Chapter 3

Developmental defects in mice lacking various p85 isoforms

ABSTRACT

It has been previously shown, that disruption of the phosphoinositide 3-kinase (PI3K) catalytic isoforms, p110α or p110β, caused early embryonic death at E10.5 and < E3.5, respectively (Bi et al., 1999), (Bi et al., 2002). Embryonic development was not impaired in mice lacking the regulatory subunit p85α (p85α-/-) or p85β (p85β-/-) suggesting redundancy between the two 85kD PI3K regulatory isoforms (Terauchi et al., 1999), (Ueki et al., 2002b). In contrast, mice lacking all p85α splice forms (p85α-/-p55α-/-p50α-/-) had a number of developmental defects, which were not manifested until birth (Fruman et al., 2000). These data suggested that intact expression of the p85α splice forms p55α and p50α can partially compensate for the loss of p85α.

We generated mice lacking all gene products of p85α and p85β (p85α-/-p55α-/-p50α-/-p85β-/-) to further analyze the role of the PI3K regulatory subunits. The mutant mice die during early embryonic development (E12.5). We further demonstrated that mice lacking the long p85 isoforms p85α and p85β (p85α-/-p55α+/+p50α+/+p85β-/-) die at embryonic day E13.5. The data presented here demonstrate that the long p85 isoforms (p85α and p85β) function in a redundant manner whereas the expression of the smaller p85 isoforms (p55α and p50α) can only partially substitute for the combined loss of p85α and p85β. The developmental defects of mice lacking p85α (with or without ablation of the smaller p85α splice forms) and p85β are highly similar to defects reported for platelet-derived growth factor (PDGF) receptor β deficient mice (Soriano, 1997). The mutant mice exhibit subepidermal blebs flanking their neural tube, hemorrhaging and facial abnormalities. Also genetic ablation of p110α results in a similar phenotype, including subepidermal blebbing and hemorrhaging. Since p110β does not compensate for loss of p110α, we conclude that p110α/p85α and p110α/p85β complexes are mediating PDGF receptor β signaling during the development of a mouse.

INTRODUCTION

Role of PI3K in development of the mouse

Higher eukaryotic organisms arise from just two cells: the egg from the mother and the sperm from the father. In order to develop into a functional animal, these two cells fuse to a zygote that then quickly proliferates. The evolving cells differentiate into multiple cell types, migrate through the constantly changing body and have to survive along the path of their journey. Some cells undergo programmed cell death (apoptosis) for example to form the digits of the hands. How does a cell know what to do and when? The decision is controlled by communication with the surrounding environment. Cells sense cues from their environment via receptors, such as the platelet derived growth factor (PDGF) receptor that span the plasma membrane and transmit external signals to the inside of the cell. Upon binding of an external ligand the PDGF receptor dimerizes and undergoes autophosphorylation on intracellular tyrosine residues. Some of these phosphotyrosine residues are within sequence contexts that allow binding to the PI3K regulatory subunit p85 and this results in recruitment of PI3K to the inner leaflet of the plasma membrane into proximity with its substrates. During this signal transduction process PI3K generates PI-3,4,5-P_3 and PI-3,4-P_2. Both PI3K lipid products are second messengers that amplify, spread and transduce the signal from the plasma membrane to the inside of the cell. In vitro studies using PI3K binding mutants of the PDGF receptor have indicated that PI3K is responsible for PDGF induced cell proliferation, cell survival and migration (Valius and Kazlauskas, 1993), (Bazenet and Kazlauskas, 1994), (Kundra et al., 1994), (Joly et al., 1994). However, these mutations have also been shown to disrupt association with other signaling molecules (Nishimura et al., 1993). Dominant negative forms of PI3K and drug inhibitors have also supported a role for PI3K in multiple responses to PDGF. But these studies are complicated by the ability of dominant negative forms of PI3K to compete with other signaling proteins for binding to the PDGF receptor and by the ability of existing PI3K inhibitors to inhibit a variety of lipid kinases

and protein kinases. Thus, to evaluate the role of PI3K isoforms in PDGF signaling during development and in isolated cells, it is critical to delete specific genes.

PI3K is a major target of PDGF receptor β during the development of the mouse. The PDGF ligand is a dimer composed of either two PDGF-A chains (AA), two PDGF-B chains (BB), two PDGF-C chains (CC) or two PDGF-D chains (DD). So far there is only evidence that PDGF-A and PDGF-B chains can heterodimerize (AB). Also two distinct genes encode PDGF receptors (PDGFR) in mammals: PDGFR α and PDGFR β. Upon binding of the dimeric PDGF ligands the PDGFR chains either homo- or heterodimerize: PDGFR-αα, PDGFR-αβ and PDGFR-ββ. The PDGFRα chain can interact with PDGF-A/-B/-C, while the PDGFRβ is able to bind to PDGF-B/-D. Experiments using transgenic mice in which the intracellular domains of the PDGF receptor α and PDGF receptor β have been exchanged, suggest that PDGF receptor α and β fulfill unique functions during embryonic development (Klinghoffer et al., 2001). Mice deficient in PDGF ligands or the β receptors exhibit embryonic lethality with cardiovascular, renal and haematological defects, demonstrating a role of PDGF signaling in muscle and vascular development (Leveen et al., 1994), (Soriano, 1994), (Bostrom et al., 1996), (Soriano, 1997), (Lindahl et al., 1998). Targeted disruption of PDGF receptor β resulted in embryonic lethality at E16 (Soriano, 1997). The mutant mice exhibited subepidermal blebs, haemorrhaging, wavy neural tubes and misformed facial structures. Taking the important role of PI3K in PDGF activated signaling *in vitro* into consideration, it was very surprising that mice lacking the PI3K binding site of the PDGF receptor β were viable, developed normally and had only minor abnormalities in their interstitial fluid homeostasis (Heuchel et al., 1999).

We show here that targeted disruption of all p85α and p85β gene products (p85α-/-p55α-/-p50α-/-p85β-/-) caused early embryonic death (E12.5) accompanied with similar defects as those seen in PDGF receptor β null mice. Furthermore, similar phenotypes were also observed p85α-/-p55α+/+p50α+/+p85β-/- and p110α-/- mice. We conclude that during development p110α fulfills unique functions downstream of the PDGF receptor β that cannot be mediated by p110β. In addition, both full-length PI3K

regulatory isoforms (p85α and p85β) can recruit p110α to the PDGF receptor β. These findings points to a p110α specific role in PDGF signaling *in vivo*.

RESULTS

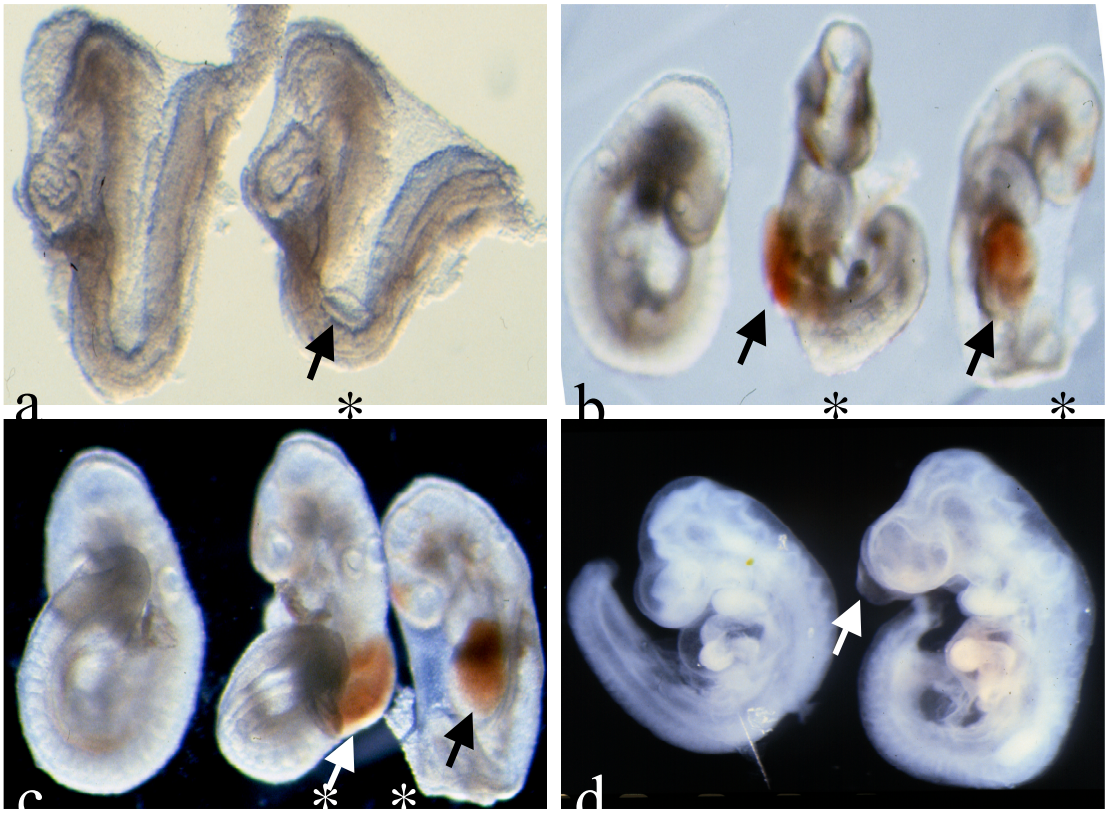
Embryonic death of mice lacking all gene products of p85α and p85β. The early embryonic lethality of mice lacking PI3K catalytic isoforms p110α or p110γ demonstrated an important non-redundant function of the PI3K catalytic isoforms in murine development (Bi et al., 1999), (Bi et al., 2002). In contrast, the PI3K regulatory isoforms p85α and p85β can compensate for each other and seem to function in a redundant manner (Terauchi et al., 1999), (Fruman et al., 2000), (Ueki et al., 2002b). In order to elucidate the role of PI3K regulatory isoforms during development, we generated mice with a combined loss of all p85α and p85β gene products. Since p85β-/- mice but not p85α-/-p55γ-/-p50α-/- mice survive until adulthood, p85α+/-p55γ+/-p50α+/- mice were crossed with p85β-/- mice to obtain p85α+/-p55γ+/-p50α+/-p85β+/- mice. Then, p85α+/-p55γ+/-p50α+/-p85β+/- mice were intercrossed to obtain p85α-/-p55γ-/-p50α-/-p85β-/- mice. No mice deficient in both p85α (and its smaller variants) and p85β were born demonstrating the requirement for the class Ia PI3K regulatory subunit (p85) in development. In order to determine at which embryonic stage the mutant mice died, timed pregnancies were analyzed. Expected Mendelian ratios of viable embryos lacking p85α, p55γ, p50α and p85β were detected until embryonic day E11.5. However, the mutant embryos exhibited severe developmental defects (Table 3). All mutant embryos displayed on the trunk one or more subepidermal blebs (detachment of the most outer epithelial cell layer) flanking the neural tube on the opposite side of the heart at a time right before the embryos turn to achieve the fetal position (E8-8.5) (Figure 4a). The mutant embryos exhibited rarely a dilated pericardium or twining. After the embryos had turned about their anterior-posterior axis (E8.5-9.5) many of the blebs were filled with blood (Figure 4b,c). At later stages (E10-11.5), blebs and other facial abnormalities (e.g clefted face) were detected on the head (Figure 4d). Some of the mutant embryos had a

dilated, wavy neural tube and exhibited multiple hemorrhages in the head region and branchial arches. At E12.5 p85□-/-p55□-/-p50□-/-p85□-/- embryos were pale and exhibited no heartbeat. The mutant embryos were delayed in their development and therefore smaller in size in comparison to their littermates.

Genotype Stage	*A-/-B-/-*	*A+/-B-/-*	*A+/+B-/-*
E8-9	**9** (60%) All unturned embryos: subepidermal blebs flanking neural tube All turned embryos: some of blebs are filled with blood	**6** normal	**5** normal
E9.5-11.5	**12** (22%, of those 2 dead) All embryos: subepidermal blebs mostly flanking neural tube, some on branchial arches or head, Some of these structures are filled with blood. Some embryos exhibit also a wavy neural tube.	**27** normal	**15** normal
E11.5-12.5	**14** (23%, of those 10 dead) Alive embryos: severe facial abnormalities,such as clefted face, blebing and hemorrhaging. A few embryos exhibit blebs on trunk and a [illegible] neural tube	**33** normal	**15** normal
>E12.5	**10** (14%, of those 9 dead) Alive embryo: developmentally delayed with small bloody dot in trunk	**30** normal	**12** normal

A-/-B-/-: p85□-/-p55□-/-p50□-/-p85□-/-
A+/-B-/-: p85□+/-p55□+/-p50□+/-p85□-/-
A+/+/B-/-: p85□+/+p55□+/+p50□+/+p85□-/-

Table 3: p85 is required for proper embryonic development
Viability of offspring from p85□+/-p55□+/-p50□+/-p85□-/- intercrosses.
Alive embryos deficient in p85□-/-p55□-/-p50□-/-p85□-/- were detected in expected Mendelian ratios until E11.5. Ten mutant embryos were found > E12.5. However, nine of those were already dead or resorbing.
Phenotype of p85□-/-p55□-/-p50□-/-p85□-/- embryos.
The defects of the mutant embryos included subepidermal blebing, hemorrhaging and wavyness of the neural tube. Facial abnormalities occurred after the appearance of blebs in the trunk.



* p85□-/-p55□-/-p50□-/-p85□-/-

Fig. 4. p85 deficiency causes similar subepidermal blebbing.
A, Subepidermal blebbing in unturned E8 day old p85□-/-p55□-/-p50□-/-p85□-/- embryo. One unturned control littermate embryo (left) and one unturned p85a-/-p85□-/-p55□-/-p50□-/-p85□-/- embryo (right) that shows a subepidermal bleb on trunk.
B/C, Blood filled bleb in turned E9.5 day old p85□-/-p55□-/-p50□-/-p85□-/- embryos. One control littermate embryo (left) and two p85□-/-p55□-/-p50□-/-p85□-/- embryos (middle and right). Both embryos lacking p85a gene products and p85b display blood filled blebs flanking their neural tube.
D. Facial abnormalities in E11.5 day old p85□-/-p55□-/-p50□-/-p85□-/- embryo. One control littermate embryo (left) and a p85□-/-p55□-/-p50□-/-p85□-/- embryo (right) which has a non blood filled subepidermal structure on the head.

Next, histological sections of p85α-/-p55γ-/-p50α-/-p85β-/- and control embryos at E8-11.5 were analyzed to elucidate defects of the mutant mice on the cellular level. The embryos were fixed, embedded in paraffin and sagittal sections were stained with hematoxilin and eosin. The sections of the mutant mice at E8.5 showed detachment of the outer epithelial cell layer (Figure 5).

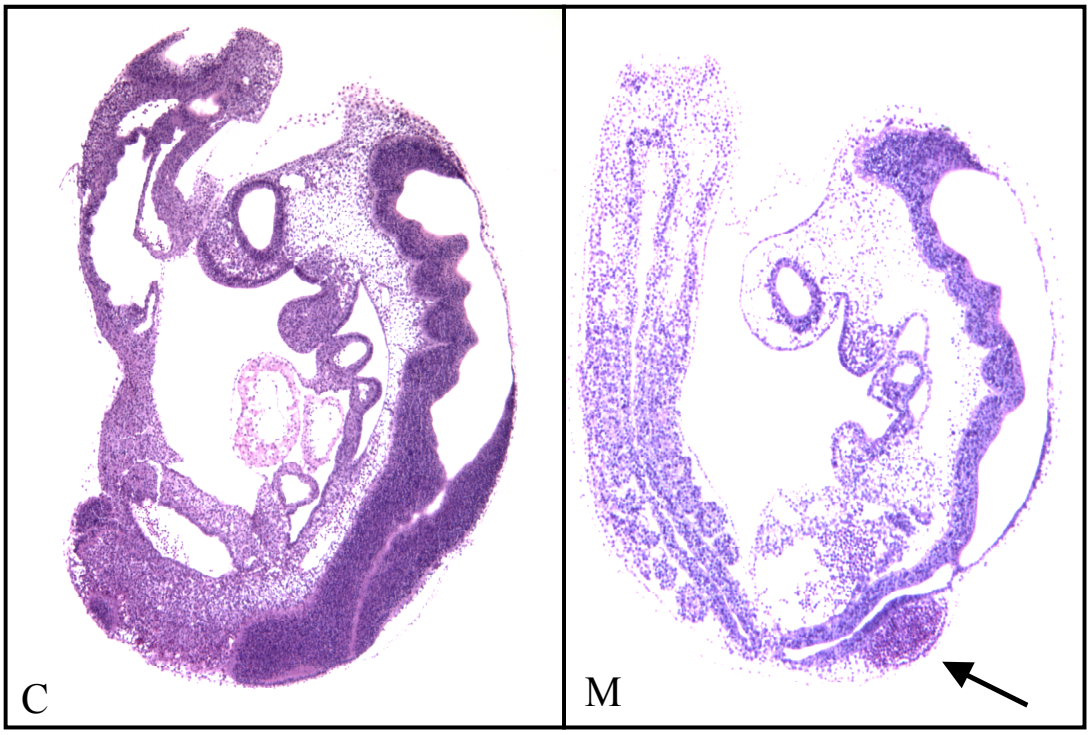
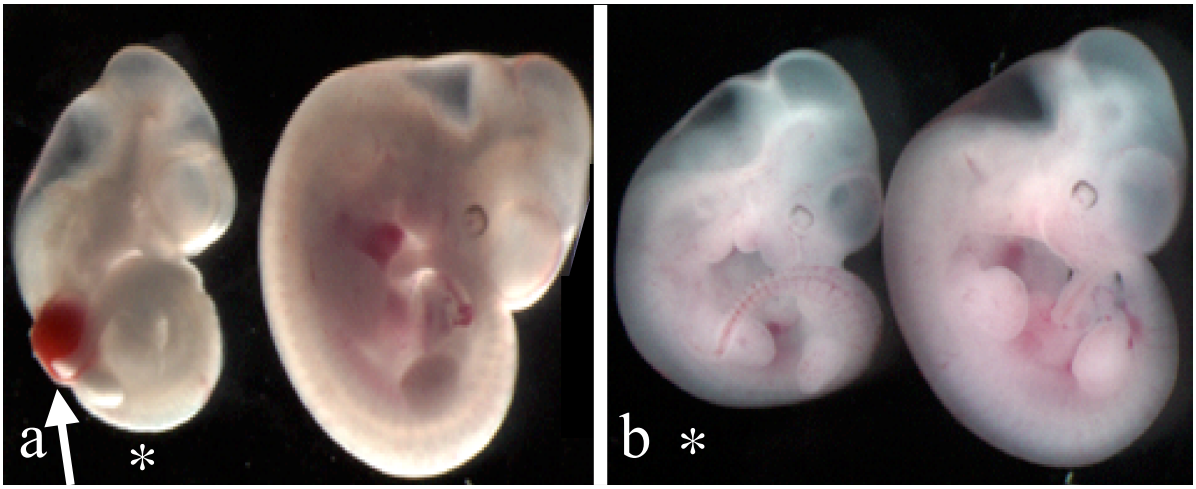


Fig. 5. Loss of class Ia PI3K causes subepidermal blebing in trunk region. Blood filled, subepidermal bleb in turned E9 day old p85α-/-p55γ-/-p50α-/-p85β-/-embryo. One control littermate embryo (C) and one p85α-/-p55γ-/-p50α-/-p85β-/- embryo (M). Embryos were dissected at E9, embedded in parafin and sagittal sections were stained with H&E to study their cell sructures.

Sections between E9.5-11.5 revealed wavy neural tubes, disturbed somites as consequences of epidermal blebbing, increased mesenchyme and hemorrhaging to various degrees (data not shown).

Partial redundancy of the smaller PI3K regulatory isoforms, p55γ and p50α in embryonic development. Next, we investigated whether intact expression of the smaller PI3K regulatory isoforms p55γ and p50α (which do not contain the N-terminal domains

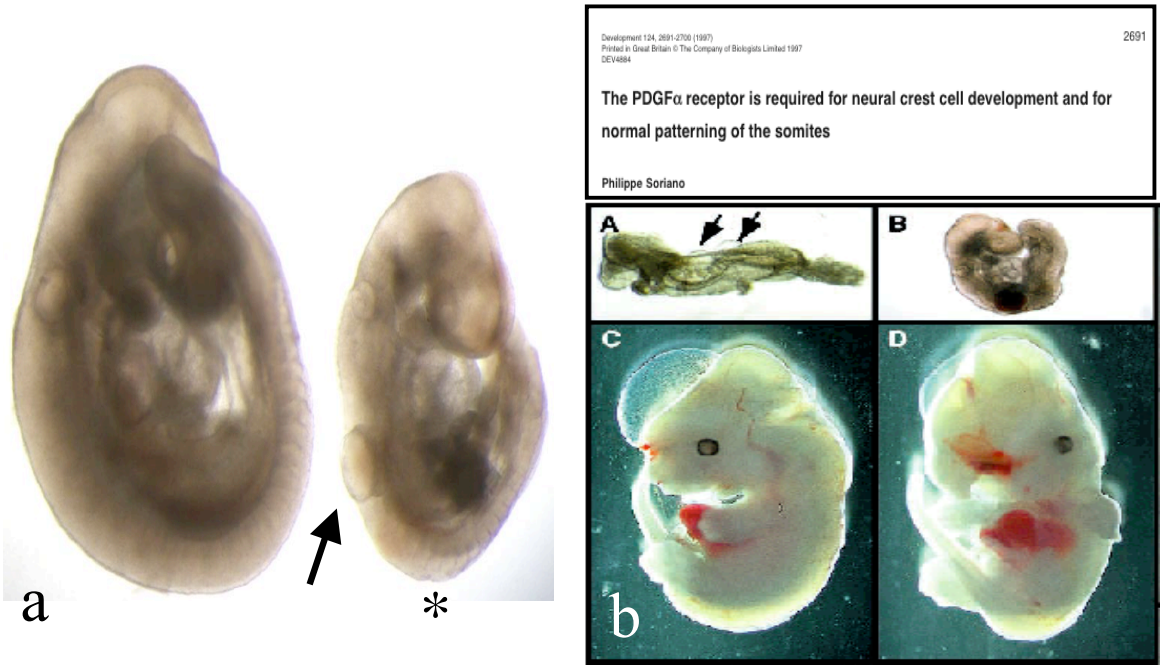
of 85kD isoforms) can rescue the defects seen in p85□-/-p55□-/-p50□-/-p85□-/- embryos. To generate mice that lack both 85kD isoforms but express the p55□ and p50□ isoforms (p85□-/-p55□+/+p50□+/+p85□-/-), p85□-/- mice (which have the first exon of p85□ deleted but retain exons that allow expression of the p55□ and p50□ isoform (Terauchi et al., 1999) were crossed with p85□-/- mice. Viable p85□-/-p55□+/+p50□+/+p85□-/- embryos were detected until E12.5, but similarly to p85□-/-p55□-/-p50□-/-p85□-/- mice, p85□-/-p55□+/+p50□+/+p85□-/- mice exhibited bloody blebs flanking their neural tube, hemorrhaging, wavy neural tubes and facial abnormalities (Figure 6a,b) and the embryos were delayed in their development. However, the phenotype of p85□-/-p55□+/+p50□+/+p85□-/- embryos was less severe (less embryos were affected) as compared to p85□-/-p55□-/-p50□-/-p85□-/- embryos. Despite of the milder phenotype, p85□-/-p55□+/+p50□+/+p85□-/- mice did not survive past E13. These data demonstrate that intact expression of p55□ and p50□ together can partially compensate for loss of PI3K 85kD isoforms during embryonic development.



* p85□-/-p55□+/+p50□+/+p85□-/-

Fig. 6. Defects in p85□-/-p55□+/+p50□+/+p85□-/- embryos.
A, Blood filled bleb in turned E10.5 day old p85□-/-p55□+/+p50□+/+p85□-/- embryo. One control littermate embryo (right) and one p85□-/-p55□+/+p50□+/+p85□-/- embryo (left). Embryos lacking both 85kD isoforms (p85□ and p85□) display blood filled bleb flanking their neural tube. Mutant embryo is smaller than control littermate.
B, Minor defects in turned E10.5 day old p85□-/-p55□+/+p50□+/+p85□-/- embryo. One control littermate embryo (right) and one p85□-/-p55□+/+p50□+/+p85□-/- embryo (left). Embryo lacking both 85kD isoforms (p85□ and p85□) is smaller than control littermate but exhibits no blebs.

Loss of p110□ developmental abnormalities similar to those due to combined loss of p85□ and p85□. Next, we compared the phenotype of p110□-/- mice to mice lacking p85□ (with or without intact expression of p55□ and p50□) and p85□. In contrast to p110□ null mice, which die at <E3.5 (Bi et al., 2002), p110□ null mice survive until day E10.5 (Bi et al., 1999) and therefore overlap temporally with the time frame of the phenotype observed in p85□-/-p55□-/-p50□-/-p85□-/- mice. We intercrossed p110□+/- mice and analyzed timed pregnancies up to E10.5. Extensive hemorrhaging and proliferative defects have been described previously for mice with targeted disruption of p110□. Interestingly, some of these mutant embryos have subepidermal blebs (some are filled with blood) in the same position as p85□-/-p55□-/-p50□-/-p85□-/- mice (Figure 7a). The frequency of this and additional defects are described in detail in the publication by Bi et al. (Bi et al., 1999).



* p110□-/-

All: PDGF receptor □ -/-

Fig. 7. Loss of class Ia PI3K causes similar phenotype as PDGF receptor □ null mice.
A, Subepidermal bleb in turned E10.5 day old p110□-/- embryo. One control littermate embryo (left) and one p110□-/- embryo (right). Embryo lacking p110□ displays subepidermal bleb flanking the neural tube. Mutant embryo is smaller than control littermate.
B, Subepidermal blebs of PDGF receptor □ null embryo at various developmental stages. (a) E8.5: bleb in trunk flanking neural tube, (b) E9.5: blood filled bleb in trunk, (c,d) E12.5: large blebs on head accompagnied by bleeding and a cleft face.

The observed phenotype of PI3K mutant embryos is highly similar to the defects seen in embryos lacking PDGF receptor β. A previous study of mice lacking the PDGF receptor β gene (Soriano, 1997) reported developmental defects similar to those we observed in p85α-/-p55γ-/-p50α-/-p85β-/- mice: subepidermal blebs flanking the neural tube starting at E8 and wavy neural tubes, hemorrhaging and facial abnormalities later in development (Figure 7b). The defects in PDGF receptor β mice were partially attributed to increased apoptosis. Since PI3K has been shown to be involved in PDGF mediated cell survival in other systems, we examined whether PI3K might be a necessary contributor to survival downstream of the PDGF receptor β during development. Disruption of p85 isoforms might also lead to increased apoptosis and hence blebbing. We tested this hypothesis by TUNEL stain of whole mount p85α-/-p55γ-/-p50α-/-p85β-/- embryos at E8. The experiments revealed no difference between mutant (12 mutant embryos) and control embryos in their apoptotic rate (experiment not shown).

DISCUSSION

PDGF signaling plays a crucial role during the development of a mouse. Targeted disruption of PDGF ligands or PDGF receptors caused early embryonic death, accompanied with severe developmental abnormalities (Leveen et al., 1994), (Soriano, 1994), (Bostrom et al., 1996), (Soriano, 1997), (Lindahl et al., 1998). *In vitro* PI3K inhibitor studies showed that class Ia PI3K is a necessary mediator of essential PDGF-induced cellular events, such as proliferation, cell survival and cell migration. Since PI3K is thought to be a major target of the PDGF receptor it was surprising that mice with tyrosine to phenylalanine mutations in the PI3K binding sites of the PDGF receptor β were viable and had only minor defects in their capillary pressure (Heuchel et al., 1999). We show here that combined loss of p85α and p85β (p85α-/-p55γ-/-p50α-/-p85β-/-) causes embryonic death at E12.5 (Table 3). These findings demonstrate the requirement for class Ia PI3K in the development of an organism. Also the genetic ablation of class Ia PI3K catalytic isoforms p110α or p110β in the mouse resulted in early embryonic lethality at E10.5 and E<3.5, respectively (Bi et al., 1999), (Bi et al., 2002). Mice lacking PTEN, a PI3K antagonist that degrades PI3K lipid products by dephosphorylating PI-3,4,5-P_3 and PI-3,4-P_2 at the D3 position, also die during early development (Stambolic et al., 1998), (Di Cristofano et al., 1998). Taken these findings together, not only loss of PI3K activity but also its misregulation results in developmental defects.

Genetic ablation of class Ia PI3K results in developmental defects similar to those due to loss of PDGF receptor β. Mice deficient in p85α/p55γ/p50α/p85β and mice deficient in PDGF receptor β (Soriano, 1997) exhibit subepidermal blebbing flanking the neural tube (Figure 4 and 7b). This finding points to a major role of class Ia PI3K downstream of the PDGF receptor β *in vivo*. In agreement with this conclusion, also p110α null mice exhibit similar developmental defects to those reported in PDGF receptor β null mice (Figure 7a). The question arises why mice with tyrosine to phenylalanine mutations in the PI3K binding sites of the PDGF receptor β had only minor defects. Mutations of the PI3K binding sites of the receptor might not completely prevent the activation of PI3K upon PDGF stimulation. A variety of adaptor proteins

become phosphorylated in response to growth factor receptors and one of these might mediate activation of PI3K in the PDGF receptor β mutant mice. Hence, the residual PI3K activity might be sufficient for proper development. The interaction between membrane bound Ras-GTP with p110 might also facilitate the recruitment of PI3K to the plasma membrane (Rodriguez-Viciana et al., 1996). Therefore, mutations of the p85 binding sites of the PDGF receptor might greatly reduce but not sufficiently block PI3K activation upon PDGF stimulation. P85α-/-p55γ+/+p50α+/+p85β-/- embryos exhibited a slightly extended lifespan (up to E13.5) combined with a slightly less severe frequency of defects in comparison to p85α-/-p55γ-/-p50α-/-p85β-/- embryos. This finding demonstrates *in vivo* that the smaller regulatory isoforms p55γ and p50α can partially compensate for the combined loss of p85α and p85β. Taken together the results indicate that during early embryonic development p110δ/p85α and p110γ/p85α complexes specifically mediate PDGF receptor β signaling that cannot be mediated by p110α or p55/p50 complexes.

Deficiency in p85α and p85β causes subepidermal blebbing in the trunk region of the embryos. TUNEL stain of whole mount embryos showed no differences in the apoptotsis -staining of mutant and control specimens suggesting that increased apoptosis in the mutant embryos was not a reason for the subepidermal structures. The blebs occur when the embryo turns to achieve the fetal position. The blebs are located quasi at the hinge region where great mechanical stress occurs. This stress might cause the detachment of the outer epithelial layer and contribute to the blebbing. Loss of p85α and p85β resulted in extensive hemorrhaging, which is most likely the cause of death in the mutant embryos as seen for p110γ-/- and PDGF receptor β -/- mice. The blebs lacking blood cells likely indicate an earlier stage abnormality that precedes the eventual breakdown of vascular integrity. Increased apoptosis of non-neuronal neural crest cells along their migration path from the somites flanking the neural tube to the brain has been attributed to the facial abnormalities in PDGF receptor β null mice. P85α-/-p55γ-/-p50α-/-p85β-/- mice do not show abnormalities in apoptosis (data not shown). However, fibroblasts derived from these mutant embryos show severe migration defects upon PDGF treatment (described in the following chapter). It is therefore possible that despite proper survival the cells do not reach the forming facial structures. Alternatively, it has

been shown that loss of p110γ results in severe proliferative defects (Bi et al). Fibroblasts derived from p85α-/-p55γ-/-p50α-/-p85β-/- mice are also defective in their proliferation since they could only be expanded by immortalization with SV40 large T (described in the following chapter). It is therefore possible that not enough cells start their journey to the forming facial structures.

The p110 catalytic subunits are thermally unstable when not bound to p85 regulatory subunit and thus deletion of the p85α subunit gene results in decreased levels of both p110α and p110β proteins (Chang et al., 1997) (Fruman et al., 2000). The embryonic death of both p110α-/- mice (E10.5) and p110β-/- mice (E<3.5) indicates that one isoform cannot fully compensate for the loss of the other and thus suggests isoform-specific functions. In contrast to p110γ, which is activated only downstream of receptor tyrosine kinases, p110δ is an integrator of both receptor tyrosine kinase signaling and G-protein coupled receptors (Kurosu et al., 1997a). This may explain why the functions are not redundant and why loss of p110δ is more severe than loss of p110γ. Therefore it is surprising that mice lacking all p85α and p85β gene products (p85α-/-p55γ-/-p50α-/-p85β-/-) die later (E12.5) than p110α-/- mice (E10.5) or p110β-/- mice (E<3.5). As seen for mouse embryonic fibroblasts derived from p85α-/-p55γ-/-p50α-/-p85β-/- embryos (see next chapter), p55γ could be upregulated in the p85α-/-p55γ-/-p50α-/-p85β-/- embryos and this could stabilize a sufficient pool of p110α and p110β for survival until E12.5. Available anti-p55γ antibodies are not sufficiently sensitive to detect p55γ in the E12 embryos. P85α-/-p55γ+/+p50α+/+p85β-/- embryos exhibit an extended lifespan (up to E13.5) in comparison to p85α-/-p55γ-/-p50α-/-p85β-/- embryos. The additional expression of p55γ and p50α might lead to stabilization of more p110 in p85α-/-p55γ+/+p50α+/+p85β-/- embryos than in p85α-/-p55γ-/-p50α-/-p85β-/- embryos. Nevertheless, p85α-/-p55γ+/+p50α+/+p85β-/- mice die during development. We therefore conclude that intact expression of p55γ and p50α in the absence of p85α and p85β is not sufficient for proper murine development. This might be because domains present in the N-terminus of p85α and p85β but absent in p55γ and p50α are necessary for development. It is also possible that the p55γ or p50α splice forms are not expressed

in the PDGF receptor responsive tissues or that their expression level is low compared to the p85 splice forms.

In summary, the data presented in this chapter demonstrate that class Ia PI3K is required for the development of an organism. In contrast to PI3K catalytic isoforms, p85 and p85 function in a redundant manner during development, whereas p55 and p50 cannot compensate for loss of p85 and p85. Furthermore, the data provide evidence for p110 specific functions in PDGF signaling, *in vivo*.