5 Discussion

PI3Kγ is a lipid kinase that is chiefly regulated by GPCRs *via* G$\beta\gamma$ complexes. Owing to this mode of activation, it is grouped separately from the other class I PI3Ks in its own subclass IB. It plays pivotal roles in several physiological settings, mostly within leukocytes but also in heart. PI3Kγ conveys signals perceived at cell surface receptors by producing the inositol lipid PIP_3, which acts as a second messenger by recruiting proteins containing specific PH domains to the plasma membrane. PI3Kγ is a heterodimeric complex consisting of the catalytic p110γ and one of the regulatory subunits p101 or p87^{PIKAP}. According to the currently accepted paradigm, G$\beta\gamma$ complexes released from heterotrimeric G proteins are bound by p101 or p87^{PIKAP}, whereby the heterodimeric complex is translocated to the plasma membrane. There, p110γ has access to its lipid substrate and is further activated again by G$\beta\gamma$.

Although many studies have emphasized the importance of p101 in the G$\beta\gamma$-mediated activation of p110γ, cellular systems relying on p110γ signaling were mostly not assayed for expression of p101, and a monomeric function of p110γ has been speculated as well. Moreover, activity of p110γ can be stimulated by G$\beta\gamma$ in the absence of p101, rendering the role of p101 still more controversial. In particular, a thorough understanding of the roles of p101 is hampered by a lack of structural information concerning the regulatory subunit itself and the heterodimeric PI3Kγ complex. Since its discovery, p101 has been considered a singular protein that appeared to be unrelated to any known protein.

This thesis aimed to clarify the role of p101 by addressing a selection of these issues. First, the characterization of deletion mutants of p101 led to the establishment of a domain structure for p101. Surprisingly, a protein homologous to p101 was identified based on this novel assignment of functional domains within the primary structure of p101. Second, the cloning and characterization of this protein – called p87^{PIKAP} – revealed that at least one of both regulatory subunits accompanies p110γ presumably in all tissues in which it is active. Moreover, the crucial requirement of a regulatory subunit *in vivo* was exemplified for the p110γ-mediated amplification of mast cell degranulation. Furthermore, the finding that p87^{PIKAP} interacts with PDE3B may point to additional modes of action for this regulatory subunit. Third, structural studies were initiated to clarify the topology of the binding interfaces and the three-dimensional structure of the heterodimeric p110γ/p87^{PIKAP} complex.

5.1 The domain structure of p101

Whereas a modular domain structure can be easily derived for the p85 regulatory subunits of the class IA PI3Ks from sequence comparison with other signaling proteins, an analogous design of the class IB regulatory subunit has not been presumed so far. This largely stemmed from the observation that p101 does not bear any similarity to known signaling proteins or other proteins. This notion was supported by a study that implied large areas of p101 to be important for the interaction with p110γ (Krugmann *et al.*, 1999). From the work described here, however, a domain structure can be deduced for p101 (Fig. 5.1). This discrepancy is most likely due to the differing suitability of the methods applied to this task. Whereas the experiments described here allowed to analyze binding to p110γ and G$\beta\gamma$ separately, Krugmann *et al.* (1999) assayed interaction with G$\beta\gamma$ only by monitoring the G$\beta\gamma$-stimulated catalytic activity of heterodimers consisting of truncated p101 and p110γ, provided either as purified protein or as immunoprecipitates from transfected COS-7 cells. In doing so, a reduction in G$\beta\gamma$-stimulated activity reflects influences on binding of p101 to p110γ as well as p101-mediated activation by G$\beta\gamma$. In agreement with the data presented here (see Fig. 4.3), a diminished responsiveness of heterodimeric PI3Kγ to G$\beta\gamma$ was observed upon deletion of either terminus of p101 (Krugmann *et al.*, 1999). However, from the observation that deletion of aa 1–161 of p101 completely abrogated activation by G$\beta\gamma$, Krugmann *et al.* (1999) concluded that the N terminus of p101 is indispensable for interaction with G$\beta\gamma$. Although the N terminus of p101 is indeed indispensable for the activation of the heterodimer by G$\beta\gamma$, its importance lies in the interaction with p110γ rather than G$\beta\gamma$.

The methods employed here allowed a separate mapping of the G$\beta\gamma$ and p110γ binding sites on p101, mostly in the context of living cells. Although FRET is routinely used in the study of protein-protein interactions, its application to the mapping of interaction domains has not been described so far. Because FRET can be measured in living cells, it may prove valuable as a method complementary to co-IP for the characterization of other protein-protein interaction domains as well. Moreover, it eliminates the need for washing steps that are necessary in co-IP protocols but generally not very well controllable. Using this novel approach combined with conventional co-IP experiments, the domain mediating heterodimerization with p110γ was mapped to the N terminus of p101. On its N-terminal border, binding was abruptly lost

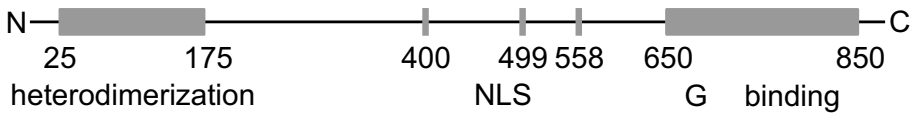

Figure 5.1: Domain structure of p101. Schematic representation of the primary structure of p101. Boxes indicate the position of interaction domains and predicted NLS. Numbering is given for porcine p101. Note that only the NLS at position 499 is conserved in orthologues of p101, suggesting that it may be functionally relevant.

upon deletion of more than 25 aa both in FRET and co-IP assays. At its C-terminal border, FRET efficiencies gradually decreased over aa 175–100, whereas co-IP signals remained comparable and were only lost completely upon deletion up to aa 100. These observations may reflect that different ranges of affinities could be resolved by the two methods. On the other hand, progressing deletions in p101 may allow the fluorescent proteins to accept a broader range of orientations that were not accessible in the presence of certain parts of the fusion partners, resulting in a decrease in FRET efficiency. However, one may argue that the fluorescent proteins should largely retain their relative orientations because the p101 fusion portion remains unchanged within at least 100 aa. If the decrease in FRET efficiency rather reflects a diminished binding affinity, these data may indicate that the core binding region within aa 25–131 is extended by further C-terminal elements that enhance binding (see Fig. 5.1). In contrast to the data presented here, Krugmann *et al.* (1999) reported that all truncated p101 constructs that they have assayed were copurified with p110γ. However, in line with the findings of this thesis, deletion of the N terminus resulted in the strongest reductions in binding.

On the side of p110γ, comparison with the other class I PI3Ks suggests that binding to p101 or p87$^{\text{PIKAP}}$ occurs *via* the N terminus of p110γ, because the N-terminal p85 binding domain of the class IA catalytic subunits is replaced by an unrelated sequence in p110γ. Accordingly, deletion of aa 1–169 in p110γ resulted in complete abrogation of binding to p101, although constructs comprising only the N terminus itself were unable to bind to p101 (Krugmann *et al.*, 1999). Therefore, other parts of p110γ are likely to contribute to binding as well. Moreover, FRET measurements indicated that the two N and two C termini of p110γ and p101 or p87$^{\text{PIKAP}}$ are in close proximity to each other within the heterodimeric complex (see Fig. 4.17 for p87$^{\text{PIKAP}}$ and Brock *et al.*, 2003, for p101), suggesting that additional contacts between the C termini of p110γ and the regulatory subunit may exist. Furthermore, it has been reported that p101 shifts the substrate specificity of p110γ from PI towards PI(4,5)P_2 (Maier *et al.*, 1999). An interaction with p101 that influences substrate specificity may require contacts close to the catalytic center of p110γ, which resides within its C terminus. Taken together, several lines of evidence suggest that some contacts with p110γ may still be established at C-terminal parts of p101. However, because the N terminus of p101 is both necessary and itself sufficient for binding to p110γ, the main binding events that provide most of the affinity between p101 and p110γ most likely take place at the N terminus of p101. Both notions are supported by the outcome of peptide SPOT array overlay experiments (discussed in section 5.3). The domains that mediate the interaction with p110γ at the N terminus of p101 and p87$^{\text{PIKAP}}$ may represent novel protein-protein interaction domains, because other proteins that exhibit significant similarity to the N termini of p101, p87$^{\text{PIKAP}}$, or p110γ cannot be identified .

The G$\beta\gamma$ binding domain was mapped to aa 650–850 of p101 (Fig. 5.1). In contrast to the behavior of the heterodimerization domain, binding to G$\beta\gamma$ was abruptly and completely lost upon deletion of additional 25 aa on either domain border. These observations may be in-

terpreted in two ways. First, the aa mediating binding to G$\beta\gamma$ may be split in two sequence stretches that are comparatively far apart in the primary structure but form a single binding site on the level of tertiary structure. Separately, each of these motifs may still bind G$\beta\gamma$ but only with very weak affinity. Synergistic binding *via* both epitopes may be a prerequisite for effective binding. Alternatively, the domain may require all of its residues to adopt a stable fold and its native conformation, such that deletion of peripheral residues interferes with folding rather than with the actual binding event. It is also conceivable that a combination of both aspects may be responsible for the observed behavior.

The structure of a G$\beta\gamma$ effector in complex with G$\beta\gamma$ has so far only been determined for one of the many G$\beta\gamma$ effectors. GRK2 interacts with G$\beta\gamma$ *via* its C-terminal PH domain (Inglese *et al.*, 1994; Lodowski *et al.*, 2003). Although PH domains are most renowned for their ability to bind different sets of phosphorylated species of PI, only ~10% of all known PH domains actually bind PI lipids with high specificity and affinity (Lemmon & Ferguson, 2000). PH domains should rather be regarded as a superfamily that shares a common fold, which provides a scaffold that can adopt variable surfaces for different recognition purposes (Blomberg *et al.*, 1999). They have a core seven-stranded β-sandwich structure, which is capped off by a C-terminal α helix on one side and by three loops connecting the β strands on the other side. Many PH domains have been shown to bind G$\beta\gamma$, some with dissociation constants in the range of 20–50 nM (Touhara *et al.*, 1994; Mahadevan *et al.*, 1995). However, besides GRK2, a clear physiological role in G$\beta\gamma$ binding has until now been ascribed only to the PH domains of PLCβ enzymes (Wang *et al.*, 1999; Rhee, 2001). In GRK2, contacts to Gβ are established by four distinct regions, located both within the N-terminal β sheets and the C-terminal α helix of the PH domain (Lodowski *et al.*, 2003). The residues mediating these contacts are not well conserved in G$\beta\gamma$-binding PH domains as *e.g.* those of PLCβ, suggesting that the specific interfaces between the domains and G$\beta\gamma$ will differ in detail, although they retain a concave surface complementary to the effector binding site on top of the Gβ propeller (Lodowski *et al.*, 2003). Considering the fact that PH domains consist of 100–200 aa, of which not a single position is rigorously conserved, a PH domain may also mediate interaction with G$\beta\gamma$ in p101 and p87$^{\text{PIKAP}}$, although it is not obvious from sequence comparison. The loss of G$\beta\gamma$ binding in p101 observed upon deletion from either end of the mapped domain would be compatible with such an assumption, because both N- and C-terminal elements of the PH domain from GRK2 are crucially involved in binding of G$\beta\gamma$. Moreover, regarding the possibility that a loss of stable folding is responsible for the loss of binding to G$\beta\gamma$ at the C-terminal domain of p101, it would be conceivable that removing a sheet from the β sandwich or the capping C-terminal α helix will impede the adoption of the PH domain fold.

To identify the actual peptide motifs responsible for binding to G$\beta\gamma$ within the binding domain of p101, peptide SPOT analysis was performed. Unfortunately, it was not possible to yield results that extend the information gained from the mapping approach (see section 5.3).

Comparison of the sequences from various p101 orthologues available in the ENSEMBL data base shows that both the heterodimerization and the G$\beta\gamma$ binding domains represent the most highly conserved parts of p101. It may be inferred that the domain structure established here for the porcine variant of p101 is functionally relevant and thus retained in its orthologues as well. Given the conservation of functional elements, it is likely that a similar mode of PI3Kγ activation is persued within a broad range of species. The functional domains may be part of an evolutionarily conserved signaling module that operates in vertebrates such as mammals, birds, amphibians, and fish.

The functional relevance of the N- and C-terminal domains of p101 is once more reflected by their conservation in the p101 homologue p87$^{\text{PIKAP}}$. As the intervening sequences differ considerably between p101 and p87$^{\text{PIKAP}}$, they may have provided means to equip both proteins with further, isotype-specific functions that are yet to be clarified. Such features may involve the interaction with as yet unknown interaction partners. Alternatively, one may argue that their divergence points to a lack of any specific function. The middle part may merely provide a scaffold for the two interaction domains, which does not require a strict conservation on the aa level as long as the overall structure is retained. A comparatively well conserved stretch is present within this part of the primary structures (aa 510–560 of porcine p101 and aa 411–461 of murine p87$^{\text{PIKAP}}$). Peptide SPOT analysis indicated that it probably does not play a role in the interaction with p110γ. It may, however, be indispensable for an as yet unknown interaction or for the establishment of structural features common to both regulatory subunits of PI3Kγ.

In contrast to p87$^{\text{PIKAP}}$, p101 contains a motif mediating nuclear import within the middle part of its primary sequence. It is, however, not clear whether nuclear localization of p101 occurs *in vivo*, where p110γ is supposably present in all cells that express p101. Moreover, it remains to be verified whether the predicted conserved NLS constitutes a functional import motif in other p101 orthologues as well. Besides the observation that the presence of monomeric p101 diminishes the expression of CFP and PDE3B encoded by cotransfected cDNA plasmids (see for example Fig. 4.24), published data concerning a possible function of monomeric p101 are unavailable. A more general role of nuclear p101 in regulating transcriptional processes may gain support from yeast two-hybrid data pointing to an interaction of p101 with transcriptional regulators (data from the Alliance for Cellular Signaling; http://www.signaling-gateway.org/). However, it is questionable whether such mechanisms are active under physiological conditions, given that p101 is probably excluded from the nucleus to a great extent by interaction with p110γ.

A minor fraction of heterodimeric PI3Kγ containing either p101 or p87$^{\text{PIKAP}}$ (see Brock *et al.*, 2003, for p110γ/p101 and Fig. 4.14 for p87$^{\text{PIKAP}}$) is present in the cell nuclei of transfected HEK293 and COS-7 cells. For HepG2 cells, a translocation of p110γ to the cell nucleus after prolonged serum stimulation has been reported (Metjian *et al.*, 1999), whereas a serum-independent but GTPγS-responsive and pertussis toxin-sensitive PI3K activity in nuclei of

VSMC has been attributed to p110γ (Bacqueville *et al.*, 2001), in both studies by the use of specific antibodies. Class IA PI3Ks have been detected in nuclei of insulin-stimulated HepG2 cells (Kim, 1998), osteoblast-like cultured cells (Martelli *et al.*, 2000), and rat liver cells (Lu *et al.*, 1998; Boylan & Gruppuso, 2002). The nuclear class IA PI3Ks may be stimulated by a nuclear GTPase termed PIKE (phosphoinositide 3-kinase enhancer, Ye *et al.*, 2000), for which PLCγ1 acts as a GEF in a lipase-independent fashion (Ye *et al.*, 2002), thereby conveying antiapoptotic effects of NGF *via* nuclear Akt (Ahn *et al.*, 2004). PTEN can also be found in cell nuclei (see for example Lachyankar *et al.*, 2000), and nuclear PTEN levels are diminished in thyroid carcinoma cells (Gimm *et al.*, 2000). However, owing to these still rather scarce sightings of nuclear PI3Ks and the poor knowledge concerning the nuclear targets of PIP_3, the relevance and mechanisms of nuclear PI3K signaling are yet not very well understood and accepted (see Irvine, 2003; Déléris *et al.*, 2006, for review). Particularly for PI3Kγ, which lacks the p85 subunit necessary for PIKE-mediated regulation, nuclear functions remain elusive.

5.2 A novel PI3Kγ regulatory subunit

Data base mining supported by knowledge of the functionally important areas of p101 allowed the identification of a novel regulatory subunit of PI3Kγ. It was termed p87$^{\text{PIKAP}}$, PI3Kγ adapter protein of 87 kDa. Based on similar data base searches, Suire *et al.* (2005) recently identified and examined this novel PI3Kγ subunit as well, giving it the name p84.

5.2.1 Genealogy of p101 and p87$^{\text{PIKAP}}$

As mentioned above, the gene encoding p87$^{\text{PIKAP}}$ is located immediately adjacent to that of p101 on chromosome 11 in mice (chromosome 17 in human, Fig. 5.2*A*). Such a vicinity of both genes can be found in all vertebrate genomes present in the ENSEMBL data base, indicating that the p101 and p87$^{\text{PIKAP}}$ genes have arosen from a gene duplication event. Strikingly, the partition of the encoded aa between exons is most similar in the p101 and p87$^{\text{PIKAP}}$genes (Fig. 5.2*B*). Moreover, the large deletions with respect to p101 in the middle part of the p87$^{\text{PIKAP}}$ protein likely stem from internal deletions and relocations of splice sites within and around exons 10–12.

A single gene related to both p87$^{\text{PIKAP}}$ and p101 can be located in the genome of the sea squirt ciona (*Ciona intestinalis*). An alignment of the ciona protein, murine p101, and murine p87$^{\text{PIKAP}}$ is shown in Fig. 5.3. The genomes of neither *C. elegans*, *Drosophila melanogaster*, nor yeast contain genes related to p87$^{\text{PIKAP}}$ or p101. Like vertebrates, ciona belongs to the phylum of chordata, but is grouped separately in the subphylum of urochordata. Therefore, it appears likely that a gene duplication event that led to the separate p101 and p87$^{\text{PIKAP}}$ genes took place subsequent to the appearance of the vertebrates. The departure of p110γ from the single class

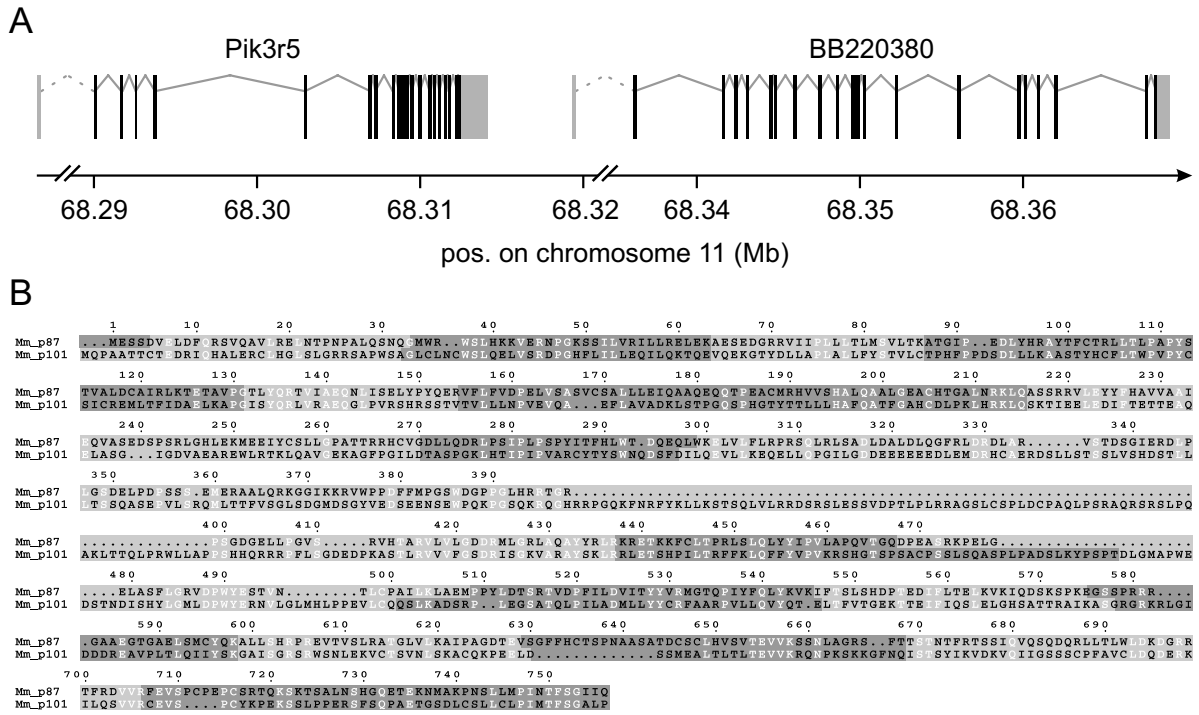

Figure 5.2: Organization of p101 and p87^{PIKAP} genes. *A*, Location of the genes encoding p101 (Pik3r5) and p87^{PIKAP} (BB220380) on murine chromosome 11. Distances are given in Mb. The 3′ end of the forward strand is indicated by an arrow. Protein-coding regions of the exons are represented by black boxes, whereas gray boxes indicate 5′ and 3′ untranslated regions. Note that the introns between exons 1 and 2 in Pik3r5 and BB220380 are not drawn to scale. Their length is ∼41.5 kb and ∼17 kb in Pik3r5 and BB220380, respectively. *B*, Alignment of murine p87^{PIKAP} (gene bank entry BC028998) and p101 (entry NM_177320) protein sequences. Alternating exons are shaded in dark and light gray. Identical residues are in white lettering.

I PI3K found in *C. elegans* (age-1, Morris *et al.*, 1996) and *Drosophila melanogaster* (Dp110, Leevers *et al.*, 1996) appears to have taken place at the level of chordata or coelomata. The fact that a gene encoding an orthologue of both p101 and p87^{PIKAP} can also be found at this stage, *i.e.* within a chordate distinct from vertebrates, suggests that p110γ and its regulatory subunit coevolved to perform their functions. The slime mold *Dictyostelium discoideum* represents a model organism in the study of chemotactic processes. Similar to neutrophils, *Dictyostelium* is dependent on G$\beta\gamma$ subunits of heterotrimeric G proteins to drive chemotaxis (van Haastert & Devreotes, 2004). However, activation of PI3Ks in *Dictyostelium* appears to be achieved *via* Ras rather than by direct interaction with G$\beta\gamma$ (Sasaki *et al.*, 2004). Accordingly, the RBDs of the three *Dictyostelium* class I PI3Ks are indispensable for chemotaxis in *Dictyostelium* (Funamoto *et al.*, 2002). Membrane localization of the PI3Ks is attained *via* their extended N-terminal domains independently of regulatory subunits, which do not exist in *Dictyostelium* (Funamoto *et al.*, 2002). It is, however, unknown which protein or lipid components of the plasma membrane are recognized by this domain. Thus, the mode of membrane localization and activa-

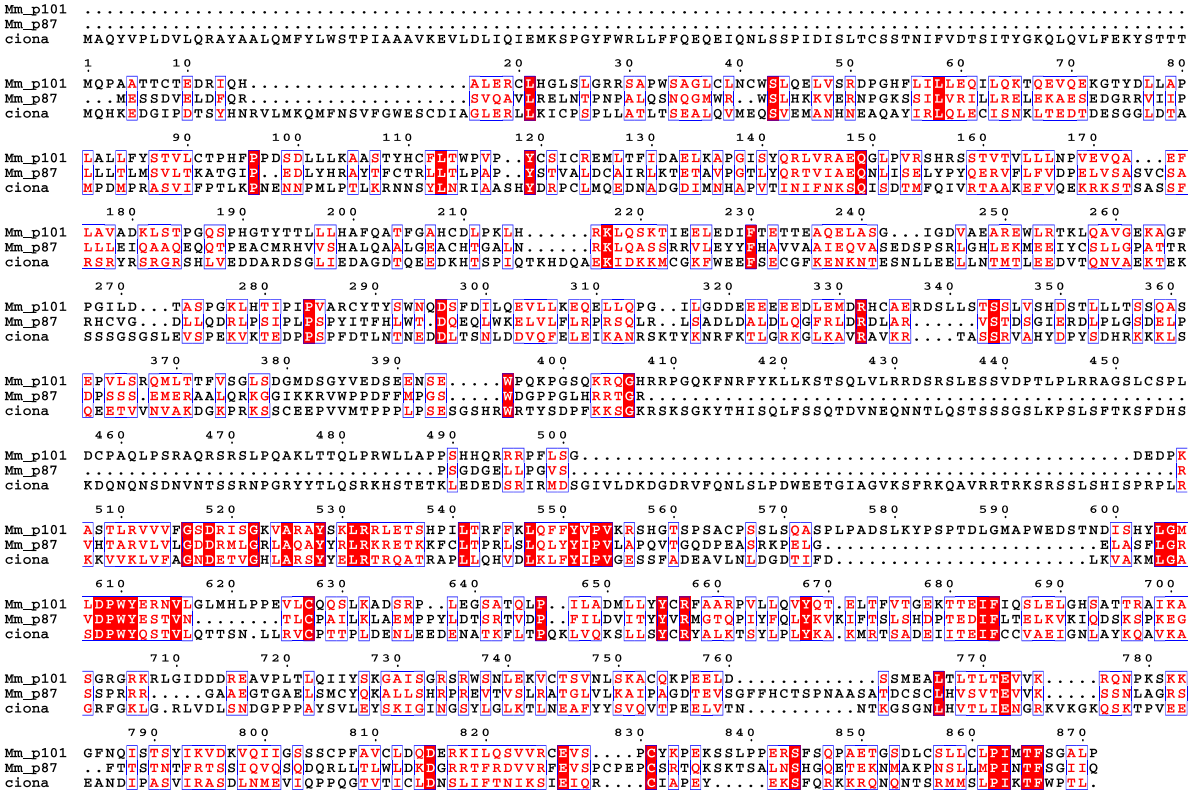

Figure 5.3: Alignment of p101 and p87^{PIKAP} with a putative orthologue from *Ciona intestinalis*. The alignment was generated with the ClustalW algorithm. It was colored using ESPript. Conserved and similar aa are highlighted by red boxes and red letters in blue boxes, respectively. For *Ciona intestinalis*, the transcript from ENSEMBL gene ENSCINP00000016882 was used. For p101 and p87^{PIKAP}, the gene bank entries NM_177320 and BC028998 were used, respectively.

tion of vertebrate PI3Kγ *via* regulatory subunits differs from that operating in *Dictyostelium*, although both systems share the activation by Ras proteins.

5.2.2 General features and expression pattern of p87^{PIKAP}

After cloning of the p87^{PIKAP} cDNA, it was first sought to characterize the interaction between p87^{PIKAP} and p110γ. In line with the findings reported by Suire *et al.* (2005), p87^{PIKAP} was readily copurified with p110γ in co-IP experiments. To further obtain insight into the conformation of the p110γ/p87^{PIKAP} complex, FRET experiments on N- or C-terminally tagged versions of p87^{PIKAP} and p110γ were carried out. As has been observed for p101 by Brock *et al.* (2003), both the N and the C termini of p87^{PIKAP} and p110γ are closer to each other than to the opposite termini. Accordingly, both heterodimeric complexes may have a similar overall architecture. This assumption is supported by the analogous design of p101 and p87^{PIKAP} on the level of primary structure. Moreover, binding of p101 and p87^{PIKAP} to p110γ was mutually exclusive, indicating that they share identical or at least overlapping binding sites on p110γ. Competitive co-IP

and FRET assays that were carried out to probe the relative affinity of the regulatory subunits towards p110γ revealed that p87$^{\text{PIKAP}}$ binds slightly more strongly to p110γ than does p101.

In the absence of p110γ, p87$^{\text{PIKAP}}$ was found to be more stable than p101. Although p110γ can be readily expressed without p101, stability of p101 appears to be dependent on coexpression of p110γ (Brock *et al.*, 2003). This finding was in contrast to the situation observed for class IA PI3Ks. There, the catalytic subunits are stabilized by their regulatory subunits (Yu *et al.*, 1998b), which even occur as monomers *in vivo* (Ueki *et al.*, 2002), although their general existence and functional relevance is still controversially discussed (Geering *et al.*, 2007). For PI3Kγ, stability of monomeric p110γ is widely accepted. Concerning the regulatory subunits, the data presented here indicate that the stability of p87$^{\text{PIKAP}}$ is largely unchanged by p110γ, whereas expression of p101 is significantly higher in the presence of p110γ. Given the observation that nuclear-localized, monomeric p101 reduces the expression of cotransfected proteins such as CFP or PDE3B, it cannot be ruled out that a similar effect is also acting on expression of p101 itself. Thus, at least p87$^{\text{PIKAP}}$ – if not p101 as well – appears to be largely unaffected by p110γ with respect to its stability. Surprisingly, neutrophils from p110$\gamma^{-/-}$ mice showed markedly reduced levels of p101 and p87$^{\text{PIKAP}}$ protein, indicating that expression of both p87$^{\text{PIKAP}}$ and p101 may depend on p110γ (Suire *et al.*, 2005). The discrepancy between these findings may point to additional or cell-type specific regulatory effects on mRNA or protein level. Moreover, albeit p87$^{\text{PIKAP}}$ may be sufficiently stable over the 48-h time span monitored in the experiments here, both regulatory subunits may experience a superimposed long-term stabilization by p110γ. It may become evident only upon prolonged withdrawal of p110γ, for example in cells derived from p110$\gamma^{-/-}$ mice. Pulse-chase analyses of p87$^{\text{PIKAP}}$ in monomeric form and in complex with p110γ may resolve these questions in future work.

In contrast to data on p101, coexpression of G$\beta\gamma$ did not result in a pronounced membrane localization of YFP-tagged p87$^{\text{PIKAP}}$. However, interaction between p87$^{\text{PIKAP}}$ and G$\beta\gamma$ was evident from activation of cotransfected p110γ under these conditions. *In vitro* PI3K lipid kinase assays performed with purified PI3Kγ dimers revealed that p110γ/p87$^{\text{PIKAP}}$ dimers are about four times less sensitive towards G$\beta\gamma$ than p110γ/p101 dimers (Suire *et al.*, 2005). The observation that p87$^{\text{PIKAP}}$ is less efficiently recruited by G$\beta\gamma$ to the plasma membrane may support these *in vitro* data. Moreover, receptor-mediated activation of p110γ/p87$^{\text{PIKAP}}$ dimers resulted in a less intense translocation of YFP-Grp1-PH, indicating that p87$^{\text{PIKAP}}$ may indeed have a lower affinity towards G$\beta\gamma$ in living cells as well. Given the existence of multiple G$\beta\gamma$ isoforms, both regulatory subunits may recognize different subsets of G$\beta\gamma$ complexes. Preferences towards certain G$\beta\gamma$ dimers have been shown for a variety of effectors, including PLCβ enzymes (Fogg *et al.*, 2001), the Rac-specific GEF P-Rex1 (Mayeenuddin *et al.*, 2006), G protein-coupled inwardly rectifying potassium channels (Lei *et al.*, 2000), and T-type calcium channels (Wolfe *et al.*, 2003). On the other hand, some GPCRs such as muscarinic receptors and βARs appear to couple specifically to G proteins composed of certain α, β, and γ subunits (see for

example Kleuss *et al.*, 1992, 1993; Kalkbrenner *et al.*, 1995; McIntire *et al.*, 2001; Gibson & Gilman, 2005). A combination of differential specificity of p101 and p87^{PIKAP} towards certain G$\beta\gamma$ subunits and preferential coupling of G$\beta\gamma$ subunits to specific GPCRs may render each PI3Kγ heterodimer selective for a subset of GPCRs. Additionally, cell type-specific expression of G$\beta\gamma$ subunits may further diversify signaling through PI3Kγ depending on its regulatory subunit. A systematic screen of a panel of G$\beta\gamma$ dimers concerning their affinity towards the p110γ/p101 heterodimer revealed that G$\beta_1\gamma_{11}$ is unable to stimulate PI3Kγ activity (Kerchner *et al.*, 2004). This observation was surprising, because G$\beta\gamma$ dimers containing the γ_{11} subunit couple well to G_i proteins (Lim *et al.*, 2001) and are highly expressed in hematopoietic cells (Morishita *et al.*, 1998). Attempts to analyze whether p87^{PIKAP} may bind to G$\beta\gamma$ subunits containing γ_{11} were hampered by an already very weak membrane localization of such dimers, precluding membrane recruitment studies with YFP-tagged p87^{PIKAP} (data not shown). Nevertheless, a screen similar to that performed by Kerchner *et al.* (2004) would be helpful to clarify whether preferences for certain G$\beta\gamma$ subtypes exist for p101 and p87^{PIKAP}.

Northern Blot analysis indicated that p87^{PIKAP} is expressed in several tissues, but most prominently in heart. Multiplex RT-PCR assays revealed that B and T cells express almost exclusively p101, whereas macrophages, neutrophils, and DCs contain p87^{PIKAP} as well. Mast cells contain barely detectable levels of p101 mRNA, but show high expression of p87^{PIKAP}. Taken together, at least one of the regulatory subunits is present in tissues and cell types that contain PI3Kγ, and therefore it appears likely that p110γ expression is always accompanied by expression of either p101 or p87^{PIKAP}. The selective induction of p110γ expression that has been observed for p110γ in U937 cells (Baier *et al.*, 1999) contradicts this notion. However, aside from technical limitations related to the insufficient avidity of common p101 antibodies, the observed induction of p110γ may have been paralleled by an enhanced expression of p87^{PIKAP}. Owing to the varying expression of p101 and p87^{PIKAP}, parameters of PI3Kγ signaling may differ between cell types. Differential regulation of PI3Kγ by its regulatory subunits may be of special interest in cell types that express both p101 and p87^{PIKAP}, such as macrophages, neutrophils, or DCs. First, the slower accumulation of PIP_3 in HEK293 cells expressing p87^{PIKAP} instead of p101 along with p110γ may suggest altered kinetics of PI3Kγ signaling events in cells expressing p101 and p87^{PIKAP}. Additionally, differences in specificities towards the upstream activator G$\beta\gamma$ may further diversify PI3Kγ signaling through its regulatory subunits (see above). In heart and mast cells, PI3Kγ may rely on additional interactions specific to p87^{PIKAP} to perform its roles in cellular signaling. Such interactions may include that with PDE3B (discussed below). Given the divergence in the sequences connecting the N- and C-terminal domains of p101 and p87^{PIKAP}, interactions specific to p101 may also be conceivable, which would further increase the potential for regulatory subunit-specific PI3Kγ signaling.

The different expression patterns may be due to variations in the range of transcription factors present in the cell types. Moreover, cell type-specific methylation of promoter regions of

the p101 and p87^{PIKAP}-encoding genes may regulate their expression in a cell type-specific fashion. For p101, a gene silencing through histone H3 methylation at Lys 9 has been reported in the context of a screen for histone-methylated genes in the SW48 colon cancer cell line (Kondo *et al.*, 2004). Similar phenomena may be active in the physiological regulation of the relative expression of p101 and p87^{PIKAP}.

A p101$^{-/-}$ mouse has recently been generated, which has so far only been characterized in depth with respect to neutrophil signaling (Suire *et al.*, 2006). p101$^{-/-}$ mice have been reported to be viable, fertile, of normal size, and with unchanged blood cell counts (Suire *et al.*, 2006). Expression of p87^{PIKAP} was elevated by ~50% in p101$^{-/-}$ neutrophils. In contrast to previously published data from the same group, which showed that ~20% of PI3Kγ acitivity in neutrophils is associated with p87^{PIKAP} (Suire *et al.*, 2005), only 5% of p110γ was reported to be bound to p87^{PIKAP} (Suire *et al.*, 2006). Accordingly, deletion of p101 resulted in reductions in PIP_3 accumulation after fMLP stimulation that were only slightly less than those observed for p110$\gamma^{-/-}$ mice. Interestingly, PIP_3 accumulation upon C5a stimulation was merely reduced to half of the level of wild-type animals (Suire *et al.*, 2006). Although expression of p87^{PIKAP} is comparatively low in neutrophils, p87^{PIKAP}-containing PI3Kγ dimers may couple more efficiently to C5a receptor-activated sets of G$\beta\gamma$ subunits than p101-containing dimers, leading to a lower decrease in C5a-mediated responses in p101$^{-/-}$ mice. Whereas phosphorylation of Akt was similarly attenuated in p101$^{-/-}$ mice, production of ROS was unchanged compared to wild-type for both fMLP and C5a stimulation. Parallel analysis of mice that express a p110γ mutant that is unable to bind to Ras unveiled that interaction with Ras is indispensable for production of ROS, whereas p101 is not essential for this response (Suire *et al.*, 2006). These mice also showed severe reductions in fMLP- and C5a-induced PIP_3 accumulation, Akt phosphorylation, and chemotaxis that were comparable to those observed in p110$\gamma^{-/-}$ and p101$^{-/-}$ mice, indicating that both G$\beta\gamma$- and Ras-mediated activation of p110γ are crucial for physiological responses of neutrophils to chemokines. Given that p110γ binds to Ras irrespective of p101 (Pacold *et al.*, 2000), it appears unlikely that p101 and p87^{PIKAP} have any differential impact on Ras-mediated activation of p110γ, although a possible interplay between Ras and p87^{PIKAP} binding to p110γ has not been analyzed yet. Besides this assessment of the dichotomy of G$\beta\gamma$- and Ras-mediated activation, further characterization of the p101$^{-/-}$ mice may also yield important insight into the relative contribution as well as redundant and unique functions of p101 and p87^{PIKAP} in PI3Kγ signaling. Moreover, knockdown and knockout studies of p87^{PIKAP} will be necessary to fully appreciate the specific role of the p87^{PIKAP} regulatory subunit.

5.2.3 Interaction of p87^{PIKAP} with PDE3B

A cardiac role for PI3Kγ has been revealed in the characterization of p110$\gamma^{-/-}$ mice , which show increased cardiac contractility (Crackower *et al.*, 2002). However, analysis of mice ex-

pressing a kinase-deficient mutant of p110γ uncovered that the impact of p110γ on contractility is mediated by a kinase-independent scaffolding interaction with PDE3B, whereas cardiac remodeling processes depend on catalytic activity of p110γ (Patrucco *et al.*, 2004). Recombinant p110γ was able to stimulate PDE3B that was immunoprecipitated from hearts of p110$\gamma^{-/-}$ mice if applied to lysates before IP but not if applied after IP (Patrucco *et al.*, 2004). These data were interpreted such that additional proteins may be involved in a macromolecular complex regulating PDE3B. It thus appeared worthwile to test whether p87$^{\text{PIKAP}}$, which is highly expressed in heart according to the RT-PCR and Northern Blot data, may interact with PDE3B. From transfected HEK293 cells, it was possible to copurify p87$^{\text{PIKAP}}$ with PDE3B and *vice versa*. Albeit to a lesser extent, p101 appeared to be capable of interacting with PDE3B as well. The observation that p87$^{\text{PIKAP}}$ was less well copurified if expressed as a heterodimer with p110γ may indicate that the heterodimeric complex bears lower affinity towards PDE3B. Attempts were made to verify this interaction in heart lysates from neonatal rats, but were unsuccessful so far (data not shown). Because the expression levels of the proteins involved are very low in these cells, disruption of the cells may dilute the proteins sufficiently to dissociate the complex.

To test whether the interaction with p87$^{\text{PIKAP}}$ is sufficient to reconstitute the PI3Kγ-mediated stimulation of PDE3B activity that is observable in heart, PDE3B activity was assayed in lysates of HEK293 cells transfected with PDE3B along with either p87$^{\text{PIKAP}}$, p110γ, or the heterodimeric p110γ/p87$^{\text{PIKAP}}$ complex. However, effects on PDE3B activity were not detectable. Most likely other myocyte-specific proteins, which are part of the complex that regulates PDE3B activity, may be necessary. p87$^{\text{PIKAP}}$ may be an essential part of this complex, although it is itself unable to stimulate PDE3B activity. As such proteins are often central to complexes regulating the activity of PKA, a member of the familiy of A-kinase anchoring proteins (AKAP, see Wong & Scott, 2004, for review) may be a likely candidate for a central factor within this PDE3B-regulating complex. For example, mAKAP has been shown to interact with PDE4D3 in heart (Dodge *et al.*, 2001).

Recently, the generation of PDE3B knockout mice has been reported, which show alterations in the regulation of energy homeostasis such as enhanced lipolysis and insulin resistance (Choi *et al.*, 2006). Cardiac phenotypes were not reported, and mice were probably not yet analyzed with respect to subtle changes in cardiac function, because the role of PDE3B classically lies more in insulin signaling (Degerman *et al.*, 1997), and PDE3A, which is highly expressed in heart, has been labeled as the heart-specific PDE3 isoform (Maurice *et al.*, 2003).

Besides heart, platelets from p110$\gamma^{-/-}$ mice showed increased cAMP levels as well, both under basal conditions and upon stimulation with prostaglandin E_1 (Hirsch *et al.*, 2001). These effects may possibly involve interactions between PI3Kγ subunits and PDE3B as well, although this remains speculative, because expression of PDE3B in rat platelets is very weak (Liu & Maurice, 1998). Expression of PDE3B (Liu & Maurice, 1998) and p110γ is detectable in VSMC. However, aortas of p110γ knockout mice did not exhibit any differences in basal or isoproterenol-

stimulated cAMP levels (Vecchione *et al.*, 2005). These observations may indicate that expression of the above mentioned elusive members of a PDE3B-regulating complex is heart-specific.

5.2.4 Role of p87^{PIKAP} in mast cell signaling

The activation of mast cells commences with the crosslinking of antigen receptors of the FcϵRI class. Downstream of this receptor complex, the class IA PI3K p110δ is activated, probably involving the tyrosine kinases Lyn, Fyn, and Syk and the adaptor molecules linker for activation of T cells (LAT) and Grb2-associated binding protein 2 (GAB2) (Gilfillan & Tkaczyk, 2006). As expected, p110δ knockout mice displayed pronounced reductions in antigen-induced phosphorylation of Akt and degranulation, and reduced responses in a passive cutaneous anaphylaxis model (Ali *et al.*, 2004). The G$\beta\gamma$-activated p110γ isoform exerts its function in the allergic response by conducting a feedback amplification of degranulation that is mediated by G_i-coupled receptors such as the adenosine A_3 receptor (Laffargue *et al.*, 2002). The relative importance of p110γ and p110δ in this context is still a matter of debate, as p110γ knockout mice showed similar reductions in degranulation and anaphylaxis tests (Laffargue *et al.*, 2002).

The data presented here support a crucial role of p110γ, which is most obvious in the response of mast cells to mediators that feed into the G_i-coupled amplification loop. In RBL-2H3 cells, which are often used as a model system for mast cell degranulation, the expression of p87^{PIKAP} was found to be significantly higher than that of p101, which was barely detectable by multiplex RT-PCR. Knockdown of p87^{PIKAP} expression by vector-encoded shRNA resulted in pronounced reductions in the phosphorylation of Akt upon stimulation of adenosine A_3 receptors. These findings indicate that p87^{PIKAP} is indispensable for G$\beta\gamma$-mediated activation of p110γ after stimulation of G_i-coupled GPCRs in RBL-2H3 cells. Although the experiments were performed only in a single cell model, it may be assumed that the same holds true also in other cell types expressing p87^{PIKAP} but not p101. Together with the data obtained on the p101$^{-/-}$ mice (Suire *et al.*, 2006), these observations are the first to demonstrate an essential role for a PI3Kγ regulatory subunit *in vivo*. Knockdown of p87^{PIKAP} not only influenced signaling events immediately downstream of p110γ but also clearly impeded the degranulation process, which leads to the release of mediators in the allergic response. Antigen-induced and IB-MECA-stimulated release of β-hexosaminidase was reduced to levels observed without co-stimulation of adenosine A_3 receptors, corresponding to an extensive loss in p110γ activity. A smaller, but still significant reduction was observed upon stimulation with DNP-BSA only, indicating that a PI3Kγ-dependent feedback loop is also triggered after initial antigen-induced degranulation by released mediators. Comparable reductions were observed for mast cells from p110$\gamma^{-/-}$ mice (Laffargue *et al.*, 2002), pointing to a clear dependence of p110γ on p87^{PIKAP} in mast cells.

5.3 Structural studies on interactions involved in activation of PI3Kγ

Several methods are available for the study of protein-protein interactions on a structural level. Of these, X-ray crystallography, NMR spectroscopy, and single-particle electron microscopy offer the most detailed information, but are on the other hand time-consuming and elaborate. However, several biophysical and biochemical approaches such as FRET, chemical crosslinking, or phage-display techniques allow to extract spatial information and to identify interacting residues more easily, albeit at a lower resolution (Russell *et al.*, 2004). Therefore, different strategies were employed to achieve more detailed structural insight into the interactions leading to PI3Kγ activation. Overlay assays on peptide SPOT arrays allow the identification of linear peptide motifs contributing to a specific interaction (Reineke *et al.*, 2001). This technique was chosen to extend and confirm the data obtained in the mapping of functional domains within p101. The novel p87$^{\text{PIKAP}}$ regulatory subunit was probed as well to find common motifs employed by both subunits in their interaction with p110γ and G$\beta\gamma$. To initiate the exploration of these interactions on the atomic level, heterodimeric PI3Kγ complexes were purified and subjected to crystallization screens.

The analysis of peptide SPOT arrays generally confirmed the data obtained by generating p101 deletion mutants. For both p101 and p87$^{\text{PIKAP}}$, peptides that interact with p110γ were obtained mostly within the N-terminal third of their primary structure. This was in good agreement with the behavior of p101 deletion mutants. For p101, four clusters of spots exhibited affinity towards His-p110γ. Of these, two were confined to the borders of the core heterodimerization domain (aa 25–175). The other two followed within the next 150 aa of the p101 primary structure. These findings support the notion that further C-terminal elements may extend the heterodimerization domain and contribute to affinity towards p110γ (see section 5.1). Moreover, epitopes at the N-terminal border of the G$\beta\gamma$ binding domain displayed affinity towards p110γ. Thus, p101 and p110γ may perhaps come into close proximity at the site of G$\beta\gamma$ recognition in p101, indicating that they may contact the same G$\beta\gamma$ molecule *via* a contiguous surface, as is suggested by other observations as well (see section 5.1). Largely the same results were obtained for the analysis of p110γ binding to p87$^{\text{PIKAP}}$-derived peptides. Thus, as expected from their homology, both proteins presumably employ similar means for interaction with p110γ. However, although interacting peptides are likewise mainly present within the N-terminal third containing the heterodimerization domain, clusters of p110γ-binding peptides in p87$^{\text{PIKAP}}$ only partially overlap with those from p101. A possible explanation for this observation may be that the N termini of p101 and p87$^{\text{PIKAP}}$ share the ability to bind p110γ but have evolved slightly different binding modes after their separation in *Ciona*.

Closer inspection of the aa that make up the peptides with affinity for p110γ revealed the presence of residues that are often found at protein-protein interaction interfaces, such as Trp, Tyr, and Arg (Bogan & Thorn, 1998; Ma *et al.*, 2003). In so-called 'hot spots', these residues

are surrounded by largely hydrophobic aa that serve to occlude water from the binding site, thereby enhancing electrostatic interactions (Bogan & Thorn, 1998; Keskin *et al.*, 2005). Hot spots provide the bulk of the energy involved in a protein-protein interaction, although they only account for a fraction of the interaction surface (Clackson & Wells, 1995). To a lesser extent, also Asp is found in hot spots. Most of the areas identified to contribute to binding of p110γ in p101 and p87$^{\text{PIKAP}}$ are consistent with these general observations. Especially the two areas at the N-terminal border of the G$\beta\gamma$ binding domain contain many Trp, Tyr, and Arg residues. The regions within the N terminus of p101 and p87$^{\text{PIKAP}}$ are comparatively large. One may thus speculate that the longer stretches at the N terminus form a large interaction surface containing many hot spot-like regions, whereas the more C-terminal epitopes provide smaller contact points with p110γ. In agreement with these data, p101 deletion mutants consisting mainly of the heterodimerization domain (aa 25–175) already display high enough affinity towards p110γ to bind it in a cellular context. Given that linear peptide motifs participating in binding events are normally rather short (Neduva & Russell, 2006) and that hot spots are generally small as well, further elucidation of the interaction sites requires probing of shorter peptides. In addition, since hot spot residues are primarily defined by their energetic contribution, alanine scanning mutagenesis of peptides should provide further information regarding residues crucially involved in the interaction.

However, data obtained from the analysis of peptide SPOT arrays has to be viewed with care. First of all, an inherent feature of this method is its confinement to linear peptide motifs. Thus, non-linear epitopes that arise upon folding into secondary and tertiary structure elements evade identification. As hot spots are defined by a clustering of aa providing high binding energy in three-dimensional space rather than in the protein sequence, it is likely that they are often not obvious from analysis of primary structure data. Still, peptides containing 15 and more aa should already form stable secondary structures to some extent. In technical terms, analysis of peptide SPOT arrays can be hampered by difficulties in the synthesis of peptides consisting of *e.g.* mainly very hydrophobic residues. In screening of p101 and p87$^{\text{PIKAP}}$-derived peptide arrays for affinity towards G$\beta\gamma$, such factors may have caused the absence of G$\beta\gamma$-interacting peptides. In addition, non-linear epitopes may be primarily responsible for binding of G$\beta\gamma$. Within p101, the same C-terminal epitopes found to bind p110γ also weakly attracted G$\beta\gamma$ complexes. A likely explanation may be that these peptides contained such a high density of residues prominent in interaction sites that the lack of structural context led to promiscuous binding. On the other hand, these regions may indeed participate in binding of both p110γ and G$\beta\gamma$. Because interaction of G$\beta\gamma$ with its binding domain in p101 was abruptly lost if the domain was truncated beyond its borders at aa 650 and 850, one may speculate that interacting epitopes may be present there (see section 5.1). Since conventional synthesis of peptides is much more efficient, synthesis of peptides covering the respective areas may allow to assay their binding to G$\beta\gamma$ in solution. Moreover, non-linear epitopes may be identified by

synthesis of peptides that would fuse two regions of the primary sequence.

Concerning the interaction with G$\beta\gamma$, however, at least some aspects could be clarified by the use of peptide SPOT arrays. Although the resolution of the G$\beta\gamma$ binding domain was not enhanced, peptides on Gβ_1 were identified that interact with heterodimeric p110γ/p87$^{\text{PIKAP}}$ complexes. These peptides mapped to various regions within the β propeller, mostly corresponding to β strands that are connected by loops on top of the propeller, but also to regions on the side of the Gβ propeller (*e.g.* region 5 within the second to last repeat unit of the propeller, see Fig. 4.42*C*). In contrast to the interaction between p110γ and its regulatory subunits, the interaction between G$\beta\gamma$ and its effectors has been very well investigated. Many different studies have demonstrated that G$\beta\gamma$ effectors bind to a region on top of the β propeller that is also addressed by the so-called switch II region of Gα subunits (Ford *et al.*, 1998; Li *et al.*, 1998; Agler *et al.*, 2003; Lodowski *et al.*, 2003). A number of peptides identified by phage display were shown to bind competitively to the same site (Scott *et al.*, 2001). Moreover, a recent crystal structure of G$\beta\gamma$ in complex with the phage display-derived SIGK peptide further supported the existence of a hot spot on top of the β propeller (Davis *et al.*, 2005). This site is thought to enable effectors featuring grossly different structures to bind G$\beta\gamma$ by employing unique binding modes, which are nonetheless all compatible with the interaction hub provided. In line with this notion, a unifying G$\beta\gamma$ binding motif has not been discovered yet, although many consensus sequences have been proposed (see *e.g.* Chen *et al.*, 1995, for the QXXER motif). Many of the residues that have been identified as important for interaction with effectors and the SIGK peptide were also covered by peptides that recognized PI3Kγ heterodimers, most notably Trp 99, Tyr 145, Asp 246, and Trp 332. These have been shown to contribute significantly to the binding energy of the SIGK peptide (Davis *et al.*, 2005) and are also found at the GRK2-G$\beta\gamma$ interface (Lodowski *et al.*, 2003). In addition, peptides corresponding to a loop within the sixth β sheet repeat were labeled by the PI3Kγ heterodimer, which may constitute a unique contact point with respect to other G$\beta\gamma$ effectors. Moreover, also the adjacent outer side of the sixth as well as of the seventh repeat unit were identified as potential sites for interaction between PI3Kγ dimers and G$\beta\gamma$. Interestingly, analysis of point-mutated G$\beta\gamma$ complexes revealed interactions of the same regions on the side of G$\beta\gamma$ with PLCβ_2 but not adenylyl cyclase II (Panchenko *et al.*, 1998). In summary, heterodimeric PI3Kγ complexes appear to interact with G$\beta\gamma$ *via* the same interface as do other G$\beta\gamma$ effectors, although additional contacts both at the top and on the side of Gβ may add to a binding mode specific for PI3Kγ. Within PI3Kγ and especially its regulatory subunits, however, the exact motifs employed in recognition of G$\beta\gamma$ remain to be clarified further in future work.

As an approach complementary to the comparatively low-resolution technique of peptide SPOT arrays, it was attempted to obtain heterodimeric complexes consisting of p87$^{\text{PIKAP}}$ and p110γ for crystallographic analysis. Although a first attempt at obtaining crystals failed, a heterodimer containing only a single His tag purified by a different approach is still under

observation in crystallization screens. As a host of factors determine the success of protein crystallization, several options are available to further modify and improve the approach used here. First, removal of the remaining His tag at the N terminus of p110γ by digestion with TEV protease may lead to protein amenable for crystallization. Moreover, limited proteolysis, mass spectrometry analysis, and N-terminal sequencing may identify flexible and unstructured regions and result in stable fragments that can be re-cloned, expressed, and purified. Similar strategies have often been successfully employed. Although the characteristics of the constructs have generally a greater impact, modifications to the purification process such as different pH and buffer conditions may also be worth to consider. As the whole PI3Kγ complex exceeds the size limitations of NMR spectroscopy-based structure resolution, the purification of the isolated functional domains of p101 may provide a vantage point for NMR-based approaches. Unfortunately, attempts at purifying the heterodimerization and G$\beta\gamma$ binding domains of p101 from *E. coli* and Sf21 cells resulted in aggregation in inclusion bodies and poor yield, respectively (data not shown). Because X-ray crystallography and NMR techniques are unmatched in providing structural insight at the atomic level, they offer the only means by which the interactions between the PI3Kγ regulatory subunits, p110γ, and G$\beta\gamma$ may be fully understood. Thus, future work on the regulation of PI3Kγ will inevitably involve further attempts towards structural elucidation of the heterodimeric PI3Kγ complex.

5.4 Potential implications for the development of isotype-specific PI3Kγ inhibitors

The critical involvement of PI3Kγ in several physiological settings that are related to inflammatory and allergic disorders has raised the attention of pharmaceutical research. p110γ is now regarded as a high-potential therapeutic target (Rückle *et al.*, 2006). The phenotype of p110$\gamma^{-/-}$ mice has been found to be generally mild. Mice are viable, fertile, and do not display any severe defects (see Introduction). They, however, show pronounced – although never complete – reductions in neutrophil ROS production, migration, and chemotaxis (Sasaki *et al.*, 2000; Li *et al.*, 2000; Hirsch *et al.*, 2000), defects in macrophage (Jones *et al.*, 2003) and DC migration (Del Prete *et al.*, 2004), and impaired mast cell degranulation (Laffargue *et al.*, 2002). Moreover, PI3Kγ deficiency protected against hypertrophy and cardiac dysfunction caused by chronic pressure overload and long-term exposure to β-adrenergic agonists (Crackower *et al.*, 2002; Patrucco *et al.*, 2004), and p110$\gamma^{-/-}$ mice were resistant to the hypertensive effect of chronic exposure to angiotensin II (Vecchione *et al.*, 2005). Based on these physiological effects of p110γ deletion, targeting PI3Kγ should be beneficial in the therapy of different acute and chronic inflammatory and allergic conditions (*e.g.* rheumatoid arthritis and asthma) as well as in the treatment of cardiovascular diseases such as hypertension and heart failure. Especially in cardiac ailments, where effects on both myocardial and inflammatory components would be

desirable, PI3Kγ appears to be an ideally suited target. The class IA PI3Kα, on the other hand, is focused on in the development of potential novel drugs in the treatment of cancer (Hennessy *et al.*, 2005), largely sparked by the observation that activating mutations in the gene encoding p110α are found in many types of human cancers (Samuels *et al.*, 2004).

Because class IA and IB PI3Ks function in fundamentally different settings, it would be deleterious if potential inhibitors against p110γ would interfere with functions of p110α or p110β in the regulation of metabolic processes or the differentiation and proliferation of cells. The inverse setting would potentially not be as harmful but still not desirable. For these reasons, broad spectrum PI3K inhibition is likely to lead to toxicity effects in patients. Both wortmannin and LY294002, which were the first inhibitors of PI3Ks to be described, lacked appreciable selectivity for a distinct PI3K isoform (Arcaro & Wymann, 1993; Vlahos *et al.*, 1994). However, both proved valuable in the eludication of the cellular functions of PI3Ks. Many attempts to identify and synthesize isotype-specific PI3K inhibitors have been persued since with varying success. Like protein kinase inhibitors, inhibitors of PI3Ks bind competitively to the ATP binding cassette at the catalytic center. Because this cassette and its environment are highly conserved between class I PI3Ks and still similar in protein kinases, it is not surprising that, to date, a strictly isotype-specific inhibitor of PI3Kγ has not been described (Rückle *et al.*, 2006). Interestingly, inhibitors whose affinity is slanted towards some isoforms almost always display selectivity either towards p110α, p110γ, and DNA-PK or towards p110β and p110δ (Knight *et al.*, 2006), corroborating the notion that development of true isotype-specific inhibitors represents a demanding task.

However, many patents covering PI3Kγ inhibitors began to emerge since 2003 (Rückle *et al.*, 2006), and a few compounds exist that show strong preferences (up to ~100-fold) for a single isotype of class I PI3Ks. IC87114 was the first isotype-specific PI3K inhibitor to be described. It inhibits p110δ with 60–1000-fold selectivity over the other class I PI3Ks (Sadhu *et al.*, 2003). It diminished degranulation of wild-type murine mast cells to an extent comparable to that caused by knockout of p110δ, but did not affect p110δ-deficient mast cells (Ali *et al.*, 2004). TGX-221 was presented as an p110β-specific inhibitor. It interfered with the formation of stable integrin $\alpha_{IIb}\beta_3$ adhesion contacts in platelets, resulting in elimination of occlusive thrombi without prolonging bleeding time in mice (Jackson *et al.*, 2005). TGX-221 is 20-fold selective over p110δ and >1,000-fold selective with regard to p110α and p110γ. A combined p110α and mTOR inhibitor (PI-103) achieved efficient proliferative arrest in glioma cells and xenografted tumors in mice (Fan *et al.*, 2006). PI-103 is, however, only 5–20-fold more active towards p110α than towards the other class I PI3Ks. In addition to several patent applications, three compounds inhibiting p110γ were recently disclosed and used in proof-of-concept studies. An orally available compound with only slight p110γ specificity (AS-605240, 30-fold selective over p110β and p110δ, 7.5-fold selective towards p110α) suppressed the progression of joint inflammation in mice models of rheumatoid arthritis (Camps *et al.*, 2005). A novel set of compounds

from the same company yielded an inhibitor which is reasonably selective within the PI3K family (IC_{50} of 30 nM, ~30-fold compared to p110α, >500-fold for p110β and p110δ), but, unfortunately, inhibits casein kinase II with similar efficacy and potency (Pomel *et al.*, 2006). Nevertheless, oral administration of the compound reduced leukocyte infiltration in a murine peritonitis model to levels comparable to p110$\gamma^{-/-}$ mice (Pomel *et al.*, 2006). TG100-115, an inhibitor with mixed p110γ/p110δ specificity (IC_{50} of 83 nM for p110γ and 235 nM for p110δ, >1 µM for p110α and p110β) has recently been shown to markedly reduce the infarct size in mice and pigs after ischemia/reperfusion injury (Doukas *et al.*, 2006). It was thus proposed for the treatment of acute myocardial infarction.

Taken together, despite promising studies using inhibitors with incomplete specificity, efforts to generate isotype-specific p110γ inhibitors are still only moderately successful. Although it has recently been proposed that combined inhibition of a class IA PI3K and p110γ may be beneficial in some settings such as thrombosis and mast cell degranulation, strict isoform selectivity would still be worth attaining to reduce potential side effects. Moreover, besides therapeutical considerations, such inhibitors would be valuable as cell-biological tools for the elucidation of isotype-specific functions of individual PI3K isoforms. The distinct mode of activation that separates PI3Kγ from the class IA PI3Ks may provide novel means to overcome the described selectivity problems. Both p101 and $p87^{PIKAP}$ are unrelated to the p85 regulatory subunits of class IA PI3Ks and, thus, offer points of attack unique to PI3Kγ. Rather than targeting the well-conserved ATP binding cassette of the catalytic subunit, small molecules may be designed that would either disrupt binding of p110γ to its regulatory subunits or interfere with recognition of G$\beta\gamma$ by p101 and $p87^{PIKAP}$. Molecules that additionally discriminate between p101 and $p87^{PIKAP}$ may provide tools to alter signaling not only specifically for a single catalytic PI3K subunit but also focused on systems that employ a specific regulatory subunit.

Compared to addressing ligand and substrate binding sites in receptors and enzymes, respectively, focusing on the disruption of protein-protein interactions marks a relatively recent development in the area of drug design (Arkin & Wells, 2004). Although it is widely regarded difficult to target protein interaction surfaces due to their flatness and extended nature, a number of interactions have been successfully adressed (see Arkin & Wells, 2004, for review). Compounds called 'nutlins' interfere with the interaction between the tumor suppressor p53 and its inactivator MDM2 by blocking a hydrophobic pocket on the surface of MDM2 with low nanomolar affinity (Vassilev *et al.*, 2004), and nutlin-3 markedly reduced growth of xenografted tumors (Tovar *et al.*, 2006). Similarly, molecules were developed that inhibit activation of anti-apoptotic Bcl-2 proteins by blocking the hydrophobic binding groove for BH3 proteins like Bad (Oltersdorf *et al.*, 2005). In relation to PI3Kγ signaling, small molecules have been described that interfere with binding of effectors to G$\beta\gamma$ in a partially effector-selective fashion (Bonacci *et al.*, 2006). These compounds displaced PLCβ, GRK2, and a p110γ/p101 heterodimer from binding to G$\beta\gamma$. Moreover, cellular effects such as the fMLP-induced phosphorylation of Akt

in differentiated HL-60 cells were markedly inhibited (Bonacci *et al.*, 2006). In all of these cases, the existence of so-called 'hot spots' on the surfaces of proteins was exploited. Hot spots are crucial for the affinity of protein-protein interactions (see also section 5.3). They are complementary on both binding partners and often contain buried charged residues that form salt bridges as well as hydrophobic residues that fit into small nooks on the opposite surface (Arkin & Wells, 2004).

To successfully target protein interactions within the PI3Kγ heterodimer and on G$\beta\gamma$, the hot spots involved in the respective interactions have to be identified first. The experiments performed here constitute important steps towards the identification of these hot spots. First, the domain mapping on p101 pointed to a crucial role of the N terminus of p101 in the interaction with p110γ, suggesting that the affinity-generating epitopes for that interaction may be found in the same region. Likewise, interaction with G$\beta\gamma$ is a property of a C-terminal domain in p101, to which G$\beta\gamma$-binding hot spots should be confined. Subsequently, peptide SPOT arrays were used to search for linear peptide sequences in p101 and p87$^{\text{PIKAP}}$ that show high affinity for p110γ or G$\beta\gamma$. Combined with the assumption that p101 and p87$^{\text{PIKAP}}$ bind to p110γ *via* conserved structural elements, a few sequences could be identified that may play a crucial role in the interaction with p110γ (see section 5.3). Although peptide SPOT arrays did not enhance the resolution of the G$\beta\gamma$ binding site either due to deficiencies in SPOT synthesis or non-linearity of the binding site, the abrupt loss of binding to G$\beta\gamma$ upon deletions between aa 650–675 and 825–850 in p101 may point to hot spots in these areas. After further experiments such as alanine-scanning of peptide epitopes, peptides showing the highest affinity for p110γ may be employed in binding and competition assays on purified protein and in cell-based assays, thereby initiating the development of peptidomimetics. However, peptides employed for displacement will have to be shortened first in order to meet the size restrictions of peptidomimetics that may be reasonably synthesized.

In a parallel approach, resolution of the three-dimensional structure of a p110γ/p87$^{\text{PIKAP}}$ complex or even a tripartite complex of a heterodimer combined with G$\beta\gamma$ will ultimately lead to a thorough understanding of the binding surfaces involved. Identification of hot spots within the structure will then allow the application of rational drug design methods such as computational docking and virtual screening of compound libraries, as well as structure-based drug design approaches. Without knowledge of the structure, high-throughput screening of compound libraries may yield interfering molecules regardless of previously available structural information. In future work, all of these approaches may be employed to yield suitable molecules. It remains to be seen, which of the interactions involved in the activation of PI3Kγ will be best to target. Concerning the interaction of G$\beta\gamma$ with p101 or p87$^{\text{PIKAP}}$, it may be advisable to develop substances binding to p101 or p87$^{\text{PIKAP}}$ rather than blocking binding sites on G$\beta\gamma$, because these are likely to overlap with binding interfaces used by other effectors (Scott *et al.*, 2001; Bonacci *et al.*, 2006). Although molecules that bind at the G$\beta\gamma$ binding site of

p101 do not block access to G$\beta\gamma$ for other effectors, specificity with regard to other G$\beta\gamma$ effectors must also be ensured for such molecules. Due to its temporary nature and the most likely smaller binding interface, interfering with G$\beta\gamma$ binding appears more promising than attempts to block the constitutive interaction between p110γ and its regulatory subunits. Nonetheless, the p101/p87$^{\text{PIKAP}}$–p110γ interaction should not be dismissed. Although it probably involves a large interface with multiple contact points that add up to a high affinity it offers advantages in terms of specificity. An inhibitor of a constitutive protein-protein interaction has been successfully generated that exerts its function during protein synthesis before binding between the partners is established. Such inhibitors of the inducible NO synthase bind to the heme moiety and disrupt dimerization of the protein, leading to effective inhibition even in animal disease models (McMillan *et al.*, 2000). Given the relative instability of the regulatory subunits if not complexed with p110γ, a similar mechanism may also provide meaningful ways to interfere with the interaction between p110γ and its regulatory subunits p101 and p87$^{\text{PIKAP}}$.