SUMMARY AND FUTURE DIRECTIONS

The data presented in this thesis show the pivotal role of class Ia PI3K in a variety of cellular responses. In chapter 3, we present evidence that loss of such a key player in the mouse causes early embryonic death. Loss of p110α or combined loss of p85α and p85β results in early embryonic lethality with similar defects as those seen in PDGF receptor β null mice. Our data suggest that during early development p110α/p85α and p110α/p85β complexes mediate PDGF signals, which cannot be accomplished by p110β or p55/p50 isoforms.

Furthermore, in chapter 4, we discuss that loss of class Ia PI3K results in defects in various PDGF-induced cellular responses. We present evidence that class Ia PI3K is necessary for proliferation, lamellipodia formation, polarization and cell migration. We show that the interaction between Rac-GTP / Cdc42-GTP with the Rho-GAP domain of p85α / p85β is surprisingly not essential for PDGF- or IGF-1-induced lamellipodia formation, since the p50α isoform lacking the Rho-GAP domain mediates this response.

As discussed in chapter 5, loss of the PI3K regulatory subunit (p85α-/-) or combined heterozygous loss of p110α and p110β (p110α+/-p110β+/-) both result in greatly diminished total PI3K activity as well as greatly reduced IRS-associated PI3K activity. However, the effects of these deletions on glucose disposal is opposite: Deletion of p85α results in increased insulin sensitivity, while deletion of p110α and p110β results in decreased insulin sensitivity. We conclude, that p85 plays a negative role in insulin signaling independent of its role in mediating activation of the p110 catalytic subunits.

Our observation that mice lacking p85α and p85β but capable of expressing the shorter isoforms of p85α are not viable indicate that the smaller PI3K regulatory subunits p55α, p55γ and p50α do not sufficiently substitute for combined loss of p85α and p85β during development. We do not know whether restricted expression of these isoforms or lack of crucial domains is the reason for lethality. It would be interesting to analyze the temporal and spatial expression of each isoform during development. For example in situ

hybridization with probes specific to sequences unique to the alternative splice forms of p85α would indicate their expression patterns.

It is also interesting to understand if the PDGF receptor β signaling to p110β is restricted to development or whether this pathway is important for events such as wound healing in adult animals. To investigate this it would be useful to analyze PDGF-induced cellular responses in p110β deficient MEFs or in inducible-tissue specific deletions. Unfortunately, p110β null cells do not proliferate (Bi et al., 1999) and are resistant to immortalization (only cell lines with great chromosomal abnormalities were obtained). However, Dr. Thomas Roberts laboratory is currently generating mice with p110β floxed alleles. MEFs could be established from the p110β$^{floxed/floxed}$ mice and both alleles could be acutely deleted in culture after Cre-recombinase addition after the cells have been expanded to the desired quantity. This approach and the use of p110 isoform-specific inhibitors (which are currently being developed by various companies) could link a distinct PI3K catalytic subunit to a particular cellular response.

The interaction between Rac-GTP/Cdc42-GTP with the Rho-GAP homology domain of p85 is not essential for PDGF- or IGF-1-induced lamellipodia formation in fibroblasts. However, this interaction might be crucial for mediating a positive feedback loop to generate an internal PIP_3 gradient that might be important for more complex cellular responses, such as cell polarization and migration. Unfortunately, the MEFs generated by the conventional knock-out approach did not allow us to address this issue, since restoration of p85α or p85β did not rescue the polarization or migration defect, although single ablation of either full-length p85 isoform (p85α-/- MEFs or p85β-/- MEFs) does not result in major polarization or migration defects. We assume that p85α-/-p55γ-/-p50α-/-p85β-/- MEFs engaged compensatory mechanisms during immortalization that allowed the mutant cells to survive and proliferate in the absence of class Ia PI3K, but that these mechanisms are not reversed by add-back of PI3K. To address if the positive feedback loop between PI3K and Rac is mediated at least in part via the Rho-GAP homology domain of p85α/β it would be useful to establish MEFs in which we acutely switch from p85α to p50α expression and then, analyze the capability of these cells to undergo PDGF-induced polarization and migration. Therefore, MEFs with floxed alleles of all p85α splice forms on a p85β null background

(p85α$^{floxed/floxed}$p55α$^{floxed/floxed}$p50α$^{floxed/floxed}$p85β-/-) could be infected with retroviral p50α and then, the endogenous p85α, p55α and p50α could be deleted by adding Cre-recombinase. At no time, would the p85α-/-p55α-/-p50α+/+p85β-/- MEFs have been deficient in class Ia PI3K and hence needed to engage compensatory mechanisms. Furthermore, the p85α-/-p55α-/-p50α+/+p85β-/- MEFs could be challenged in a chemotactic gradient instead of applying a uniform stimulus to induce cell polarization and migration. These experiments are currently in progress.

Although studies with inhibitors and genetic ablation of p110γ (class Ib PI3K) implicated PI3K in migration of neutrophils and macrophages the role of class Ia PI3K is still unclear (Hirsch et al., 2000). Although, many studies address the function of class Ia PI3K in cell motility in fibroblasts, neutrophils/macrophages are more "professional" in cell polarization and migration. Their main function is to quickly move through the body to sites of infections. Neutrophils/macrophages are much more flexible and faster than fibroblasts. It might be possible that the PI3K-Rac-PI3K positive feedback loop is playing a more important role in neutrophils than fibroblasts. Recent studies with a p110δ-specific inhibitor showed defects in fMLP-stimulated cell polarization and chemotaxis in neutrophils despite the presence of the class Ib PI3K p110γ isoform (Sadhu et al., 2003). Neutrophils and macrophages could not be obtained from p85α-/-p55α-/-p50α-/-p85β-/- mice since these mutant mice die before their fetal liver is sufficiently developed. However, p85α$^{floxed/floxed}$p55α$^{floxed/floxed}$p50α$^{floxed/floxed}$p85β-/- mice are viable (Ji Luo, personal communication) and therefore a great source for the desired blood cells. Thus, neutrophils and macrophages with floxed alleles of all p85α splice forms on a p85β null background (p85α$^{floxed/floxed}$p55α$^{floxed/floxed}$p50α$^{floxed/floxed}$p85β-/-) could be infected with retroviral p85α, p85β or p50α and then, the endogenous p85α, p55α and p50α could be deleted by adding Cre-recombinase. By doing so, the role of the various class Ia PI3K isoforms as well as the importance of the Rho-GAP domain of p85α and p85β in cell polarization and migration could be studied in neutrophils and macrophages.

Alternatively, the hypothesis of Rac activating PI3K via the Rho-GAP homology domain of p85α/β could be tested by acutely inducing the expression of a constitutively active form of Rac (RacV12) in the mutant cells (p85α-/-p55α-/-p50α-/-p85β-/-) that

were restored for expression of either p85α, p85β or p50α (lacking the Rho-GAP homology domain).

Finally, the p85α-/-p55γ-/-p50α+/+p85β-/- MEFs did not proliferate unless immortalized with SV40 large T. Therefore, careful analysis of the role of class Ia PI3K in the regulation of the cell cycle could not be conducted. However, the MEFs generated from p85α$^{floxed/floxed}$p55γ$^{floxed/floxed}$p50α$^{floxed/floxed}$p85β-/- mice offer a great tool for such studies.

In chapter 5, we provide evidence that loss of PI3K catalytic subunits (p110α+/-p110β+/-) has the opposite effect on insulin signaling to the loss of PI3K regulatory isoforms (p85-/-). Heterozygous loss of p110α or p110β alone had no significant effect on glucose homeostasis. In order to further address the roles of these two subunits in insulin signaling it would be interesting to engineer mice that allow deletion of either p110α or p110β in insulin-responsive tissues. Homozygous deletion of p110α or p110β in just one organ (liver, muscle, fat, brain, pancreas, heart) most likely would not result in death and could allow analysis of glucose homeostasis without compensatory effects of the remaining p110 protein like in the heterozygous mice.

Various models could explain the negative role of p85 in insulin signaling (Fig. 35). As discussed in chapter 5, p85 could bind to and activate a PIP_3 phosphatase. It has been previously reported that p85 binds via its SH2 domain to the 5'- phosphatase SHIP-1 (Gupta et al., 1999). However, SHIP-1 is only expressed in hematopoetic cells whereas SHIP-2 is more widely expressed, including in insulin-sensitive tissues. Indeed, SHIP-2 plays an important role in glucose homeostasis. Genetic ablation of SHIP-2 in the mouse results in improved insulin signaling (Clement et al., 2001). It would be interesting to determine whether p85 recruits SHIP-2 to sites of PIP_3 production. In a biochemical approach we observed that the SH3 domain of p85β binds to SHIP-2 *in vitro* (data not shown). In addition, we determined the optimal binding motifs of the SH3 domains of p85α and p85β by using a peptide library approach developed by Songyang in the Cantley laboratory (Songyang et al., 1993) and subsequently modified by Mary Lynch in the Cantley laboratory (Grabs et al., 1997). The consensus sequence for the optimal binding motif for the SH3 domain of p85α is present in both SHIP-2 and SHIP-1 and this might explain the *in vitro* interaction we observed. There is evidence that p85 interacts

with signaling molecules simultaneously through its SH2 and SH3 domains, e.g. the interaction between p85 and c-cbl (Soltoff and Cantley, 1996). Therefore it is likely that p85 interacts with SHIP isoforms through both the SH2 and SH3 domains. It remains to be determined whether p85α interacts with SHIP-2 *in vivo*. To do so, co-immunoprecipitations could be investigated.

Furthermore, we detected c-cbl in the GST-pulldowns with the SH3 domain of p85α. It has been reported that cbl binds preferentially to p85β (Hartley et al., 1995). The optimal binding motifs for the SH3 domains of p85α and p85β derived from the peptide library approach also suggest that p85α and p85β SH3 domains have different specificities. There is the possibility that cbl is mediating a negative role of p85 in insulin signaling. Cbl has been implicated in mediating endocytosis and degradation of receptor complexes. The interaction between cbl and p85 could recruit cbl to the phosphorylated insulin receptor substrate and result in the mislocalization of the signaling complex. There is evidence from our lab that upon IGF-1 stimulation of CHO-IR cells, IRS-1/p85 complexes are accumulating away from the plasma membrane into speckles (Ji Luo, personal communication). It is not clear whether this compartmentalization of the signaling complex serves as a shut off mechanism or a mechanism of localizing the signal. Finally, as discussed in chapter 5, p85 seems to regulate JNK activity independently of p110. JNK activity has been implicated in a negative feedback loop that ultimate shuts off insulin-dependent glucose uptake *in vivo*.

The protein levels of the catalytic and regulatory subunits of PI3K are interdependent. Loss of p85 causes reduction of p110 due to p110's thermal instability and, very surprisingly, heterozygous loss of both p110α and p110β (p110α+/-p110β+/-) results in reduction of the regulatory isoforms. It is not clear yet how p110 influences the p85 protein levels, but we have preliminary evidence that the mRNA levels of p85 are not affected upon loss of p110. Therefore the p85 protein levels might be controlled at the posttranslational level. It seems that insulin signaling is regulated by a delicate balance between the positive mediator p85-p110 and the negative regulator p85. Since subtle changes in the p85/p85-p110 ratio have a dramatic effect on insulin sensitivity, it is not surprising that the protein levels of the catalytic and regulatory subunits of PI3K are tightly controlled. However, the interpretation of PI3K deficient mice is complicated by

the interdependence of p110 and p85 expression levels. It is important to delete p110 subunits without influencing the expression of p85. To do so an enzyme-deficient knock-in approach is most promising. The p110 protein should be present but lacking enzymatic activity. In the case that p85 expression is independent of PI3K downstream signaling but dependent on p110 protein the p85 levels should not change in this system.

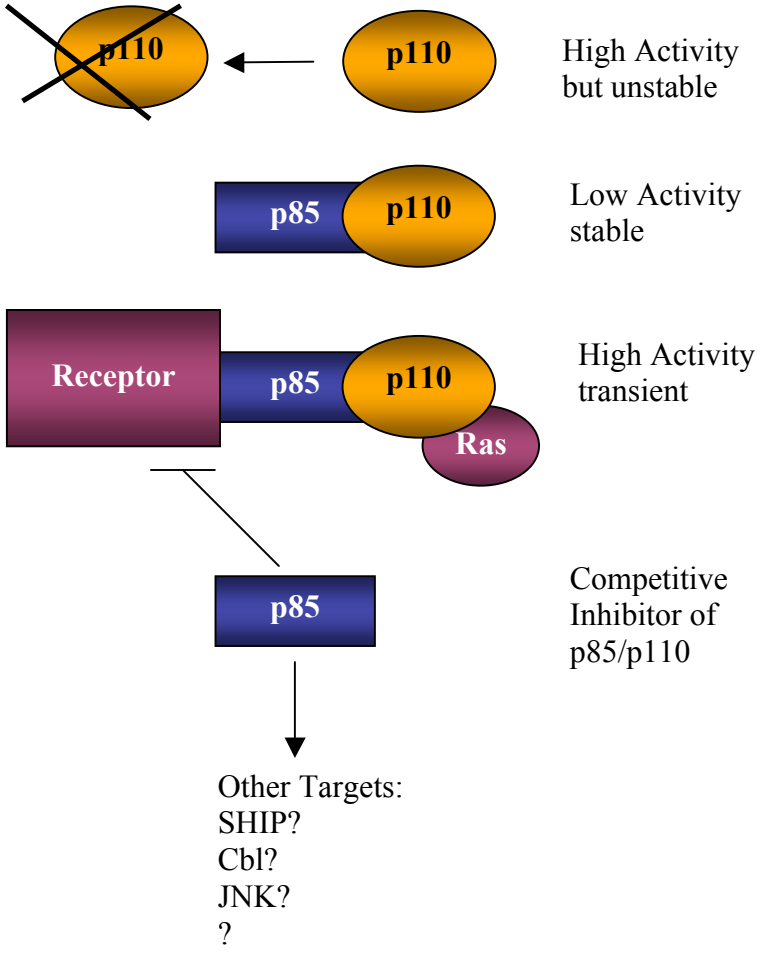

Fig. 35. Roles of p85. The monomeric PI3K catalytic subunit p110 has a higher lipid kinase activity than p110 that is bound to p85. However, monomeric p110 is thermally unstable at 37°C. The binding of p110 to p85 causes a drop in p110's lipid kinase activity of 65-85%. However, this "inhibitory" effect of p85 on p110 is overcome by engagement of both SH2 domains of p85 with phosphotyrosine containing containing peptides. Furthermore, the interaction between p85 and phosphotyrosine motifs on receptors or receptor substrates recruits the p85 associated p110 into proximity with its membrane bound substrates. PI3K activity is further augmented by the interaction between p110 and Rac-GTP. P85 seems to play an inhibitory role on insulin signaling. Monomeric p85 could compete with p85-p110 complexes for binding sites on the activated receptors or potentially fulfill inhibitory functions via the PIP_3 -phosphatase SHIP-2, Cbl or JNK.

Finally, we have provided the p85α-/-p55α-/-p50α-/-p85β-/- MEFs to a number of collaborators for use in determining the role of class Ia PI3K in various cellular responses. Eight publications have resulted from these collaborations (see CV). The major findings of these papers are listed below:

1) Class Ia PI3K is required for optimal phagocytosis by promoting the formation of the nascent phagosomal cup (Vieira et al., JCB, 2001).

2) P85 is in excess over p110 and reduction of p85 (in p85α+/-p55α+/-p50α+/- cell lines) results in improved IGF-1/insulin-mediated signaling whereas complete loss of p85α (p85α/p55α/p50α-/- cell lines) results in increased apoptosis (Ueki et al., MCB. 2002).

3) The mitotic inhibition of autophagy is not relieved by loss of class Ia PI3K (Eskelinen et al., Traffic, 2002).

4) Class Ia PI3K activates Rac via the Eps8-Abi1-Sos-1 complex (Innocenti et al., JCB 2003).

5) The Jaagsiekte sheep retrovirus–induced transformation (JSRV) does not require class Ia PI3K (Maeda et al., J Virol. 2003).

6) The protozoan parasite, Trypanosoma cruzi, invades the host cell via two distinct PI3K-dependent pathways: a Ca^{2+} -regulated lysosome-mediated and an alternative actin-independent entry pathway (Woolsey et al., J Cell Sci. 2003).

7) IFNα and IFNβ mediate mRNA translation in a class Ia PI3K dependent manner (Lekmine et al., JCB. 2003).

8) Loss of the class Ia PI3K regulatory subunit p85α results in improved insulin sensitivity (Ueki et al., PNAS. 2002).

Genetic ablation of catalytic and regulatory subunits of PI3K in the mouse allowed us to analyze *in vivo* the complex role of class Ia PI3K in glucose homeostasis, development and cell migration.