Chapter 1

General Introduction

In this introduction I want to invite the reader to a historical journey that reveals the discovery of a signaling pathway that regulates many different cellular functions and whose dysregulation had been shown to cause various types of cancer and diabetes mellitus: the phosphoinositide 3-kinase (PI3K) signaling pathway.

Discovery of the PI3K pathway.

The early research on phosphorylated forms of phosphatidylinositol (PI) focused on the conversion of phosphatidylinositol-4,5-bisphosphate (PI-4,5-P_2) by phospholipase C (PLC) into diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3), which ultimately causes Ca^{2+} release from internal Ca^{2+} stores and protein kinase C (PKC) activation (Hawkins et al., 1984). In the mid 80's researchers discovered that the lipid PI-4,5-P_2 can alternatively serve as substrate for the lipid kinase PI3K, which phosphorylates phosphoinositides at the D-3 position of the inositol ring thereby producing PI-3,4,5-P_3 (PIP_3). PIP_3 and its precursors are components of the cytosolic leaflet of membrane bilayers of all eurkaryotic cells. PI accounts for about 10% of the cellular membrane phospholipids (Canivenc-Gansel et al., 1998). PI and its phosphorylated derivates (phosphoinositides) are less abundant than phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylserine (PS), but play an essential role in growth factor activated signal transduction, regulation of the actin cytoskeleton and intracellular membrane trafficking. The inositol head group can be phosphorylated in the 3'-, 4'-, and 5'- positions, forming the phosphoinositides illustrated in Figure 1. Despite the low cellular levels of PIP_3 (0.05μM in unstimulated neutrophils and 2μM after treatment with an agonist peptide for 10 seconds) in comparison to more abundant phosphoinositides, PI-4-P (30-40μM) and PI-4,5-P_2 (50-35μM), this lipid has caught the attention of the scientific community, since its abundance correlates with cellular transformation and oncogenesis. PI3-kinase activity was found associated with viral

oncogenes, such as pp60$^{v\text{-}src}$ (Sugimoto et al., 1984), polyoma middle T (mT) antigen (Fukui and Hanafusa, 1989; Whitman et al., 1985), and pp68v-ros (Macara et al., 1984)(reviewed in Carpenter and Cantley 1990). Transformation of mammalian cells by polyoma mT absolutely requires its association with PI3K activity and the consequent elevated PIP_3 levels (Serunian et al., 1990), (Ling et al., 1992).

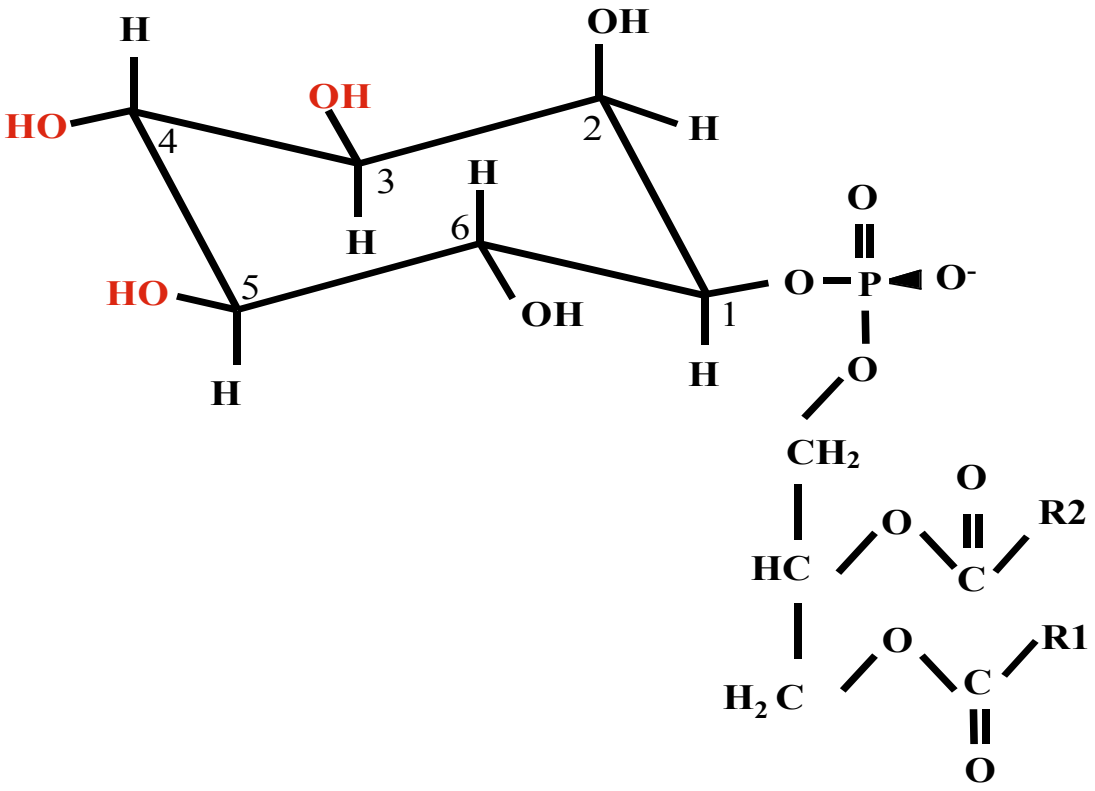

Fig. 1. Chemical Structure of phosphatidylinositol (PtdIns). The myo-D-enantiomer of inositol is shown, in which the 2'hydroxyl is axial and the other hydroxyls are equatorial. The hydroxyl groups labeled in red can be phosphorylated by phosphoinositide kinases. The fatty acids that form esters at the 1 and 2 position of the glycerol are typically stearate and arachidonate.

In normal cells, PIP_3 can be transiently detected upon stimulation by a variety of growth factors (Whitman et al., 1988), (Varticovski et al., 1989). The level of PIP_3 can increase by more than 50-fold, peaking between 10 - 60 seconds after PDGF stimulation and lasting 30-60 minutes (Auger et al., 1989). The platelet derived growth factor (PDGF) receptor was the first receptor shown to associate with PI3K (Kaplan et al., 1987) and activate PI3K (Auger, 1989). In addition to the rapid response just described, PDGF

treatment causes a delayed, sustained phase of PIP_3 production between 3-8hours after addition. The production of PIP_3 also increases upon treatment with insulin, insulin-like growth factor 1 (IGF-1), colony stimulating growth factor (CSF), epidermal growth factor (EGF) and hepatocyte growth factor (HGF) (Coughlin et al., 1989), (Whitman et al., 1988), (Varticovski et al., 1989), (Ruderman et al., 1990). In contrast to PI-4,5-P_2, PIP_3 is not a substrate for PLC (Serunian et al., 1989). PIP_3 acts as a second messenger that recruits intracellular signaling molecules such as the Ser/Thr protein kinase AKT / PKB, Tec family tyrosine kinases and the Grp1 / Arf exchange factor to the plasma membrane. These and other PIP_3-interacting proteins coordinate complex cellular events, such as glucose uptake, cell proliferation, survival and migration. The latter three processes are pivotal in the development of an organism and can promote carcinogenesis and metastasis if they are dysregulated. The dual phosphatase PTEN antagonizes PI3K signaling by dephosphorylating the 3 position of PIP_3 and PI-3,4-P_2 (Figure 2). In this thesis I describe the consequences of genetic ablation of PI3K in the mouse. In chapter 3 I discuss the *in vivo* role of PI3K in development of an organism. Interestingly, loss of the regulatory subunits of class Ia PI3K results in similar developmental defects as loss of the PDGF receptor alpha. In chapter 4, I focus on the role of PI3K in cell migration and chapter 5 is dedicated to the *in vivo* role of PI3K in regulating glucose homeostasis.

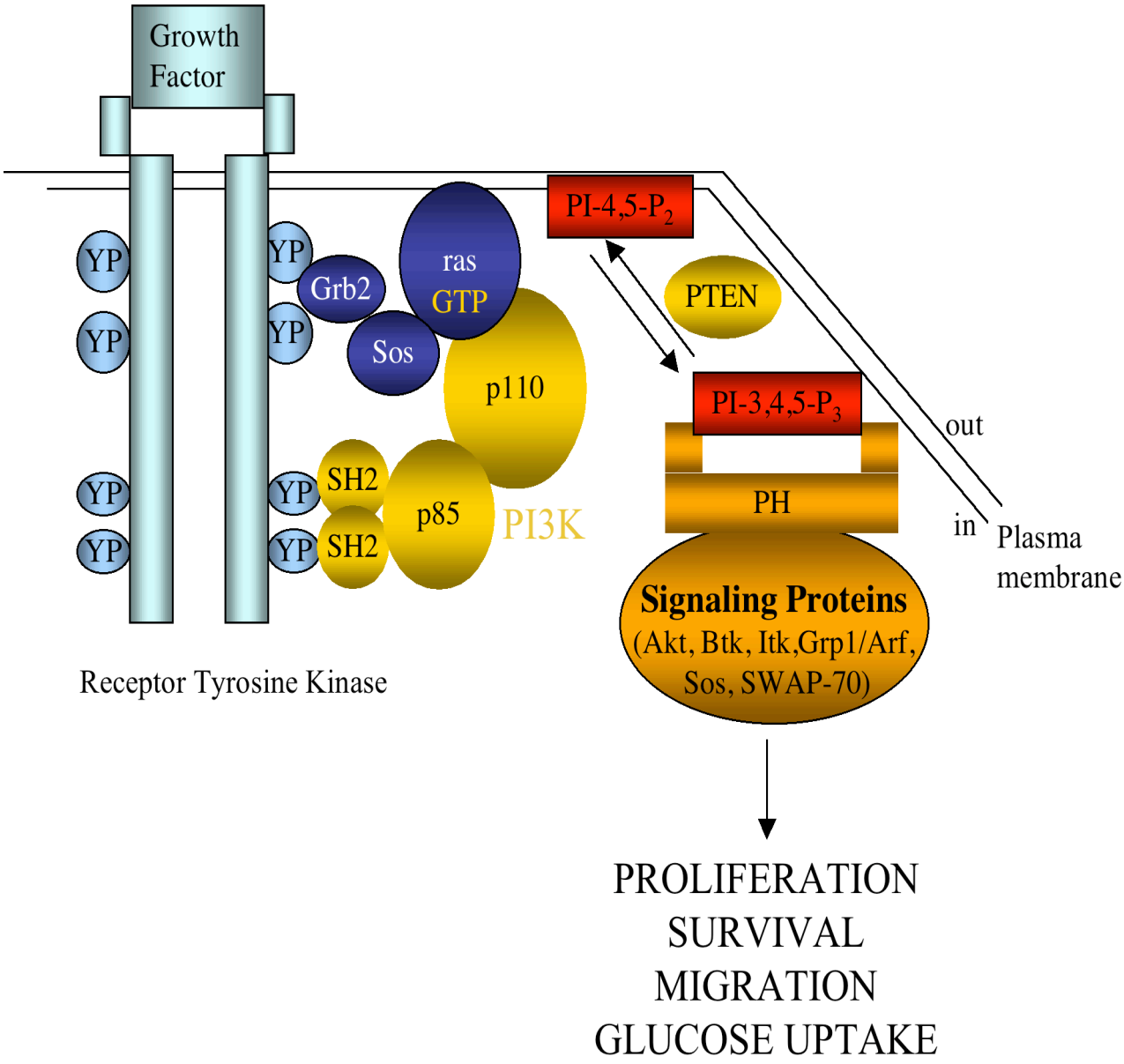

Fig. 2. Class Ia PI3K signaling pathway. Upon growth factor stimulation, the receptor tyrosine kinase dimerizes and autophosphorylates on tyrosine residues that resemble binding sites for the SH2 domains of the PI3K regulatory subunit p85. In addition, the small GTPase ras gets activated and can bind to the PI3K catalytic subunit p110. By binding to the receptor and ras, PI3K gets recruited to the plasma membrane where it phosphorylates PI-4,5-P_2 to produce PIP_3. PTEN antagonizes PI3K signaling by dephosphorylating PIP_3 back to PI-4.5-P_2. PIP_3 acts as a second messenger that recruits signaling molecules that contain a subgroup of PH domains to the plasma membrane. These signaling molecules get activated and mediate cellular events, such as proliferation, survival, migration or glucose uptake.

Classes of PI3K

PI3K belongs to a family of lipid kinases that are grouped into three classes according to their sequence homologies and substrate specificities. Class Ia PI3K is a heterodimer consisting of a catalytic subunit, p110, and an associated regulatory subunit, so called p85 (even though smaller isoforms are known). In vitro, the catalytic subunit phosphorylates PIs at the D3 position of their inositol ring to produce PI3P, PI-3, 4-P_2 and PI-3,4,5-P_3. Kinetic studies in ^{32}P –labelled cells suggested that PI-3,4-P_2 may also be produced from PIP_3 by a 5-phosphatase. In contrast to class Ia PI3K which is activated by receptor tyrosine kinases, class Ib PI3K generates PIP3 upon activation of G-protein coupled receptors. Class Ib PI3K also consists of a regulatory subunit (p101) and a catalytic subunit (p110).

Class II PI3K contains a C-terminal region with homology to C2 domains and has therefore been termed PI3KC2. PI3KC2 isoforms produce mainly PI3P and PI-3,4-P_2 *in vitro*, although human PI3KC2 converts PI-4,5-P_2 to PIP_3 in the presence of phosphatidylserine (McDougall et al., 1995). There is evidence that PI3KC2 isoforms are also activated upon PDGF, insulin and EGF (Arcaro et al., 2000).

Class III PI3K phosphorylates PI and thereby generates PI3P, *in vivo*. Total cellular levels PI3P levels do not change much upon growth factor stimulation (Toker et al., 1995). Subcellular visualization of PI3P via the fusion protein of the green fluorescent protein (GFP) to the PI3P binding FYVE domain of EEA-1 (GFP-FYVE) reveals that most of this lipid is at early endosomes (Ellson et al., 2001).

Studies in which PI3K inhibitors were used revealed that PI3K regulates crucial cellular processes. However, these inhibitors block all three classes of PI3K and can also inhibit a subset of protein Ser/Thr kinases. In this thesis, a genetic approach was taken to analyze the *in vivo* functions of class Ia PI3K. By genetic ablation of class Ia PI3K in the mouse, the pivotal role of class Ia PI3K in embryonic development (described in chapter 3). I also show that this class of enzymes are required for membrane ruffling and cell migration in response to PDGF (described in chapter 4). Finally, I address the relative role of the p85 and p110 subunits in glucose homeostasis (described in chapter 5). In collaboration with Vieira et al, we could demonstrate the requirement for class Ia PI3K in the formation of the nascent phagosome (Vieira et al., 2001). By using GFP-labeled PIP_3-

or PI3P-binding probes we showed that PIP_3 accumulates at the nascent phagosome, while PI3P is transiently generated at the maturing phagolysosome.

Isoforms of class Ia PI3K.

In mammals, the class Ia family of PI3Ks comprises multiple isoforms for the catalytic as well as the regulatory subunit. In mammals, three genes have been identified to encode the class Ia catalytic isoforms, termed p110α, p110β and p110δ (Hiles et al., 1992), (Hu et al., 1993), (Vanhaesebroeck et al., 1997b). Whereas p110α and p110β are ubiquitously expressed, p110δ is specific to leukocytes. Also, for the regulatory subunit (often simply refered to as p85) three genes have been described. Two genes encode isoforms of 85kD, named p85α and p85β (Carpenter et al., 1990), (Escobedo et al., 1991), (Skolnik et al., 1991). In addition, two smaller proteins (p55α and p50α) arise from alternative splicing of the p85α gene (Fruman et al., 1996), (Antonetti et al., 1996), (Inukai et al., 1996), (Inukai et al., 1997). The p55γ protein has a similar structure to the p55γprotein, which is encoded by a third gene (Pons et al., 1995). In contrast to the 85kD proteins, which are ubiquitously expressed, the smaller isoforms are distributed in a tissue specific manner. Extensive studies using PI3K inhibitors or overexpression of dominant negative mutants showed that PI3K is involved in various cellular processes. However, these studies did not distinguish between the different isoforms of the catalytic and regulatory subunit of class Ia PI3K. In chapter 3 of this thesis, I show that p110β/p85α and p110δ/p85α specifically mediate PDGF receptor β dependent developmental processes that cannot be mediated by p110β/p85 or p110δ/p55 complexes. In chapter 4, I will show that all p85 isoforms are able to mediate PDGF induced membrane ruffling. Finally, in chapter 5, I evaluate the effect of heterozygous loss of p110α and/or p110β on insulin mediated glucose homeostasis and compare these results to previous studies of mice lacking p85 genes.

Domain structure of PI3K subunits.

The three PI3K catalytic isoforms are molecules of about 110-120kD that share 42-58% amino acid sequence identity (Hiles et al., 1992), (Hu et al., 1993). At their N-terminus they contain a p85-binding site, followed by a Ras interaction site that interacts

with Ras in its GTP bound (active) form. Adjacent to a C2-related domain that binds phospholipids in a Ca^{2+} dependent manner, p110s possess a PIK homology region that is conserved between members of the PI3-kinase and PI4-kinase superfamily and members of a more distant related family of protein kinases, that includes mTOR, DNA-PK, ATM and ATR. At the C-terminus, p110s each contain a catalytic domain that shares distant homology to protein kinase domains (Table 1).

The 85kD regulatory proteins also share an overall domain structure (Table 2). All PI3K regulatory isoforms contain one or two proline rich motifs, two SH2 domains that bind to phosphorylated tyrosines in the context of YXXM motifs (which are found in the activated PDGF receptor or insulin receptor substrates (IRS)) and a coiled coiled region that tightly binds to the catalytic subunit. A huge body of evidence focuses on the role of p85 to recruit p110 to the plasma membrane into proximity to the membrane bound substrates. This function of p85 is conserved back to Drosophila melanogaster which expresses a 60kD regulatory subunit comprising the SH2 domains and the p110-binding region (Weinkove et al., 1997). However, in contrast to the conserved smaller p85 isoforms (p55/p50), p85α and p85β contain additional domains: one src homology (SH) 3 domain, one proline rich region and an area with homology to GTPase-activating proteins for the rho family of small G proteins (Rho-GAP domain). The Rho-GAP domain does not possess a catalytic activity since it lacks five residues that are conserved among functional Rho-GAP domains (Musacchio et al., 1996). The smaller isoforms do not possess the N-terminal domains; instead they contain unique short N-terminal sequences (35 amino acids for p55α/γ and 6 amino acids for p50α). Various signaling molecules interact exclusively with p85α and p85β by binding to the additional domains in the N-terminus of p85α and p85β. For example, the E3 ubiquitin ligase c-cbl binds to the SH3 domain of p85, the small GTPases Cdc42 and Rac bind in their activated state to the Rho-GAP homology domain of p85 and src isoforms interact with the proline rich regions of p85. These interactions might regulate PI3K signaling by changing the subcellular localization or activity of PI3K and therefore result in PI3K isoform-specific functions. Since the small p85 isoforms lack the N-terminal domains, they might not be able to fulfill the same functions as the 85kD isoforms.

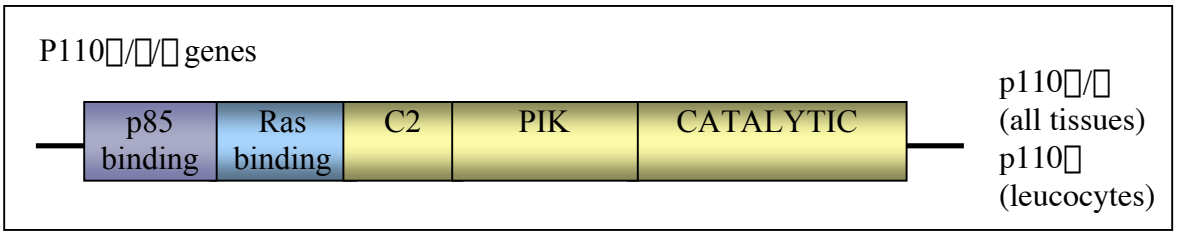

Table 1: Domain structure and tissue distribution of p110 isoforms

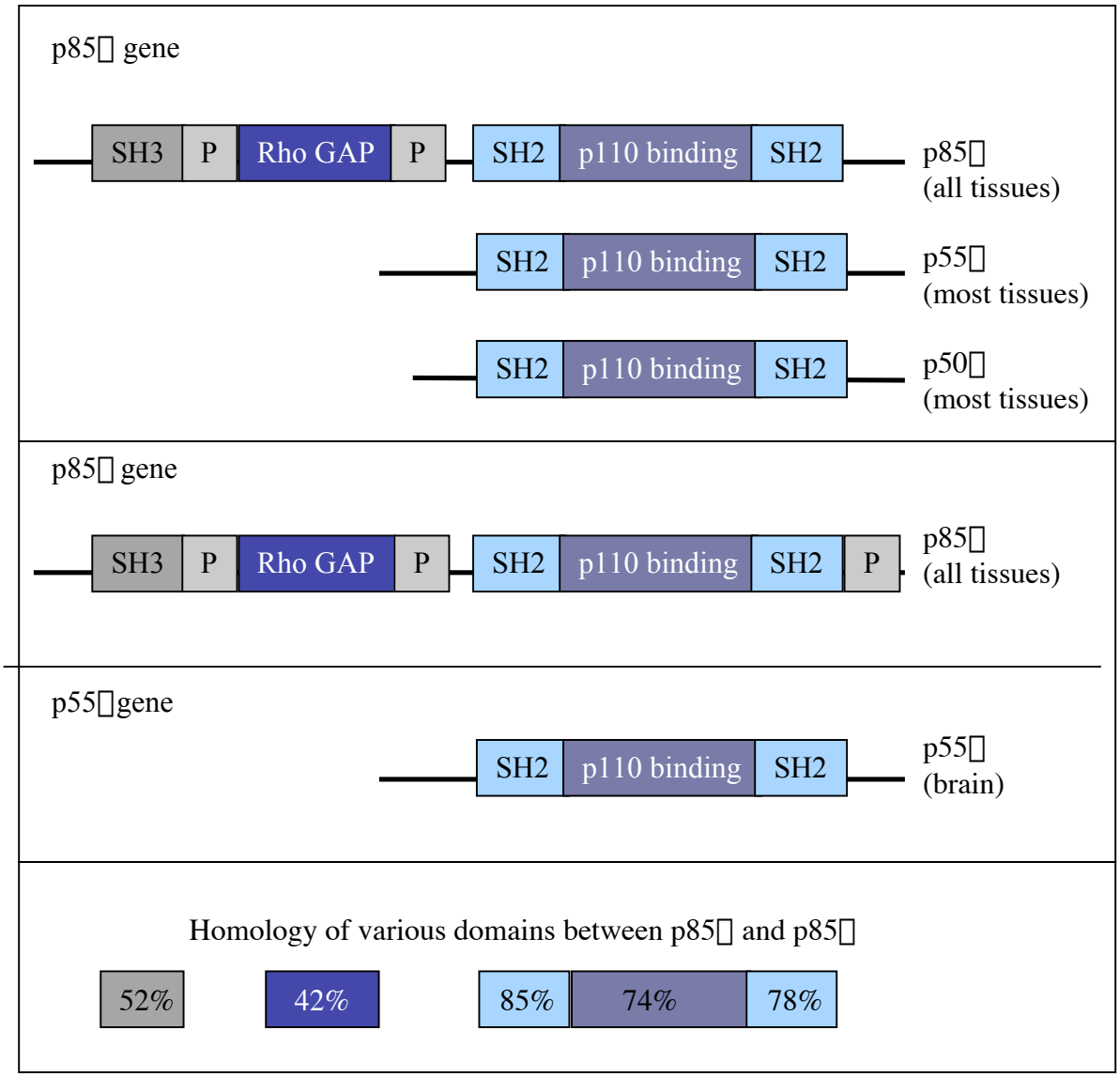

Table 2: Domain structure, homology and tissue distribution of p85 isoforms

In chapter 3, I present evidence that the smaller p85 isoforms p55α and p50α are not fully compensating for the loss of 85kD isoforms in murine development. In contrast, similarly to p85α and p85β, p50α is sufficient to mediate PDGF-induced membrane ruffling indicating that the N-terminal domains of p85 are not required for this particular event (described in chapter 4). Furthermore, the identity between the N-terminus of p85α and p85β is only about 50% and therefore, some interaction partners might bind preferable to either p85α or p85β. Therefore, I determined (described in chapter 6) the optimal binding motifs for the SH3 domains of p85α and p85β.

Activity of PI3K.

The catalytic domains of PI3Ks apparently evolved from closely related protein Ser/Thr kinases (Tor, DNA-PK, ATM, ATR). Indeed, the class Ia PI3K catalytic subunits can catalyze phosphorylation of a serine residue on the p85 regulatory subunit (Carpenter et al., 1993). The phosphorylation of the regulatory subunit seems to play a role in a shut off mechanism of PI3K. P110α phosphorylates p85α at serine 608 thereby downregulating the lipid kinase activity of p110 three to seven fold (Carpenter et al., 1993), (Dhand et al., 1994)). Both p110α and p110β phosphorylate the p85 regulatory subunit, whereas p110γ and p110δ do not seem to possess this activity. In contrast, p110β autophosphorylates at Ser1039 at its C-terminus, and this modification also inhibits the kinase activity (Vanhaesebroeck et al., 1997b). Autophosphorylation of p110γ has also been demonstrated, however no effect on the enzymatic activity has been shown (Stoyanova et al., 1997). Besides autophosphorylation, PI3K has been shown to phosphorylate the insulin receptor substrate 1 (IRS1) (Lam et al., 1994). In order to understand the role of the protein kinase activity in signaling, researchers specifically abolished the lipid kinase activity. A PI3K (p110γ) mutant that lacks lipid kinase activity but exhibits intact protein kinase activity is able to activate Erk, however, fails to activate Akt (Bondeva et al., 1998).

There is recent evidence that p85 plays a negative role in insulin mediated signalling. Loss of any p85 isoform results in improved insulin signalling. One way to mediate this negative function could be by shutting off p110 activity after being phosphorylated on Serine 608. In chapter 5, I tested if the negative role of p85 in insulin

signaling is dependent on p110 by reducing the level of class Ia p110 in the mouse. We concluded that p85 mediates its inhibitory function by a mechanism not involving p110.

Inhibitors of PI3K.

Both, lipid and serine kinase activities can be inhibited by the PI3K inhibitors Wortmannin (WM, IC_{50}=4.2nM) and LY294002 (LY, IC_{50}=1.4μM) (Norman et al., 1996), (Wymann et al., 1996), (Vlahos et al., 1994). The quick reduction of 3'-phosphoinositides (about 80% decline of PI3P within 5min) upon treatment of cells with WM or LY implies a rapid turnover of these lipids. The fungal metabolite WM reacts covalently with lysine 802 in the ATP binding site of p110□; this residue is highly conserved among PI-kinases as well as protein kinases and is indispensable for the catalytic activity. In contrast to the non-competitive irreversible inhibitor WM, the pharmacological compound LY acts in a competitive and reversible manner. Both inhibitors have been very useful in demonstrating the PI3K dependence of a given cellular response. However, these studies should be evaluated with some caution since neither of these chemicals are specific for class Ia PI3K. These compounds can inhibit class II and III PI3K, PI4K□ (Meyers and Cantley, 1997), mTor (Brunn et al., 1996) and DNA-PK (Hartley et al., 1995b). In contrast, the knock-out approach we have taken in this thesis allows analysis of the role of class Ia PI3K specifically.

Activation of PI3K.

In insect cells grown at low temperatures, the monomeric PI3K catalytic subunit p110 has a higher lipid kinase activity than p110 that is bound to p85; however monomeric p110 is thermally unstable at 37°C. In chapter 3 of this thesis I describe decreased p110 levels in p85 null cells and that retroviral reexpression of p85 isoforms led to normalized levels of p110. The binding of p110 to p85 causes a drop in p110's lipid kinase activity of 65-85% (Yu et al., 1998). However, this "inhibitory" effect of p85 on p110 is overcome by engagement of both SH2 domains of p85 with phosphotyrosine containing peptides (Yu et al., 1998). For example, binding of p85 via its two SH2 domains to the activated PDGF receptor or IRS proteins causes a conformational change and releases the inhibitory effect. In addition, these interactions are thought to recruit

PI3K into the proximity of its membrane bound substrates. Many growth factors that lead to engagement of the SH2 domains of p85 also activate ras (exchange of GDP to GTP). PI3K activity is augmented by the interaction between p110 and the GTP bound form of ras (150nM for Ras-GTP with p85α/p110α) in vitro and in vivo and can therefore be seen as an effector of ras (Kodaki et al., 1994), (Rodriguez-Viciana et al., 1996).

Role of PI3K in lower organisms.

The slime mold ***Dictyostelium discoideum*** has served as a useful model system to study the molecular pathways that establish cell polarity and control chemotaxis. Starving amoebae of Dictyostelium discoideum communicate through extracellular cAMP signals, which promote chemotaxis. Ultimately the concerted movement results in the formation of fruit bodies, multicellular aggregates, of thousands of amoebae. Multiple events during this process, such as polarization, chemotaxis and aggregation have been shown to be dependent on class I PI3K signaling. Dictyostelium discoideum expresses three PI3K orthologues, refered to as DdPIK1, DdPIK2 and DdPIK3. Loss of one PI3K isoform does not result in any growth or developmental abnormalities and therefore points to redundancy among the three different isoforms. In contrast, cells with combined loss of DdPIK1 and DdPIK2 exhibit both growth and developmental defects (Zhou et al., 1995). Combined loss of DdPIK3 with any other DdPI3K isoform is lethal (Zhou et al., 1995). ΔDdPIK1/ ΔDdPIK2 cells also show defects in their endosomal pathway, pinocytosis, cell shape, polarization and chemotaxis (Buczynski et al., 1997), (Funamoto et al., 2001), (Rupper et al., 2001). In chapter 4 of this thesis I show the requirement for class Ia PI3K in membrane ruffling, cell polarization and cell migration. This body of evidence demonstrates that the role of PI3K in cell motility is conserved from the mouse back to the slime mold.

Another aspect of class I PI3K signaling was revealed through analysis of PI3K mutants in the nematode ***Caenorhabditis elegans***. In times of food shortage, C. elegans undergoe a reversible developmental arrest at the long-lived dauer diapause stage instead of entering a reproductive life cycle. A conserved insulin-like signaling pathway that includes DAF-2 (insulin receptor homolog), AGE-1 (p110 P3K homolog), the akt-1/akt-2

ser/thr protein kinases and DAF-16 (Fork head transcription factor), governs this decision (Kimura et al., 1997), (Morris et al., 1996), (Paradis and Ruvkun, 1998). Inhibition of PI3K signaling induces dauer formation (PI3K signaling is necessary for non-dauer development) (Morris et al., 1996). In cases where maternal AGE-1 is present the C.elegans reach the adult stage but still have a 2-3 fold increased life span. Both the dauer and life span phenotypes can be suppressed by mutations in DAF-18, the PTEN homolog in C. elegans (Mihaylova et al., 1999), (Rouault et al., 1999), (Ogg and Ruvkun, 1998). In addition, mutations in the DAF-2/AGE-1 PI3K signaling pathway affect reproduction and embryonic development (Tissenbaum and Ruvkun, 1998). New evidence points to a role of PI3K signaling in concert with mPar3/mPar6/aPKC in establishing neuronal polarity in C. elegans (Shi et al., 2003). A recent report demonstrates the role of insulin signaling in longevity also in mammals: targeted disruption of the insulin receptor in adipocytes results in extended longevity (Bluher et al., 2003). These data demonstrate the impact of the insulin receptor/PI3K signaling pathway on metabolism, development and longevity and that this pathway is conserved back to the nematode.

Role of PI3K signaling in cancer

The understanding that misregulation of PI3K signaling correlates with carcinogenesis drew great attention to this pathway. Augmented PIP_3 levels correlate with transformation by viral oncogenes, such as v-src, polyoma middle T antigen and v-ros (Auger et al., 1992; Whitman et al., 1985), (Whitman et al., 1988), (Serunian et al., 1990), reviewed in: (Cantley et al., 1991). Transformation-defective mutants of v-src, v-abl, v-fms and polyoma middle T lack the ability to activate PI3K, although a subset of these mutants are not impaired in binding to PI3K. However, they fail to target PI3K to the membrane, where the substrates of the lipid kinase are located (Varticovski et al., 1991). The avian sarcoma virus 16 (ASV16) encodes the oncogene v-p3k, which acts as a potent transforming gene in cultured chicken embryo fibroblasts. The gene is derived from the cellular gene for p110 (Chang et al., 1997). Gene copy amplification of p110 has frequently been found in ovarian cancers and therefore implicates PI3K as an oncogene itself (Shayesteh et al., 1999).

The lipid phosphatase activity of PTEN/MMAC antagonizes PI3K signaling and can therefore be seen as a counterpart of the PI3K oncogene. PTEN is a tumor suppressor gene that is homozygously mutated in a variety of human cancers, most prominently in advanced glial tumors, but also in prostate, endometrial, renal, small lung carcinomas, melanoma and meningioma (Cantley and Neel, 1999). Targeted disruption of PTEN results in early embryonic lethality, whereas all mice heterozygous for PTEN exhibit hyperplastic-dysplastic features and a high tumor occurrence (Di Cristofano et al., 1998). PTEN is second only to p53 as the most frequently deleted tumor suppressor gene in human cancers.

Recent evidence shows that mutations in the p85α gene promotes tumorigenesis. Using single-strand conformational polymorphism / heteroduplex analysis Philp et al. showed the presence of an activating deletion in the inter-SH2 region of the p85α gene in primary human colon and ovarian tumors as well as cancer cell lines (Philp et al., 2001), (Jimenez et al., 1998), (Borlado et al., 2000).

Also downstream effectors of PI3K signaling have been linked to cancer, such as the Ser/Thr kinase Akt/PKB. Reports show human cancers with either amplified or activated AKT1 and AKT2 (Marte and Downward, 1997), (Cheng et al., 1992), (Sun et al., 2001). Akt contains a PH domain that specifically interacts with PI-3,4-P_2 and PIP_3. Binding to these lipids recruits Akt to the plasma membrane where it becomes phosphorylated by phosphoinositide-dependent kinase (PDK) -1, a protein kinase that itself contains a PI-3,4-P_2 and PIP_3-specific PH domain. Akt activity promotes S-phase entry (via p27/KIP downregulation and Cyclin D stabilization) and induces survival by suppressing apoptosis (via activation of NFkB cascade, sequestering of pro-apoptotic BAD in the cytosol and inhibition of Forkhead mediated transcription of pro-apoptotic genes, such as Fas ligand) (Romashkova and Makarov, 1999), (Datta et al., 1997), (Kops and Burgering, 1999). While increased engagement of the PI3K pathway results in augmented cell proliferation and cancer, in chapter 4 of this thesis I describe that reduction of PI3K signaling (reduced p85-p110 levels) results in decreased cell proliferation and senescence. Furthermore, in collaboration with Ueki et al. we showed decreased IGF-1 induced survival in fibroblasts lacking all p85α gene products (p85α-/-p55α-/-p50α-/-) (Ueki et al., 2002).

P110 Isoform-specific Functions

Early embryonic lethality of p110α null or p110β null mice indicate p110-isoform specific functions during development (Bi et al., 2002; Bi et al., 1999). Microinjection of specific, inhibitory antibodies that block the activity of one p110 isoform without influencing the signaling capacity of the others has recently been used to distinguish isoform specific functions of the catalytic isoform. These studies implied that p110α mediates PDGF induced actin rearrangement, whereas p110β mediates insulin responses (Hooshmand-Rad et al., 2000). In contrast, using a different cell type, another lab reported p110β to be necessary for insulin induced membrane ruffling (Siddhanta et al., 1998). Using the same approach, Roche et al. showed that p110β seems to be responsible for insulin- and LPA- induced cell cycle entry, but not for the PDGF- mediated mitogenic response (Roche et al., 1998). Whereas p110α activity seems to be involved in CSF-1 induced DNA synthesis, p110β might be engaged in the actin rearrangement processes induced by this growth factor. Interpretation of these results are complicatd by the inherent limitations of antibody injection experiments. Christoforidis et al. reported that Rab5 selectively binds to p110β but not p110α implying a differential role in intracellular membrane trafficking (Christoforidis et al., 1999). Distinct roles for p110α and p110β might be explained if they each dimerized with a distinct p85 isoform and the p85 isoforms mediated preferential complexes with distinct receptors and adaptors. However, each p110 isoform can associate with all the p85 isoforms, at least when overexpressed (Vanhaesebroeck et al., 1997a). However, there are several reports showing that the different p85 isoforms bind to distinct subsets of target proteins that therefore might allow specific signaling (Hartley et al., 1995a).

An additional difference between p110α and p110β arises from the fact that p110β but not p110α can be substantially activated by $\beta\gamma$ subunits of heterotrimeric GTPases provided it is also bound to a tyrosine phosphorylated peptide/proteine (Kurosu et al., 1997b). This important finding attributes p110β the capability of integrating signals from receptor tyrosine kinases as well as G-protein coupled receptors. The two catalytic isoforms also differ in their kinetic properties (Vmax, Km) for both the lipid and protein kinase activity. Beeton at al. hypothesize that p110β works better in areas with low

substrate densitiy whereas p110δ favors membranes with a high substrate density (Beeton et al., 2000).

An alternative approach to distinguish isoform specific functions of PI3K isoform is the analysis of mouse models with targeted disruption of single or multiple isoforms. The advantage of this method is the assessment of the *in vivo* functions – the developmental or physiological role - of a given isoform. Furthermore, some studies can only be done on the whole-body level, for example the regulation of glucose homeostasis requires the proper interplay of various endocrine organs.

P85 functions

The main function of p85 is most likely the recruitment of p110 to the growth factor - stimulated receptors and their substrates (e.g. IRS, Gab) where the lipid kinase can phosphorylate the membrane bound substrates. Subsequently, PIP_3 is produced. In agreement with this being the major role of p85, the p85 orthologues in the fly, worm and slime mold are only comprised of those domains that are necessary for this function. Furthermore, p85 functions as an adapter that interacts with a variety of other signaling molecules besides p110. The interaction between p85 and src was shown to augment the PI3K activity (Pleiman et al., 1994), whereas it is still controversial if the interaction between p85 and the activated forms of Cdc42 and Rac influences PI3K activity (Tolias et al., 1995; Zheng et al., 1994). However, these interactions might also influence the subcellular localization of PI3K.

There is recent evidence from this laboratory that p85 plays an inhibitory role on insulin-dependent processes. PI3K is necessary but not sufficient for the insulin-induced translocation of the glucose transporter GLUT4 from an intracellular compartment to the plasma membrane and the subsequent glucose uptake from the blood into the cell. Thus, it was very unexpected and surprising that genetic ablation of any PI3K regulatory subunit (p85α-/- or p85β-/- or p85α-/-p55γ-/-p50α+/- or p85α-/-p55γ-/-p50α-/-) in the mouse caused actually improved insulin sensitivity and glucose tolerance (Terauchi et al., 1999) (Fruman et al., 2000; Mauvais-Jarvis et al., 2002; Ueki et al., 2002b). One could conceive of several mechanisms by which p85 inhibits signaling; some of them are addressed in this thesis (Figure 3):

- PI3K Competitior

In collaboration with Ueki et al., we detected in mouse embryonic fibroblasts excess amounts of p85 over p110 (Ueki et al., 2000). The monomeric p85 might compete with p85/p110 complexes for binding sites at the activated receptors and their substrates.

- Negative Feedback – p110 dependent

PI3K dependent serine phosphorylation of the insulin receptor substrate 1 (IRS-1) results in decreased insulin signaling. Several PI3K downstream kinases have been implicated in the inhibitory serine phosphorylation, such as Akt, mTor, p70S6K and JNK (Mamay et al., 2003). In chapter 4 of this thesis I test this model by analyzing insulin signaling upon loss of p110 isoforms and conclude that p85's negative role on insulin signaling is at least partially p110 independent.

- Negative Feedback –p110 independent

Phosphorylation of the insulin receptor substrate 1 (IRS-1) by JNK1 results in decreased insulin signaling. Upon loss of p85□/p55□/p50□ JNK activity is decreased and can be restored by p85 mutants that lack the p110 binding domain (Ueki et al., 2003).

- Degradation or mislocalization of signaling proteins

P85 binds the E3 ubiquitin ligase c-Cbl and could thereby modulate internalization and degradation of p85-associated signaling proteins (Sanjay et al., 2001).

- PIP_3 Degradation

P85 recruits the SH2 domain-containing inositol 5'-phosphatase 1 (SHIP-1) to the activated B cell receptor which results in the dephosphorylation of PIP_3 into PI-3,4-P_2 (Gupta et al., 1999). SHIP-2 is more broadly expressed than SHIP-1 and genetic ablation of SHIP-2 in the mouse results in improved insulin sensitivity (Clement et al., 2001). Thus, p85 could play a role both in recruiting p110 subunits for PIP_3 production and SHIP-2 for PIP_3 degradation.

These models are discussed in more detail in chapter 5 and 6.

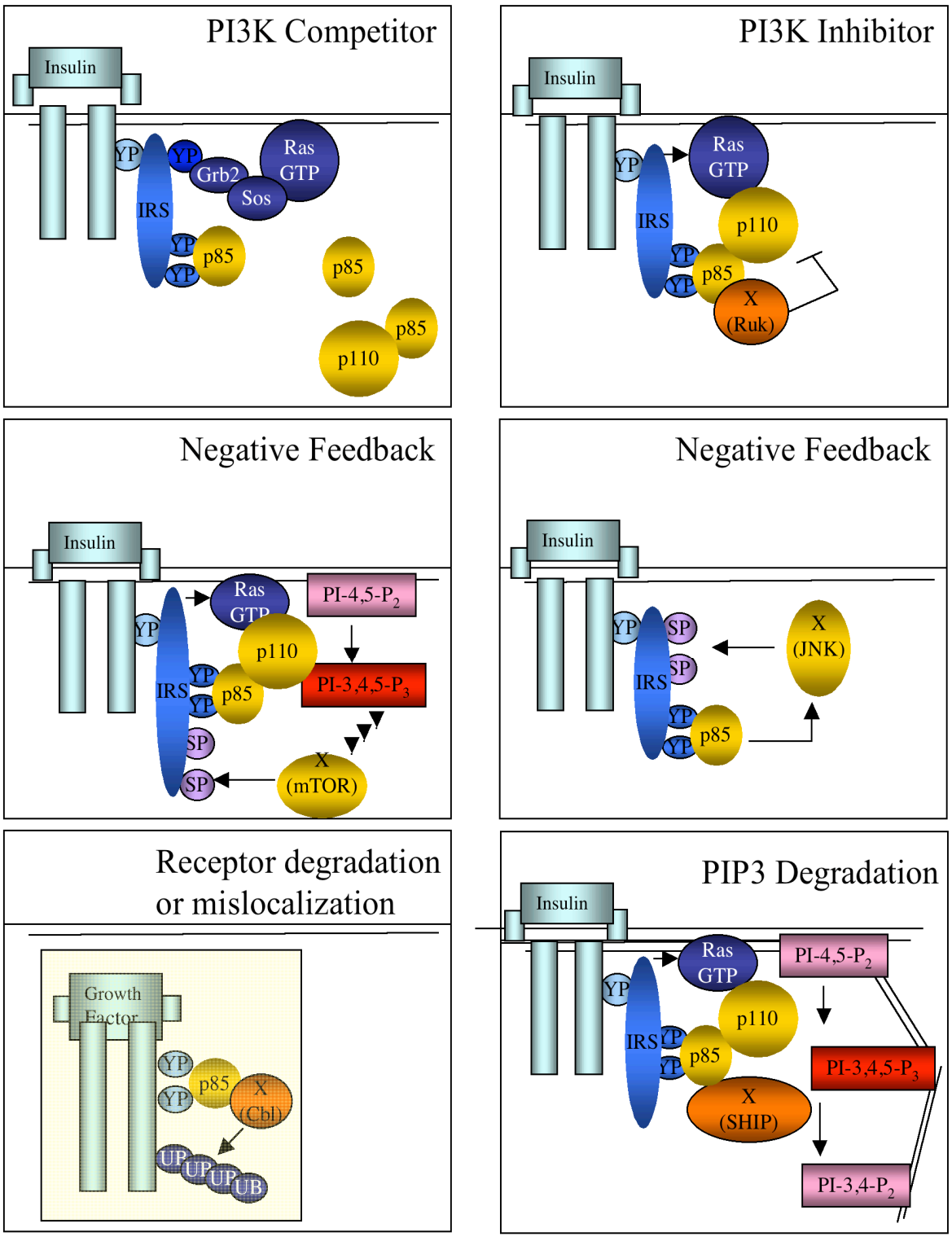

Fig. 3. Models how p85 could inhibit receptor signaling

GOAL OF THESIS

The goal of this thesis is to understand the *in vivo* role of PI3K catalytic and regulatory isoforms in development and glucose homeostasis. Furthermore, I wanted to compare the role of p85 isoforms in actin rearrangement processes. In order to do this, I analyzed and compared defects in mice lacking all p85α and p85β gene products (p85α-/-p55γ-/-p50α-/-p85β-/-), mice lacking only 85kD isoforms (p85α-/-p55γ+/+p50α+/+p85β-/-) and mice lacking the catalytic subunit p110α (p110α-/-) at various developmental stages. The results are described in Chapter 3. Secondly, I assessed the capability of various p85 isoforms to mediate actin rearrangement processes, such as cell ruffling and migration in mouse embryonic fibroblasts derived from p85α-/-p55γ+/+p50α+/+p85β-/- mice by retroviral reintroduction of the individual isoforms. The findings are discussed in Chapter 4. Thirdly, I examined the consequence of single or combined heterozygous loss of PI3K catalytic isoforms (p110α+/- vs p110β+/- vs p110α+/-p110β+/-) on insulin and glucose metabolism. These results are discussed in Chapter 5. In Chapter 6, I describe the determination of the optimal binding motifs for the SH3 domains of p85α and p85β via a peptide library approach and the identification of potential isoform-specific interaction partners via GST-pulldown and mass spectrometry.