Characterization of p87\textsuperscript{PIKAP}, a Novel Regulatory Subunit of Phosphoinositide 3-Kinase γ That Is Highly Expressed in Heart and Interacts with PDE3B\textsuperscript{*}

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Phosphoinositide 3-kinase (PI3K) γ has been implicated in a vast array of physiological settings including the activation of different leukocyte species and the regulation of myocardial contractility. Activation of PI3K γ is primarily mediated by Gβγ subunits of heterotrimeric G proteins, which are recognized by a p101 regulatory subunit. Here, we describe the identification and characterization of a novel regulatory subunit of PI3K γ, which we termed p87\textsuperscript{PIKAP} (PI3K-γ adapter protein of 87 kDa). It is homologous to p101 in areas that we have recently shown that they mediate binding to the catalytic p110γ subunit and to Gβγ. Like p101, p87\textsuperscript{PIKAP} binds to both p110γ and Gβγ and mediates activation of p110γ downstream of G protein-coupled receptors. In contrast to p101, p87\textsuperscript{PIKAP} is highly expressed in heart and may therefore be crucial to PI3K γ cardiac function. Moreover, p87\textsuperscript{PIKAP} and p101 are both expressed in dendritic cells, macrophages, and neutrophils, raising the possibility of regulatory subunit-dependent differences in PI3K γ signaling within the same cell type. We further provide evidence that p87\textsuperscript{PIKAP} physically interacts with phosphodiesterase (PDE) 3B, suggesting that p87\textsuperscript{PIKAP} is also involved in the recently described noncatalytic scaffolding interaction of p110γ with PDE3B. However, coexpression of PDE3B and PI3K-γ subunits was not sufficient to reconstitute the regulatory effect of PI3K γ on PDE3B activity observed in heart, implying further molecules to be present in the complex regulating PDE3B in heart.

Receptor-regulated class I phosphoinositide 3-kinases (PI3K)\textsuperscript{2} are lipid kinases that produce the 3'-phosphorylated inositol lipid phosphatidylinositol 3,4,5-trisphosphate (PtdIns 3,4,5-P\textsubscript{3}). It acts as a lipid second messenger by recruiting proteins containing pleckstrin homology (PH) domains to cellular membranes, thereby initiating various cellular responses (1, 2). Class I PI3K are further subdivided into classes IA and IB according to their mode of activation. Class IB PI3K γ is chiefly activated by G protein-coupled receptors (GPCR) and therefore grouped separately from the class IA PI3K that are activated downstream of receptor tyrosine kinases. Insight into the physiological role of PI3K γ has been mostly derived from the characterization of p110γ knockout mice, which show defects in chemottractant-induced neutrophil migration and oxidative burst, thymocyte development (3–6), macrophage and dendritic cell (DC) migration (7), and the GPCR-dependent autocrine amplification of FceRI-mediated mast cell degranulation (8). Moreover, characterization of p110γ knockout mice revealed a role for PI3K γ both in the regulation of myocardial contractility and in cardiac remodeling processes (9, 10). Recently, characterization of mice with a knockin of a catalytically inactive mutant of p110γ revealed that the impact on contractility is probably mediated by a scaffolding interaction with phosphodiesterase (PDE) 3B, whereas remodeling processes are governed by pathways depending on catalytic activity of p110γ (11).

Besides the catalytic p110γ subunit, PI3K γ consists of a p101 regulatory subunit that binds both p110γ and Gβγ (12). Although lipid kinase activity of p110γ can be stimulated by Gβγ in the absence of the regulatory p101 subunit (13), p110 appears to be necessary for Gβγ-mediated activation of PI3K γ in living cells. In a heterologous reconstitution system, p101 binds to Gβγ subunits and thereby recruits p110γ to the plasma membrane, whereas p110γ was neither recruited to the plasma membrane by Gβγ nor activated by GPCR stimulation in the absence of p101 (14). However, p110γ is functional and physiologically important in tissues where neither expressed sequence tag data nor direct experimental evidence validate an expression of p110γ, rendering the role of p110γ still controversial. Recently, we were able to map the determinants relevant for interaction with p110γ and Gβγ to distinct areas within the p101 primary structure (15). These findings enabled us to identify an mRNA sequence within the DDB/EMBL/GenBank\textsuperscript{TM} data base that encodes a distantly related p101 homologue (15). Whereas the encoded putative protein showed little overall sequence similarity to p110γ, a higher degree of conservation was observed within the regions that correspond to the p101 functional domains. Meanwhile, an initial characterization of this gene product was published by Suire et al. (16), who showed that it indeed interacts with p110γ and Gβγ.

Here we report a different cloning strategy and further functional characterization of this novel PI3K γ regulatory subunit, which we designated as p87\textsuperscript{PIKAP} (p87 PI3K adapter protein) (17, 18). Similarly to p101, p87\textsuperscript{PIKAP} interacts with p110γ and Gβγ. p87\textsuperscript{PIKAP} and p101 bind to p110γ in a mutually exclusive fashion with a similar orientation within the dimeric complex. p87\textsuperscript{PIKAP} was necessary and sufficient to reconstitute a PI3K γ signaling pathway in transfected HEK293 cells, mediating Gβγ-dependent activation of p110γ downstream of a G\textsubscript{βγ}-coupled receptor. p87\textsuperscript{PIKAP} mRNA was detected in various tissues, albeit most prominently in the heart. By contrast, p101 is only weakly expressed in the heart, whereas B and T cells feature p101 as the only p110γ regulatory subunit. In DCs, neutrophils, and macrophages, both p87\textsuperscript{PIKAP} and p101 are coexpressed, raising the possibility of isoformal...
specific signaling with respect to the PI3Kγ regulatory subunit. Moreover, we present evidence that p87PIKAP interacts with PDE3B, pointing to an additional involvement of p87PIKAP in the recently described non-catalytic scaffolding interaction of p110γ with PDE3B.

**EXPERIMENTAL PROCEDURES**

Cloning of Murine p87PIKAP cDNA and Generation of Expression Plasmids—Total RNA of murine CD11c+ DC was reverse-transcribed using oligo(dT) primers and Superscript II reverse transcriptase (Invitrogen). Murine p87PIKAP cDNA was then amplified by PCR (Expand HF; Roche Applied Science) using the primers 5’-CCCTC-CCCCATAAGGACAGA-3’ and 5’-GTGGGCTGTGCTGTA-AATG-3’ and subcloned into pcDNA3.1/V5-HIS-TOPO (Invitrogen). The p87PIKAP cDNA nucleotide sequence of three independently amplified and subcloned clones was determined by sequencing (DYEnamic ET kit; Amersham Biosciences). These sequence data have been submitted to the DDBJ/EMBL/GenBank™ data base under accession number AJ753194. Expression plasmids encoding p87PIKAP N- or C-terminally tagged with cyan (CFP) or yellow (YFP) fluorescent proteins were generated by subcloning the p87PIKAP open reading frame into the custom-made vectors pcDNA3-NCFP, pcDNA3-NYFP, pcDNA3-CFP, or pcDNA3-YFP (19). In p87PIKAP-FLAG, a C-terminal FLAG epitope tag was added by subcloning into a pcDNA3-FLAG vector (20). A C-terminally tagged version of the C terminus of β-adrenergic receptor kinase 1 (βARK-CT-CFP) was generated by PCR using the primers 5’-GCCACCATGGGCACCAAAAAAACGAGCTTG-3’ and 5’-TTAATCTAGACCTGGCTGCGG-3’ and subcloned first into pcDNA3.1/V5-HIS-TOPO and then into pcDNA3-CFP-HIVIII and XbaI. Construction of expression plasmids coding for p101, CFP-p101, YFP-p101, p110γ-YFP, CFP-p110γ(K833R), p110γ-CAAX, YFP-p110β, YFP-Grp1-PH, Gβγ, Gγ, and the human IMLP receptor has been described elsewhere (14). The generation of the plasmid encoding murine PDE3B-FLAG has been published (21). For the generation of CFP-PDE3B, the PDE3B open reading frame was amplified by PCR with the primers 5’-GGACGGACGGCGGACTCCCTCTCTCTGGTGTGTCTTCTCTCC-3’ and 5’-TTAGCTCGAGTCGCTTCTTTTGTTTGTTTTCTTC-3’ and subcloned into pcDNA3.1/V5-HIS-TOPO. The CFP tag was inserted into this construct via Ndel and HindIII.

**Cell Culture and Transfection—**HEK293 cells were grown at 37 °C and 5% CO2 in Dulbecco’s modified Eagle’s medium (Invitrogen). Murine p87PIKAP cDNA was then amplified by PCR (Expand HF; Roche Applied Science) using the primers 5’-CCTC-CCCCATAAGGACAGA-3’ and 5’-GTGGGCTGTGCTGTA-AATG-3’ and subcloned into pcDNA3.1/V5-HIS-TOPO (Invitrogen). The p87PIKAP cDNA nucleotide sequence of three independently amplified and subcloned clones was determined by sequencing (DYEnamic ET kit; Amersham Biosciences). These sequence data have been submitted to the DDBJ/EMBL/GenBank™ data base under accession number AJ753194. Expression plasmids encoding p87PIKAP N- or C-terminally tagged with cyan (CFP) or yellow (YFP) fluorescent proteins were generated by subcloning the p87PIKAP open reading frame into the custom-made vectors pcDNA3-NCFP, pcDNA3-NYFP, pcDNA3-CFP, or pcDNA3-YFP (19). In p87PIKAP-FLAG, a C-terminal FLAG epitope tag was added by subcloning into a pcDNA3-FLAG vector (20). A C-terminally tagged version of the C terminus of β-adrenergic receptor kinase 1 (βARK-CT-CFP) was generated by PCR using the primers 5’-GCCACCATGGGCACCAAAAAAACGAGCTTG-3’ and 5’-TTAATCTAGACCTGGCTGCGG-3’ and subcloned first into pcDNA3.1/V5-HIS-TOPO and then into pcDNA3-CFP-HIVIII and XbaI. Construction of expression plasmids coding for p101, CFP-p101, YFP-p101, p110γ-YFP, CFP-p110γ(K833R), p110γ-CAAX, YFP-p110β, YFP-Grp1-PH, Gβγ, Gγ, and the human IMLP receptor has been described elsewhere (14). The generation of the plasmid encoding murine PDE3B-FLAG has been published (21). For the generation of CFP-PDE3B, the PDE3B open reading frame was amplified by PCR with the primers 5’-GGACGGACGGCGGACTCCCTCTCTCTGGTGTGTCTTCTCTCC-3’ and 5’-TTAGCTCGAGTCGCTTCTTTTGTTTGTTTTCTTC-3’ and subcloned into pcDNA3.1/V5-HIS-TOPO. The CFP tag was inserted into this construct via Ndel and HindIII.

**Cell Culture and Transfection—**HEK293 cells were grown at 37 °C and 5% CO2 in Dulbecco’s modified Eagle’s medium or minimal essential medium with Earle’s salts, supplemented with 10% fetal calf serum, 2 mM glutamine, 100 μg/ml streptomycin, and 100 units/ml penicillin. COS-7 cells were cultivated at 37 °C and 7% CO2 in Dulbecco’s modified Eagles medium containing 4.5 g/liter glucose supplemented with 10% fetal calf serum, 2 mM glutamine, 100 μg/ml streptomycin, and 100 units/ml penicillin. Transfection of HEK293 and COS-7 cells was performed 24–48 h prior to the experiments.

**Immunoprecipitation and Immunoblot Analysis—**Immunoprecipitations and immunoblot analyses were carried out as described previously (15) using anti-FLAG M2 (Sigma) and anti-GFP antibodies (BD Biosciences) and suitable secondary antibodies (Sigma). For the analysis of p87PIKAP and p101 stability, whole cell lysates were prepared by lysing the cells directly into Laemmli sample buffer followed by sonication (5 s) to ensure complete lysis. The lysates were analyzed by SDS-PAGE and immunoblot with anti-FLAG M2 antibody. Akt phosphorylation was analyzed in whole cell lysates using anti-Akt and anti-phospho-Akt (Ser473) antibodies (Cell Signaling Technology).

**Fluorescence Imaging and Confocal Microscopy—**FRET efficiencies were determined using the acceptor photobleaching method as described previously (15). FRET efficiencies E were calculated using the equation $E = 1 - (F_{DA}/F_D)$, with $F_{DA}$ representing the CFP fluorescence measured before bleaching YFP and $F_D$ representing the CFP fluorescence in absence of YFP acceptor. $F_{DA}$ was obtained by linear regression of the increase in CFP fluorescence with the decrease in YFP fluorescence and extrapolation to zero YFP fluorescence, i.e. complete YFP photobleach (22). The expression levels of YFP- and CFP-tagged proteins were assessed by calibration of fluorescence intensities using an intramolecularly fused CFP-YFP construct. A 1.5–3-fold excess of YFP-over CFP-tagged proteins was maintained to ensure comparability and to avoid situations where availability of the FRET acceptor may limit FRET efficiencies. For FRET competition studies, the excess of YFP-over CFP-tagged proteins was limited to 1.2–1.5-fold, allowing for an effective competition with untagged proteins. For the analysis of p87PIKAP and p101 expression, fluorescence intensities of YFP-tagged regulatory subunits of PI3Kγ and of a cotransfected free CFP (to identify transfected cells) were quantified using a 40×/1.3 F-Fluar objective and CFP- and YFP-selective band pass filters used for FRET microscopy (15). Pixel fluorescence intensities were integrated over single cells and corrected for background. Confocal microscopy was performed essentially as described (15). All of the imaging experiments were performed at room temperature in 10 mM HEPEs, pH 7.4, 128 mM NaCl, 6 mM KCl, 1 mM MgCl2, 1 mM CaCl2, 5.5 mM glucose, and 0.2% bovine serum albumin.

**Northern Blot Analysis—**Multiple tissue Northern blots were purchased from BD Biosciences. RNA probes were generated with the StripEz-SP6 kit (Ambion) using either BglII-linearized p87PIKAP-FLAG or the supplied β-actin control plasmid as a template and α-[32P]UTP (10 mCi/ml; PerkinElmer Life Sciences) as the radioactive label. Hybridizations were performed overnight at 68 °C in UltraHyb hybridization buffer (Ambion) according to the manufacturer’s protocol. The signals were detected on an image plate (Fujiﬁlm), which was read by a phosholimaging device (Fujiﬁlm BAS Reader 1500).

**Multiplex and Competitive PCR—**Bone marrow-derived macrophages and splenocytes from C57BL/6 mice were obtained and cultured as described (23). Magnetic cell sorting of leukocyte subtypes from splenocytes of C57BL/6 mice was performed according to the manufacturer’s conditions (Miltenyi Biotec). Purity of sorted cell populations was assessed with appropriate antibodies by flow cytometry on a LSR II cytometer (BD Biosciences) using the FlowJo analysis software (TreeStar Inc.) as described (24). Total RNA was prepared using the High pure RNA kit (Roche Applied Science), and poly(A)+ RNA was prepared with the μMACS mRNA kit (Miltenyi Biotec). Both were carried out according to the manufacturer’s protocols.

Total RNA (0.5–1.5 μg) or mRNA (5–50 ng) was reverse-transcribed using oligo(dT) primers and Superscript III reverse transcriptase (Invitrogen) according to the manufacturer’s instructions. Multiplex PCR was performed using Taq DNA polymerase (Promega) in the supplied buffer supplemented with 1.5 mM MgCl2. Initial denaturation at 94 °C for 2 min was followed by 24 amplification cycles (94 °C for 20 s, 60 °C for 45 s, and 72 °C for 45 s) and a final extension time of 5 min at 72 °C. The following primers were used: GAPDH forward (5’-TTAGCCCGCCACTTTTGATGTTCTTCTC-3’) and GAPDH reverse (5’-CCTCCTGGAGCCATG-3’) to amplify bases 521–1061 (541 bp) of NM_001001303; p87PIKAP forward (5’-GGACGGACGGCGGACTCCCTCTCTCTGGTGTGTCTTCTCTCC-3’) and GAPDH reverse (5’-CCTCCTGGAGCCATG-3’) to amplify bases 521–1061 (541 bp) of NM_001001303; p87PIKAP forward (5’-GGACGGACGGCGGACTCCCTCTCTCTGGTGTGTCTTCTCTCC-3’).
Identification and Cloning of p87PIKAP—As previously reported, we have identified an mRNA (GenBank™ accession number BC028998), whose encoded putative protein shows homology to p101 within the p110γ- and Gβγ-interacting domains (15), although the overall sequence similarity is relatively low (about 24% amino acid similarity). Based on expressed sequence tag data and the origin of the identified sequence, we chose murine DCs as a suitable source for RT-PCR-based amplification and cloning of the coding sequence of the p101 homologue. We could amplify and subclone the expected 2.3-kb fragment from total RNA of CD11c+ DCs of C57BL/6 mice. Clones derived from three independent PCRs were sequenced and yielded the coding sequence deposited in the DDBJ/EMBL/GenBank™ data base (accession code AY753194). It corresponds to the predicted coding region in BC028998, which was also used in the study of Suire et al. (16). Multiple clones were obtained containing a 12-bp insertion at the boundary of exons 12 and 13 (DDBJ/EMBL/GenBank™ accession number DQ295832). This insertion, however, did not result in obvious functional differences to the protein encoded by the deposited sequence (data not shown). The p87PIKAP coding sequence consists of 20 exons, and the gene is located on murine chromosome 11 immediately next to the p101 gene (see the Ensembl data base at www.ensembl.org for further information on gene structure). An alignment of the protein sequence has been published previously (15). Although Suire et al. (16) proposed p84 as a name for the novel regulatory subunit, we intend to stick to our previously introduced nomenclature that is also used in the DDBJ/EMBL/GenBank™ data base entries pertaining to this gene (Refs. 17 and 18; see also GenBank™ entry AY753194).

Subcellular Distribution and Stability of p87PIKAP—The subcellular distribution of p87PIKAP was assayed in living cells by confocal microscopy. Unlike p101 fusion proteins, YFP-tagged p87PIKAP localized almost exclusively to the cytosol of HEK293 and COS-7 cells regardless of the position of the fluorescent tag (Fig. 1A, upper panels). Because p110γ also localizes to the cytosol in these cell types, it is not surprising that coexpression of p110γ did not change the localization of p87PIKAP (Fig. 1A, lower panels). In contrast, YFP-p101 was predominantly localized within the nucleus of both COS-7 and HEK293 cells in the absence of p110γ, whereas coexpression of p110γ led to a redistribution to the cytosol (Fig. 1A, right panels; see also Ref. 14). For p101, a conserved nuclear localization signal can be located at positions 499–502 (residues numbered as in pig p101). The p87PIKAP sequence, however, lacks such nuclear targeting signals, and the protein partitions to the cytosol.

Furthermore, as observed previously (14), the overall fluorescence intensity of YFP-tagged p101 was substantially lower if it was expressed without p110γ. If cotransfected with free CFP as a control for transfection efficiency, expression levels of p101-YFP were reduced to 36 ± 3% (n = 3, 75–120 cells each) upon expression without p110γ. In contrast, expression levels of p87PIKAP-YFP remained largely unchanged (reduction to 75 ± 2%). To further test a dependence on p110γ expression, HEK293 cells were transfected with different ratios of FLAG-tagged P13Kγ subunits, whose expression was assayed in whole cell lysates. A dependence on p110γ expression was observed for p101-FLAG (Fig. 1B), which was comparable with previous results with CFP-p101 (14). We thus conclude that these p101 fusion proteins are stabilized by coexpression of p110γ. In contrast, the expression levels of p87PIKAP-FLAG were largely independent of the amount of p110γ-FLAG coexpressed (Fig. 1B), indicating a higher stability of the p87PIKAP protein in the absence of p110γ.

Interaction between p87PIKAP and p110γ—Next, we tested the interaction between p87PIKAP and the catalytic p110γ subunit of PI3Kγ by employing co-IP assays. CFP-fused p87PIKAP copurified with p110γ-FLAG from lysates of HEK293 cells transfected with both proteins (Fig. 2), supporting the earlier observation of Suire et al. (16). Likewise,
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FIGURE 1. Subcellular distribution and stability of fluorescent p87\textsuperscript{PIKAP}. A, HEK293 and COS-7 cells were transfected with the indicated plasmids and analyzed by confocal microscopy. Representative images are shown. Bars, 10 μm. B, HEK293 cells were transfected with the indicated amounts of plasmids encoding p110γ-FLAG and either p101-FLAG (upper panels) or p87\textsuperscript{PIKAP}-FLAG (lower panels). The total amount of 2 μg of plasmid/transfection was kept constant by the addition of empty pcDNA3 expression vector. Equal amounts of whole cell lysates prepared 2 days after transfection were analyzed with an anti-FLAG antibody.

CFP-p101 copurified with p110γ-FLAG under the same conditions. A CFP-YFP fusion protein employed as a control for unspecific binding to the fluorescent protein moiety was not detectable in immunoprecipitates containing p110γ-FLAG. To validate the interaction between p87\textsuperscript{PIKAP} and p110γ in living cells and to obtain further spatial information about the p110γ-p87\textsuperscript{PIKAP} complex, FRET measurements were performed. A representative acceptor photobleaching FRET experiment on HEK293 cells expressing YFP-p110γ and CFP-p87\textsuperscript{PIKAP} is shown in Fig. 3A. A FRET efficiency of about 17% was determined (22) as depicted in Fig. 3B. To verify the specificity of the FRET signals, FRET competition assays were performed. If FRET is due to a specific interaction between two proteins, coexpression of untagged protein is expected to displace the tagged protein from its interaction partner, thereby leading to a reduction in FRET efficiency. Indeed, such a reduction in FRET efficiencies was observed if CFP-p87\textsuperscript{PIKAP} and YFP-p110γ were coexpressed with either untagged p87\textsuperscript{PIKAP} or p110γ (Fig. 3, C and D), showing that the FRET signals measured arise from a specific protein-protein interaction.

FRET efficiencies were further determined for all combinations of CFP- and YFP-tagged p110γ and p87\textsuperscript{PIKAP} in living HEK293 cells (Fig. 3, E and F). All of the FRET efficiencies were significantly higher than those measured for CFP- and YFP-tagged p87\textsuperscript{PIKAP} coexpressed with free YFP and CFP, respectively. FRET efficiencies comparable with those of the negative controls were obtained if YFP-p110B or CFP-p110B were used instead of p110γ fusion proteins, indicating that p87\textsuperscript{PIKAP} is a class IB PI3K adapter protein that does not interact with class IA catalytic subunits. Similar to the situation observed for p110γ and p101 (14), higher FRET efficiencies were obtained if fluorescent proteins were fused to the same termini in both p110γ and p87\textsuperscript{PIKAP}. Assuming unhindered rotation of the fluorochromes, this indicates that the relative orientation of the polypeptide chains within the complex of p87\textsuperscript{PIKAP} and p110γ is similar to that of p101 and p110γ.

Binding of p87\textsuperscript{PIKAP} and p101 to p110γ Is Mutually Exclusive—To test whether p101 and p87\textsuperscript{PIKAP} bind to p110γ in a pairwise or in a mutually exclusive fashion, we further employed FRET competition assays. The interaction between p110γ-YFP and p101-CFP was monitored by determining FRET efficiencies under conditions of cotransfection with either a control vector or plasmids encoding p87\textsuperscript{PIKAP}, p101, or p85α. We observed that p101-CFP was displaced from its interaction with p110γ upon coexpression of either p87\textsuperscript{PIKAP} or p101, whereas coexpression of p85α had no effect on the FRET efficiency (Fig. 4A). Similar results were obtained if the interaction between p87\textsuperscript{PIKAP}-CFP and p110γ-YFP was assayed under the same conditions (Fig. 4B). Thus, p87\textsuperscript{PIKAP} and p101 bind to p110γ in a mutually exclusive fashion, indicating overlapping binding surfaces of p87\textsuperscript{PIKAP} and p101 on p110γ. Also, a rough measure of the relative binding affinities can be deduced from the competition assays. Because both p87\textsuperscript{PIKAP} and p101 competed with p101-CFP and p87\textsuperscript{PIKAP}-CFP with comparable efficiency (i.e. bring about comparable reductions in FRET efficiencies), both proteins should have a comparable affinity for the common interaction partner p110γ.

To more directly assess their relative affinity for p110γ, various amounts of CFP-tagged p87\textsuperscript{PIKAP} and p101 were cotransfected with p110γ-FLAG. p110γ-YFP was immunoprecipitated, and the recovery of fluorescently tagged p87\textsuperscript{PIKAP} and p101 was analyzed by comparing the signals obtained in immunoprecipitates with those obtained in the lysates. If comparable amounts of CFP-p87\textsuperscript{PIKAP} and CFP-p101 were expressed, an excess of CFP-p87\textsuperscript{PIKAP} was detected in the immunoprecipitate (Fig. 4C, lane 3). Under conditions of a slight excess of CFP-p101, CFP-p87\textsuperscript{PIKAP} was still enriched to a greater extent within the immunoprecipitate (Fig. 4C, lanes 4 and 5). Still, CFP-p101 was able to
displace CFP-p87\textsuperscript{PIKAP} from its binding to p110\textgamma-FLAG (Fig 4C, lanes 1 and 2). Therefore, p87\textsuperscript{PIKAP} may bind to p110\textgamma-FLAG with a slightly higher affinity than does p101.

**Interaction between p87\textsuperscript{PIKAP} and G\textbeta y**—According to the crucial role of p101 in the activation of p110\textgamma by G\textbeta y, we examined the ability of p87\textsuperscript{PIKAP} to bind to G\textbeta y in living cells. Suire et al. (16) reported that p87\textsuperscript{PIKAP} has an ~4-fold lower affinity for G\textbeta y as compared with p101 in *in vitro* lipid kinase assays using purified recombinant protein. Probably in line with these findings, we failed to observe a predominant membrane staining in HEK293 or COS-7 cells coexpressing YFP-p87\textsuperscript{PIKAP} and an excess of G\beta y\textgamma, although a membrane accumulation can be observed for YFP-p101 or the fluorescent protein alone instead of p110\textgamma fusions were used as controls. C–F, means and S.E. of four independent measurements with at least six cells each are shown.

**FIGURE 3.** FRET between fluorescent fusion proteins of p110\textgamma and p87\textsuperscript{PIKAP}. FRET was measured in living HEK293 cells transfected with the indicated plasmids. A, single cell (gray lines) and mean (black lines) fluorescence traces for CFP and YFP during selective photobleaching of the acceptor with 512-nm light. Initial, initial. B, regression analysis of the mean fluorescence traces from the experiment shown was performed to calculate FRET efficiencies using the formula given under "Experimental Procedures." C and D, FRET competition assays were performed to verify the specificity of the obtained FRET signals. The indicated amounts of expression plasmids for wild-type untagged p87\textsuperscript{PIKAP} (C) or p110\textgamma (D) were transfected together with constant amounts of plasmids encoding the fluorescent fusion proteins. E and F, FRET efficiencies (E) for different combinations of N- and C-terminally CFP- and YFP-fused p110\textgamma and p87\textsuperscript{PIKAP}. The donor fluorochrome CFP was either fused to p87\textsuperscript{PIKAP} (E) or to p110\textgamma (F). The cells transfected with fluorescent p110\textgamma or the fluorescent protein alone instead of p110\textgamma fusions were used as controls. C–F, means and S.E. of four independent measurements with at least six cells each are shown.

**FIGURE 4.** Competition of FRET between P13K\textgamma subunits and competitive coimmunoprecipitation of p87\textsuperscript{PIKAP} and p101. A and B, FRET was measured in living HEK293 cells transfected with the plasmids encoding either p101-CFP and p110\textgamma-YFP (A) or p87\textsuperscript{PIKAP}-CFP and p110\textgamma-YFP (B) as well as either empty pcDNA3 vector or vectors encoding p87\textsuperscript{PIKAP}, p101, or p85\textalpha at the amounts indicated. The means and S.E. of four independent measurements with at least six cells each are shown. C, HEK293 cells were transfected with plasmids encoding p110\textgamma-FLAG and CFP-tagged p101 and p85\textalpha at the relative amounts indicated below. p110\textgamma-FLAG was immunoprecipitated with an anti-FLAG antibody, and copurified CFP-p101 and CFP-p87\textsuperscript{PIKAP} was detected with an anti-GFP antibody (IP). The cell lysates were analyzed with anti-FLAG and anti-GFP antibodies to measure expression (load). The lanes are numbered from left to right. The data shown are from a representative experiment of three. IB, immunoblot.
p87\textsuperscript{PIKAP}, a Novel Regulatory Subunit of PI3Kγ

FIGURE 5. Interaction between Gβγ and p87\textsuperscript{PIKAP} mediates PI3Kγ activation. A, the subcellular localization of the YFP-tagged PtdIns 3,4,5-P\textsubscript{3}-binding PH domain of Grp1 (YFP-Grp1-PH) was monitored by confocal microscopy in living HEK293 cells 24 h after transfection with plasmids encoding YFP-Grp1-PH, Gβ\textsubscript{172}, p110\textsubscript{γ}, p101, and p87\textsuperscript{PIKAP} in the combinations indicated in the figure. The images are representative of at least three independent transfection experiments each. Bars, 10 μm. B, analysis of Akt phosphorylation in HEK293 cells transfected as in A. Whole cell lysates were probed with an anti-phospho-Akt (Ser473) antibody to assess the phosphorylation status of Akt. Probing with anti-Akt antibody was performed to verify equal loading.

shown; for YFP-p101 see Ref. 14). To test whether the interaction between p87\textsuperscript{PIKAP} and Gβγ is still sufficient to drive p110γ activation, we assayed Gβγ interaction based on PI3Kγ activity. The YFP-fused PtdIns 3,4,5-P\textsubscript{3}-binding PH domain of Grp1 (YFP-Grp1-PH) acts as a translocating biosensor for class I PI3K activity in living cells (27). In HEK293 cells transfected with plasmids encoding Gβ\textsubscript{172}, YFP-Grp1-PH was almost exclusively located within the cytosol (Fig. 5A, left panels). Coexpression of wild-type p110γ led to a membrane localization of a minor fraction of YFP-Grp1-PH. An almost quantitative membrane localization pattern was only observed in cells that were additionally cotransfected with either wild-type p101 or p87\textsuperscript{PIKAP}, indicating a strong and sustained activation of PI3Kγ in this context (Fig. 5A, middle panels). Thus, like p101, p87\textsuperscript{PIKAP} functions as an adapter to drive activation of p110γ by Gβγ. The degree of activation appeared to be slightly higher with p101 than with p87\textsuperscript{PIKAP} (Fig. 5), corresponding to the observation that p101 probably has a higher affinity for Gβγ. Gβγ is necessary for a p101- or p87\textsuperscript{PIKAP}-mediated activation, because coexpression of the Gβγ-scapenging C terminus of β-adrenergic receptor kinase (βARK-CT-CFP) reduced the degree of YFP-Grp1-PH membrane association and because omission of Gβγ completely abolished the translocation signal in the presence of p87\textsuperscript{PIKAP} and p110γ (Fig. 5A, right panels; only shown for p87\textsuperscript{PIKAP}). These results could be confirmed by analyzing the phosphorylation state of Akt, which is a primary downstream effector of PI3K signaling. Gβγ-mediated activation of p110γ and subsequent phosphorylation of Akt on Ser\textsuperscript{473} was only observed if either adapter protein p101 or p87\textsuperscript{PIKAP} was present (Fig. 5B).

p87\textsuperscript{PIKAP} Mediates Activation of PI3Kγ Downstream of GPCR Stimulation—To assess the role of p87\textsuperscript{PIKAP} in the activation of PI3Kγ downstream of chemokine receptor stimulation, YFP-Grp1-PH translocation upon treatment with fMLP was monitored in HEK293 cells expressing a reconstituted PI3Kγ signaling cascade consisting of the fMLP receptor, wild-type PI3Kγ subunits, and YFP-Grp1-PH. In agreement with the findings shown in Fig. 5, expression of either p101 or p87\textsuperscript{PIKAP} was required for fMLP-induced PI3Kγ activation (Fig. 6A). Expression of p101 resulted in a slightly more pronounced translocation of YFP-Grp1-PH, which was reminiscent of the results obtained for static overexpression of Gβγ (Fig. 5). The fMLP-induced translocation of YFP-Grp1-PH was disrupted in the absence of p110γ or upon expression of a kinase-deficient p110γ (CFP-p110γ(K833R); Fig. 6B). Additionally, the fMLP-induced PI3Kγ activation mediated by p87\textsuperscript{PIKAP} was significantly reduced upon coexpression of βARK-CT-CFP (Fig. 6B). Thus, we conclude that the observed translocation is due to catalytic activity of p110γ and depends on the release of Gβγ complexes and is mediated by either adapter p101 or p87\textsuperscript{PIKAP}. Essentially the same results were obtained using Akt phosphorylation as an independent read-out system (Fig. 6, C and D).

Expression Pattern of p87\textsuperscript{PIKAP} and p101—To explore in which physiological context p87\textsuperscript{PIKAP} may be important for the activation of PI3Kγ, we examined the expression of p87\textsuperscript{PIKAP} mRNA using Northern blot analysis and semi-quantitative multiplex RT-PCR. The Northern blots containing 2 μg of poly(A\textsuperscript{+}) RNA isolated from tissues of 8–10-week-old mice showed that transcripts of the expected size of 3.2 kb are most prominent in heart, but weaker signals in the other lanes indicate that p87\textsuperscript{PIKAP} is also broadly expressed in a variety of tissues including brain, spleen, lung, liver, kidney, prostate, thyroid, and salivary glands (Fig. 7). Expression in thymus was barely detectable, probably because of the adult age of the mice. In testis, a shorter transcript variant was detected, which is too short to encode a full-length protein and has therefore so far not been characterized further. Additional information can be obtained from expressed sequence tag data bases, which corroborate the results of Northern blots and also extend the expression pattern by bone marrow (see the UniGene entry Mm.234573).

We then went on to assay the relative expression of the PI3Kγ subunits p110γ, p101, and p87\textsuperscript{PIKAP} in heart and various leukocyte species, i.e. tissues that are known to harbor physiologically important PI3Kγ signaling cascades. In multiplex PCRs on reverse-transcribed RNA, fragments of GAPDH, p110γ, p101, and p87\textsuperscript{PIKAP} cDNA were simultaneously amplified and then analyzed by agarose gel electrophoresis. As expected, all cell types assayed showed expression of p110γ, albeit to a varying extent (Fig. 8). In agreement with the Northern blot data, p87\textsuperscript{PIKAP} was highly expressed in heart, where p101 was only marginally present. By contrast, in thymus and spleen, expression of p101 was more
abundant than that of p87PIKAP, which was also barely detectable in thymus on the Northern blot (Fig. 7). However, examination of leukocyte subpopulations revealed that p87PIKAP and p101 are differentially expressed in leukocyte subpopulations. Although B and T cells feature p101 as the only p110γ regulatory subunit, p87PIKAP is clearly expressed, along with p101, in macrophages, neutrophils, and DCs.

To quantify the relative expression of p101 and p87PIKAP, competitive PCR was performed. The amount of amplified cDNA fragments was compared with the amount of product obtained from an internal standard template of known copy number that has identical sequence except for an ~40-bp deletion with respect to the native cDNA fragments. By varying the copy number of internal standard, conditions can be found where amplification for the cDNA fragment and the internal standard are equally efficient. In such reactions, the number of cDNA fragments equals the number of internal standard molecules initially introduced as template. Such sets of PCRs were generated for heart, neutrophils, and CD11b+ DC, which all contain different ratios of p101 and p87PIKAP. Based on these assays, p87PIKAP mRNA is expressed in heart at an about 5-fold higher level than p101 (about 21,500 and 4,400 copies of p87PIKAP and p101 mRNA, respectively, per 100 ng of total RNA; Fig. 8B). In neutrophils, less p87PIKAP than p101 mRNA was detectable (37,900 and 107,000 copies, respectively, per ng of poly(A+)-containing RNA). An even higher excess of p101 mRNA was detected in CD11b+ DC (about 30,800 and 120,000 copies of p87PIKAP and p101, respectively, per 100 ng of total RNA).
Equal amounts of reverse-transcribed RNA were used as template in each PCR, whereas FLAG immunoprecipitates (Fig. 9, the presence of p110 in the immunoprecipitates (Fig. 9) resulted in very faint to undetectable signals of CFP-tagged proteins in RIPA buffer (data not shown). To control for cell lysis artifacts, cells obtained with a different protocol for lysis and co-IP that employs a panel of each PCR set for p87PIKAP and p101 contains 10^6 copies of internal standard but no RT. The last reaction does not activate PDE3B-FLAG, thus explaining the reduced PDE3B activity in these samples (see blot in Fig. 10). Furthermore, no change in PDE3B activity was observed if cell lysates of PDE3B-FLAG-expressing cells were assayed for PDE activity in the presence of 100 nM recombinant hexahistidine-tagged p87PIKAP affinity-purified from baculovirus-infected Sf21 cells (Fig. 10, right panel). We therefore conclude that the interaction of p87PIKAP or of a p87PIKAP/p110y dimer with PDE3B is not sufficient for modulating PDE3B activity.

**DISCUSSION**

Here we report the cloning and characterization of a novel p101 homologue. We previously suggested its existence and possible functional relevance based on its amino acid similarity to the N- and C-terminal functional domains of p101 (15). Based on a similar notion, Suije et al. (16) also identified and initially characterized this novel PI3K regulatory subunit and termed it p84. Because we previously presented data on and submitted sequence data pertaining to this regulatory subunit, we used our previously introduced name p87PIKAP within this publication (Ref. 17; see also GenBank™ entry AT753194).

We could confirm that p87PIKAP interacts with p110y and extend knowledge about this interaction using co-IP and FRET assays. Both p101 and p87PIKAP bind to the same surface or to at least overlapping binding surfaces on p110y because their binding is mutually exclusive. The results of the FRET measurements on N- or C-terminally tagged p87PIKAP and p110y constructs indicate that the complex between p87PIKAP and p110y probably resembles that of p101 and p110y in that both N termini and both C termini are closer to each other than to the opposite termini (14). Based on competitive FRET and co-IP assays, the affinity of both p101 and p87PIKAP for p110y is in a similar range and perhaps slightly higher in the case of p87PIKAP. In contrast to p101, p87PIKAP is lacking nuclear localization signals and remains mostly within the cytosol also in the absence of p110y. Furthermore, in the absence of p110y, p87PIKAP was found to be more stable than p101.

Neutrophil lysates from p110y knockout mice, however, showed strong reductions in protein levels for both p101 and p87PIKAP (16). The difference between these findings probably results from additional or cell type-specific regulatory effects on the mRNA or protein level within the native context. Moreover, p87PIKAP may have a somewhat extended half-life compared with p101, which is visible 48 h after transfection.
but negligible compared with an additional long term stabilization by p110γ. We further observed that expression of monomeric p101 results in reduced expression of cotransfected cDNAs (for example of PDE3B-FLAG in Figs. 9 and 10, or of free CFP (data not shown)). A nuclear function of monomeric p101 in regulating the expression of other proteins may be supported by yeast two-hybrid data showing the interaction of p101 with transcriptional regulators (Alliance for Cellular Signaling; www.signaling-gateway.org/).

Although overexpression of Gβγ did not result in a marked plasma membrane localization of p87PIKAP, its interaction with Gβγ could nevertheless be revealed via Gβγ stimulation of p110γ activity in the presence of p87PIKAP. These findings may extend the in vitro data of Suire et al. (16) to a context of living cells; in vitro lipid kinase assays showed 4-fold stronger stimulation by Gβγ for the p101/p110γ than for the p87PIKAP/p110γ heterodimer. In accordance with this finding, we could demonstrate that, in living cells, receptor-mediated activation of p87PIKAP/p110γ heterodimer results in a less pronounced translocation of YFP-Grp1-PH, which may be explained by the lower affinity of p87PIKAP for Gβγ.

The relative level of expression of p87PIKAP and p101 in subtypes of leukocytes differs between cell types. The presence of p87PIKAP in macrophages, neutrophils, and DCs may render their PI3Kγ signaling different from that in B and T cells, from which p87PIKAP is virtually absent. Both regulatory subunits may differ in specificity for subtypes of the upstream activator Gβγ. A systematic screen for activation by various Gβγ dimers performed on the p101/p110γ heterodimer revealed that the Gβγ11 dimer, although highly expressed in tissues containing p110γ, is ineffective in stimulating the p101/p110γ heterodimer (28). A similar screen on the p87PIKAP/p110γ heterodimer may be helpful to settle this question of different Gβγ specificities. Moreover, the slower

FIGURE 9. p87PIKAP interacts with PDE3B. HEK293 cells were transfected with plasmids encoding the indicated proteins. The cells were lysed, and FLAG-tagged proteins were precipitated with an anti-FLAG antibody. The recovery of FLAG-tagged protein was tested by probing with an anti-FLAG antibody, and copurification of CFP-tagged protein was analyzed with an anti-GFP antibody (IP). Aliquots of cell lysates used for IP were probed with anti-FLAG or anti-GFP antibodies to assay for expression of FLAG- and CFP-tagged proteins (load). Left panels, IP of PDE3B-FLAG; middle panels, IP of p87PIKAP-FLAG; right panels, IP of TRPV1-FLAG employed as a negative control. Prominent bands of copurified protein can be observed for p87PIKAP in the PDE3B-FLAG immunoprecipitate as well as for CFP-PDE3B in the p87PIKAP-FLAG immunoprecipitate. The experiments shown are representative of three each. IB, immunoblot.

FIGURE 10. Effect of PI3Kγ subunits on PDE3B activity. Left panel, HEK293 cells were transfected with plasmids encoding FLAG-tagged versions of the indicated proteins (0.2 µg of PDE3B-encoding plasmid and a total of 1.8 µg of plasmid cDNA encoding the indicated PI3Kγ subunits). The cells were lysed, and PDE activity was determined in the presence and absence of 10 µM cilostamide to assess PDE3 activity. To maintain comparability between assays from different transfection experiments, PDE3B activities (in pmol/min/mg protein) were normalized to the activity in lysates of cells transfected with only PDE3B-FLAG. The means and S.E. of three independent transfection experiments are given. The amount of recombinant PDE3B-FLAG in cell lysates was analyzed by immunoblotting (IB) with anti-FLAG antibody. A blot from a representative experiment is shown. Right panel, lysates of HEK293 cells transfected with a plasmid encoding PDE3B-FLAG were incubated with 100 nM purified recombinant p87PIKAP in either native (nat.) or denatured (boiled for 5 min, denat.) state as a control. The means and S.E. of two independent experiments are given.
accumulation of PtdIns 3,4,5-P_3 observed in cells expressing the 
$p87^{\mathrm{PIKAP}}/\mathrm{p}110\gamma$ heterodimer instead of the $p101/p110\gamma$ heterodimer (see above) may result in different kinetics of P13K\gamma signaling events in 
cells expressing both $p87^{\mathrm{PIKAP}}$ and $p101$.

Based on data showing an indirect interaction of $p110\gamma$ with PDE3B 
that regulates PDE3B activity in heart, a PDE3B-regulating multi-protein 
complex, which is disrupted upon genetic ablation of $p110\gamma$, has 
been postulated (11). According to our Northern blot and RT-PCR data, 
$p87^{\mathrm{PIKAP}}$ is highly expressed in the heart. Because the expression of 
$p87^{\mathrm{PIKAP}}$ and $p101$ is strongly reduced in $p110\gamma$ knockout mice (16), the absence of $p87^{\mathrm{PIKAP}}$ in hearts of $p110\gamma$ knockout mice may explain why 
recombinant $p110\gamma$ is unable to reconstitute regulation of PDE3B 
immunoprecipitated from hearts of $p110\gamma$ knockout mice. Therefore, we 
asked whether $p87^{\mathrm{PIKAP}}$ is a component of the $p110\gamma$-containing 
complex regulating PDE3B activity in the heart. We could show that 
$p87^{\mathrm{PIKAP}}$ and also $p101$ interact with PDE3B.

To test whether the regulation of PDE3B observed in heart can be 
reconstituted by $p87^{\mathrm{PIKAP}}$ in vitro, we assayed PDE3B activity in 
the presence of $p87^{\mathrm{PIKAP}}$, $p110\gamma$, or the $p87^{\mathrm{PIKAP}}/p110\gamma$ heterodimer. However, for both monomeric $p87^{\mathrm{PIKAP}}$ and the $p87^{\mathrm{PIKAP}}/p110\gamma$ het-
erodimer, the effects on PDE3B activity were not observed in vitro. 
Because $p87^{\mathrm{PIKAP}}$ interacts with PDE3B, it may be an essential part of 
a PDE3B-regulating protein complex in the heart, although the lack of 
effects on PDE3B activity indicates that additional proteins are required. Gene 
knockdown and knockout studies on regulatory subunits of P13K\gamma will be 
necessary to obtain further knowledge on the role of $p87^{\mathrm{PIKAP}}$ in 
cardiac P13K\gamma signaling and to reveal the relative contributions of 
$p87^{\mathrm{PIKAP}}$ and $p101$ to P13K\gamma signaling.

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