6 Summary

PI3Kγ is a lipid kinase that is chiefly activated downstream of GPCRs. By producing the lipid second messenger PIP_3 it addresses a plethora of effector proteins that contain PIP_3-binding PH domains. Among these effectors are the serine/threonine kinases Akt and PDK-1, tyrosine kinases such as Btk, and both GEFs and GAPs for small GTPases of the Arf and Rac families. By virtue of these effectors, PI3Kγ controls many cellular processes ranging from cell survival and proliferation to chemotaxis and the production of ROS. In line with its expression pattern, genetic ablation of the catalytic p110γ subunit of PI3Kγ results in defects in various inflammatory and allergic responses such as neutrophil chemotaxis, oxidative burst, and degranulation of mast cells. Moreover, recent studies have revealed a crucial role of PI3Kγ in the regulation of cardiac contractility and the development of cardiac hypertrophy.

PI3Kγ consists of a catalytic p110γ and a regulatory p101 subunit. The regulatory subunit is indispensable for activation of PI3Kγ in cellular systems. p101 binds to G$\beta\gamma$ complexes released from heterotrimeric G proteins upon stimulation mainly of G_i-coupled GPCRs. Thereby, p101 co-translocates the bound catalytic p110γ subunit to the plasma membrane, where it has access to its substrate $PI(4,5)P_2$. There, G$\beta\gamma$ and Ras proteins may further stimulate the catalytic activity of p110γ by allosteric mechanisms. Despite the crucial role of the interactions mediated by p101, its binding sites for p110γ and G$\beta\gamma$ are largely elusive. Therefore, this thesis aimed first at elucidating the elements within the primary structure of p101 that are responsible for interaction with p110γ and G$\beta\gamma$.

To this end, deletion mutants of p101 were constructed and analyzed with respect to their ability to bind p110γ and G$\beta\gamma$. By employing co-immunoprecipitation, FRET, and recruitment assays, the heterodimerization domain, which interacts with p110γ, could be ascribed to an N-terminal region of p101 (aa 25–175, supported by further affinity-generating areas within the extended N terminus). The G$\beta\gamma$ binding domain resides within the C terminus of p101 (aa 650-850). These domains were found to be highly conserved between orthologues of p101, whereas the intervening sequence stretches display a less stringent conservation.

The primary sequences of the functional domains of p101 were then used to search for potential novel interaction partners for p110γ and as yet unrecognized G$\beta\gamma$ effectors. Strikingly, a cDNA was retrieved in data base searches that showed significant similarity to p101 within the p110γ and G$\beta\gamma$ binding regions. Cloning and subsequent characterization of the corresponding mRNA from dendritic cells yielded a novel regulatory subunit of PI3Kγ. According to its molecular weight and function, it was termed PI3Kγ adapter protein of 87 kDa ($p87^{PIKAP}$).

Interaction of $p87^{PIKAP}$ and p101 with p110γ was found to be of comparable affinity and mutually exclusive, indicating that both proteins presumably bind to the same binding site on p110γ. $p87^{PIKAP}$ displayed a lower affinity towards G$\beta_1\gamma_2$ dimers than p101, resulting also in reduced PI3Kγ activity upon stimulation of a G_i-coupled GPCR in a reconstituted system in living cells, as determined by monitoring the translocation of a YFP-tagged PIP_3-binding PH domain and the phosphorylation of Akt. $p87^{PIKAP}$ is, thus, able to adopt the role of p101 in PI3Kγ signaling, although it confers different properties to the heterodimer. Analysis of the expression pattern of p101, $p87^{PIKAP}$, and p110γ demonstrated that at least one regulatory subunit accompanies p110γ in all cell types assayed. Whereas expression of p101 dominates in B and T cells, both subunits can be found in dendritic cells, macrophages, and neutrophils. In heart and mast cells, however, $p87^{PIKAP}$ is expressed almost exclusively.

Therefore, it was focused on these two systems for further analysis of the role played by $p87^{PIKAP}$ in PI3Kγ signaling. RBL-2H3 cells resemble mast cells in their ability to degranulate upon stimulation with antigen. Knockdown of $p87^{PIKAP}$ expression in RBL-2H3 cells by shRNA led to diminished phosphorylation of Akt upon stimulation of the endogenous G_i-coupled adenosine A_3 receptor. Moreover, the p110γ-dependent feedback loop, which amplifies antigen-induced degranulation *via* autocrine stimulation of G_i-coupled GPCRs, was severely blunted if $p87^{PIKAP}$ expression was diminished, indicating an essential role for $p87^{PIKAP}$ in this physiological setting. The negative inotropic impact of PI3Kγ on cardiac contractility has recently been shown to be mediated by stimulation of PDE3B within a macromolecular complex that contains p110γ as an essential component. However, p110γ itself was reported to be unable to bind PDE3B. Therefore, it was analyzed whether $p87^{PIKAP}$ may bridge p110γ and PDE3B. $p87^{PIKAP}$ and p110γ/$p87^{PIKAP}$ heterodimers could indeed be copurified with PDE3B and *vice versa* from co-transfected cells. However, $p87^{PIKAP}$ and p110γ/$p87^{PIKAP}$ complexes were unable to stimulate PDE3B activity. These findings indicate that $p87^{PIKAP}$ may equally be an essential component of the PDE3B regulatory complex, but further cofactors are necessary for efficient PDE3B stimulation.

To obtain a more detailed structural view on the interactions involved in activation of PI3Kγ, peptide SPOT arrays were used to identify linear peptide sequences within the regulatory subunits that exhibit affinity towards purified p110γ or G$\beta\gamma$. Confirming the results obtained on p101 deletion mutants, N-terminal areas in both p101 and $p87^{PIKAP}$ were recognized by p110γ in overlay assays. Moreover, contacts with p110γ may also be established close to the G$\beta\gamma$ binding domain. Heterodimeric p110γ/$p87^{PIKAP}$ complexes were used to probe Gβ-derived peptides, revealing that PI3Kγ binds to the common effector binding hot spot in G$\beta\gamma$, but presumably establishes contacts to other parts of Gβ as well. Crystallization studies on the purified p110γ/$p87^{PIKAP}$ heterodimer were initiated to describe the interaction between $p87^{PIKAP}$ and p110γ in atomic detail. However, further crystallization attempts will be necessary to achieve this goal.