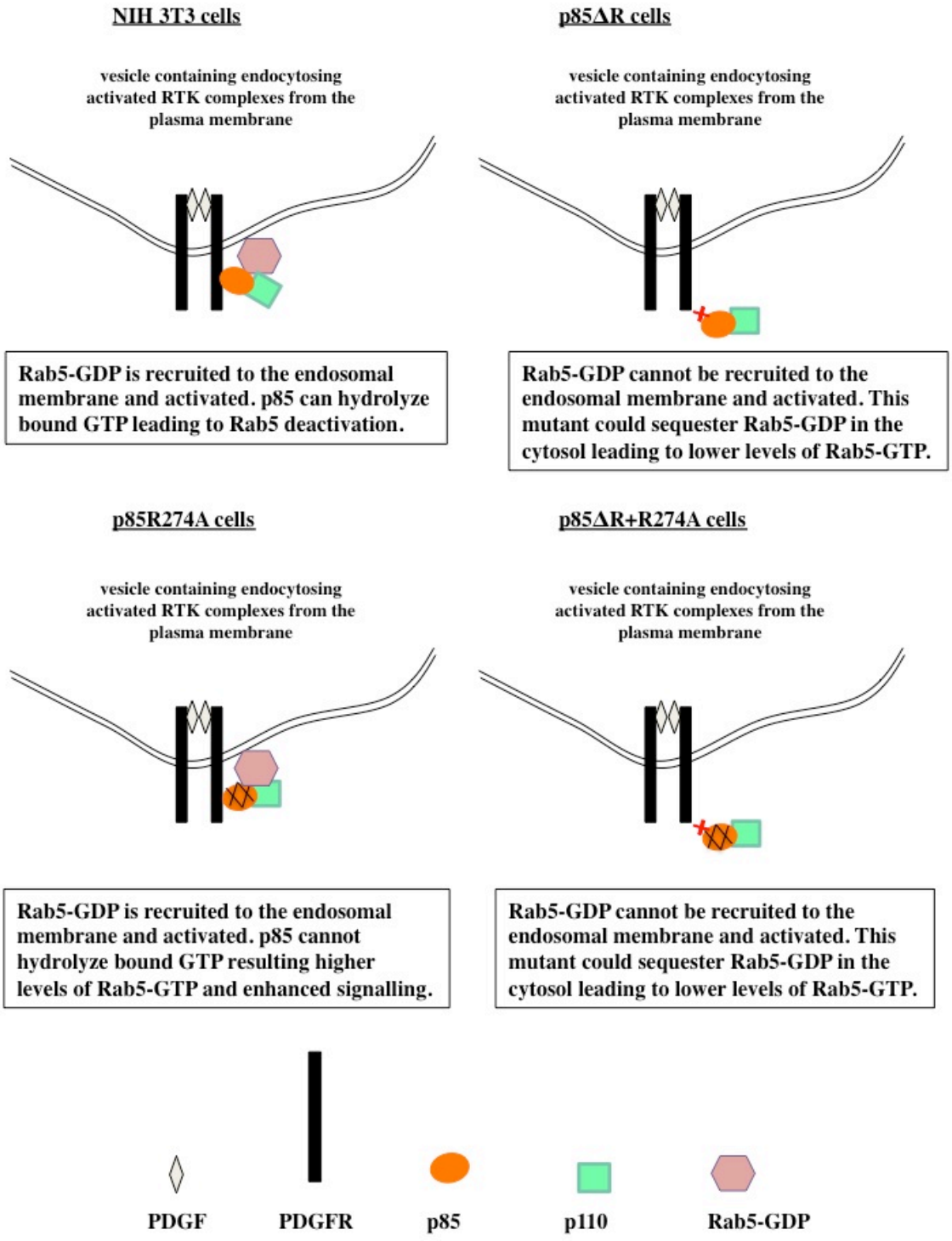
NIH 3T3 cells
vesicle containing endocytosing activated RTK complexes from the plasma membrane
Rab5-GDP is recruited to the endosomal membrane and activated. p85 can hydrolyze bound GTP leading to Rab5 deactivation.
p85ΔR cells
vesicle containing endocytosing activated RTK complexes from the plasma membrane
Rab5-GDP cannot be recruited to the endosomal membrane and activated. This mutant could sequester Rab5-GDP in the cytosol leading to lower levels of Rab5-GTP.
p85R274A cells
vesicle containing endocytosing activated RTK complexes from the plasma membrane
Rab5-GDP is recruited to the endosomal membrane and activated. p85 cannot hydrolyze bound GTP resulting higher levels of Rab5-GTP and enhanced signalling.
p85ΔR+R274A cells
vesicle containing endocytosing activated RTK complexes from the plasma membrane
Rab5-GDP cannot be recruited to the endosomal membrane and activated. This mutant could sequester Rab5-GDP in the cytosol leading to lower levels of Rab5-GTP.
PDGF
PDGFR
p85
p110
Rab5-GDP

Figure 5.2: Model for cells expressing p85ΔR or p85ΔR+R274A. *NIH3T3 cells*: Activated PDGFR complexes are internalized and loaded onto the early/sorting endosome *via* a Rab5-mediated pathway. Rab5-GDP is delivered to the early endosomal membrane. PDGFR-associated p85 may aid in Rab5-GDP recruitment. A GEF promotes the exchange of GDP for GTP resulting in an active form of Rab5. The RabGAP function of p85 enhances the GTPase activity of Rab5. Observed phenotype: Normal signalling events, non-transformed cells. *p85ΔR-expressing cells*: p85 is unable to bind to the PDGFR preventing the recruitment of Rab5-GDP to the endosomal membrane. Rab5-GDP is sequestered in the cytosol. Observed phenotype: Normal signalling events, non-transformed cells. (Note: There are normal endogenous levels of p85 present in these cells). *p85R274A-expressing cells*: PDGFR-associated p85 may aid in Rab5-GDP recruitment, however, p85 is RabGAP defective leading to an inability to inactivate Rab5. Accumulation of Rab5-GTP results in enhanced signalling events. Observed phenotype: Prolonged PDGFR activation, enhanced signalling (MAPK/Erk), reduced PDGFR degradation, transformed cells. (Note: There are normal endogenous levels of p85 present in these cells. It appears that the level of p85R274A present in the cells is enough to cause such a strong response in cells). *p85ΔR+R274A-expressing cells*: p85 is unable to bind to the PDGFR preventing the recruitment of Rab5-GDP to the endosomal membrane. Rab5-GDP is sequestered in the cytosol. Observed phenotype: Prolonged PDGFR activation, enhanced signalling (MAPK/Erk), reduced PDGFR degradation, transformed cells. (Note: There are normal endogenous levels of p85 present in these cells. It appears that the level of p85ΔR+R274A present in the cells is enough to cause such a strong response in cells indicating that binding of p85 to PDGFR is not required for Rab5-GDP recruitment to endosomal membranes).

5.3.1 Role of p110β in the RabGAP function of p85-p110β complex

It was shown in this thesis that expression of p85Δ110+R274A in cells leads to sustained PDGFR signalling that does not result in cellular transformation. This effect was attributed to the varying functions of the PI3K complex (i.e. p85-mediated GAP activity (arginine finger), p110β-mediated GAP activity (switch stabilization) and lipid generation). To determine whether p110β does indeed contribute GAP activity towards Rab5, a GAP assay (Chamberlain *et al*., 2004) can be performed using a recombinant stabilized p110β (p85iSH2-p110β; the iSH2 domain of p85 bound to p110β) generated by Dielle Detillieux. In order to tease out possible functions of p110β in the RabGAP activity of the PI3K complex, p110β can be knocked down from cells expressing p85wt, p85Δ110, p85R274A or p85Δ110+R274A using shRNA-p110β lentiviral particles. After the knock-down experiments, p110β lacking kinase activity (p110Δkinase, K805R) (Yart *et al*., 2002) or p110β unable to bind Rab generated by Dielle Detillieux (p110ΔRab: E238R and

Y244A, unpublished data) can be introduced into cells to determine the effects of p110β-mediated lipid generation or p110β-mediated GAP activity on receptor signalling and trafficking.

5.3.2 p85 as a GDI displacement factor (GDF)

It was postulated that p85 binds to Rab5-GDP as well as activated PDGFR and may help recruit Rab5 to vesicle membranes containing activated PDGFR signalling complexes. This hypothesis was to be tested by examining the importance of p85-PDGFR binding for the normal functioning of the Rab5 pathway by measuring the levels of Rab5-GTP using an activation specific antibody for Rab5. However, the antibody used for these measurements immunoprecipitated both GDP and GTP bound Rab5. The Rab5-GTP (active Rab5) can be measured using a glutathione S-transferase (GST) pull down technique (Chamberlain *et al*., 2010; Dou *et al*., 2013). In this assay a GST-tagged Rab5-binding domain of Rabaptin5 (residues 739–862, R5BD) that binds specifically to Rab5-GTP (Liu *et al*., 2007) can be used to pull down and measure Rab5-GTP levels in response to growth factor treatment. The model predicts that cells expressing p85R274A will have higher levels of Rab5-GTP, whereas, cells expressing p85ΔR or p85ΔR+R274A will have lower levels of Rab5-GTP.

Sequestration of Rab5-GDP by p85R274A, for example, can be further tested by immunoprecipitating FLAG-tagged p85 and Western blotting for bound Rab5.

5.3.3 Monitor PDGFR trafficking

The working model for p85-p110β-Rab5 binding suggests that cells in which p85 is unable to bind to p110β, the internalization of activated receptor complexes is increased due to the increase in levels of Rab5-GTP as a result of a loss of the switch stabilization function of p110β. In cells expressing the RabGAP defective p85, both internalization and recycling of receptor are increased due to the increase in levels of Rab5-GTP as a result of a reduction in RabGAP activity of p85 (Chamberlain *et al*., 2010). Based on these observations, it is proposed that the loss of RabGAP activity in a p85 molecule that is unable to bind to p110β (and in which the capacity for switch stabilization is presumably lost) leads to an even greater increase in internalization over the either one of these individual effects. It is also hypothesized that recycling of the receptor to the plasma membrane is also increased in these cells to the same extent as cells expressing the RabGAP defective p85. In order to validate this model a biotin

assay that involves cell-surface protein biotinylation, induction of endocytosis by growth factor stimulation and analysis of internalized receptors can be performed. Internalized biotinylated proteins can be immunoprecipitated using strepdavidin beads, followed by anti-PDGFR Western blot analysis (Chamberlain *et al*., 2010).

Immunofluorescence experiments can also be performed using anti-PDGFR, anti-Rab5, anti-Rab4 or anti-Rab7 antibodies to determine the localization of the receptor with respect to its association with internalization, recycling or degradation endosomes. During these immunofluorescence experiments, the size of the early endosomes can also be measured as an indication of active Rab5 levels since an increase in Rab5-GTP results in enlarged early endosomes (Barbieri *et al*., 2000; Stenmark *et al*., 1994).

5.3.4 Constitutive PDGFR ubiquitination in p85R274A-expressing cells

As mentioned above, the rate of PDGFR degradation is decreased in cells that express p85R274A. As this mutation disrupts the ability of p85 to enhance the GTPase activity of Rab5 and Rab4, which would allow these Rab proteins to remain active, i.e. in their 'on' state for longer (Chamberlain *et al*., 2010), this would translate into a more rapid cycling and recycling of receptor-ligand complexes through the early/sorting endosome, with very few of these complexes being sorted into the late endosomal/lysosomal degradative pathway (Chamberlain *et al*., 2010). Monoubiquitination marks the receptor for late endosomal sorting and lysosomal degradation (Marmor and Yarden, 2004). The ubiquitinated receptor is sorted into multivesicular bodies within the lumen of the late endosome; these bodies are then delivered to the lysosome where the proteins are degraded by lysosomal enzymes (Marmor and Yarden, 2004). Even though PDGFR in cells expressing p85R274A are ubiquitinated, it is likely that the receptor-ligand complexes are cycling through the early/sorting endosomes so quickly that they are unable to be sorted into the late endosome (Chamberlain *et al*., 2010). As such a constitutively ubiquitinated PDGF receptor may be sufficient to drive receptor-ligand complexes into the lysosomal degradation pathway, and restore normal degradation. This should decrease the downstream signalling observed in cells expressing the RabGAP defective p85R274A mutant since more of the receptor complexes would be expected to go down the lysosomal degradative pathway. To investigate this hypothesis, degradation rates (or half-lives) of a control PDGFR and its ubiquitin (Ub) chimera, with ubiquitin linked directly onto the receptor, could be determined.

Receptor trafficking events and signalling kinetics in cells expressing these chimeras could also be studied and would be expected to impart a shorter half-life to the PDGFR-ubiquitin chimera than the half-life observed with the control PDGFR in p85R274A-expressing cells.

5.4 Conclusions

Expression of a RabGAP defective mutant p85R274A acts as a dominant negative mutant and blocks degradation of activated PDGFR, alters receptor trafficking and transforms cells (Chamberlain *et al*., 2004; Chamberlain *et al*., 2008). These observations suggest that the RabGAP activity of p85 is associated with PDGF receptor down-regulation. The properties of cells expressing this mutant were further characterized by investigating whether normal PDGFR signalling events can be restored by abolishing the binding of p85 to PDGFR or to p110β. In summary, expression of a RabGAP defective p85 mutant unable to bind PDGFR shows that p85-PDGFR binding is not necessary for the RabGAP function of p85, since these cells had phenotypes similar to cells expressing the RabGAP defective mutant p85R274A. On the other hand, expression of a RabGAP defective p85 mutant unable to bind p110β resulted in the reversal of the tumorigenic properties observed in cells expressing the RabGAP defective mutant p85R274A, but not the enhanced signalling events in response to growth factor. This suggests some role of the p110β subunit in the RabGAP activity of p85. A better understanding of the role(s) that p110β has on p85's non-canonical function would contribute to our understanding of the progression of cancers that result from specific mutations in both p85 and p110β, within regions implicated in regulation of RabGAP activity. In fact, in recent years a number of different p85 mutations have been reported in the following cancers; glioblastomas, colorectal, urothelial, ovarian, colon, breast and pancreatic cancers (Mellor *et al*., 2012; Ross *et al*., 2013). Some of these mutations occur within the nSH2, iSH2 and cSH2 domains of p85 and disrupt the physical interaction between p85 and p110 resulting in increased PI3K signalling (Mellor *et al*., 2012; Ross *et al*., 2013). Other mutations occur within the GAP domain and may disrupt the GAP functions of p85 (Cheung *et al*., 2011; Ross *et al*., 2013). The findings reported in this thesis corroborate these reports and provide a mechanism to explain how mutations within the GAP domain can alter signalling events and lead to the enhancement of tumour-associated phenotypes. This knowledge not only broadens out basic understanding of tumour progression,

but also could provide the groundwork for the development of small molecule inhibitors targeted at these specific cancers for better cancer prognosis.

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