Assigning Functional Domains within the p101 Regulatory Subunit of Phosphoinositide 3-Kinase \(\gamma^p\)^{\#}

Phosphoinositide 3-Kinase (PI3K) \(\gamma\) is a lipid kinase that is regulated by G-protein-coupled receptors. It plays a crucial role in inflammatory and allergic processes. Activation of PI3K\(\gamma\) is primarily mediated by G\(\beta\gamma\) subunits. The regulatory p101 subunit of PI3K binds to G\(\beta\gamma\) and, thereby, recruits the catalytic p110\(\gamma\) subunit to the plasma membrane. Despite its crucial role in the activation of PI3K\(\gamma\), the structural organization of p101 is still largely elusive. Employing fluorescence resonance energy transfer (FRET) and coimmunoprecipitation (co-IP) studies, we show here that distinct regions within the p101 primary structure are responsible for interaction with p110\(\gamma\) and G\(\beta\gamma\). The p110\(\gamma\) binding site is confined to the N terminus, whereas binding to G\(\beta\gamma\) is mediated by a C-terminal domain of p101. These domains appear to be highly conserved among various species ranging from Xenopus to men. In addition to establishing a domain structure for p101, our results point to the existence of a previously unknown, p101-related regulatory subunit for PI3K\(\gamma\).

Phosphoinositide 3-kinases (PI3Ks) control a vast array of cellular processes through the generation of lipid second messengers that recruit proteins containing binding domains for 3-phosphorylated phosphatidylinositol (PtdIns) lipids to cellular membranes. The receptor-activated class I PI3K regulate cellular functions such as differentiation, survival, proliferation, and chemotaxis (1, 2). At the plasma membrane, they phosphorylate PtdIns-4,5-P2 to PtdIns-3,4,5-P3, which is recognized by a subclass of PH domains (3). Proteins featuring such PH domains include Akt/protein kinase B and phosphoinositide-dependent kinase-1, which initiate the major signaling pathways downstream of class I PI3K, accounting for most known cellular effects of class I PI3K activation. Class II and III PI3K are involved in membrane homeostasis and trafficking events within the endomembrane system, presumably in a constitutive manner (4). Effectors of class II and III PI3K are proteins containing FYVE and PX domains, binding PtdIns-3-P and PtdIns-3,4,5-P3, respectively (5, 6).

The receptor-regulated class I PI3K are heterodimeric enzymes, consisting of a 110-kDa catalytic subunit and a regulatory subunit. The p110\(\alpha\), -\(\beta\), and -\(\delta\) isoforms bind to regulatory subunits of the p85/p55 family and are referred to as class IA PI3K. The p85/p55 regulatory subunits consist of modular interaction domains (such as SH2 and SH3) and mediate the activation of these PI3K downstream of receptor tyrosine kinases by membrane translocation and des inhibition of the bound catalytic subunit (7, 8). The p110\(\gamma\) isoform tightly binds to the p101 regulatory subunit, which is unrelated to the p85/p55 subunits as well as to other known proteins (9). In contrast to class IA PI3K, PI3K\(\gamma\) is primarily regulated by G-protein-coupled receptors (GPCR) and grouped separately into class IB (10, 11). According to its prominent expression in leukocytes, PI3K\(\gamma\) has been implicated in processes such as leukocyte chemotaxis, mast cell degranulation, and oxidative burst, rendering it a crucial regulator of allergic and inflammatory processes (12). Characterization of p110\(\gamma\) knock-out mice (13–16) fostered this role while additionally revealing PI3K\(\gamma\) to be a negative regulator of myocardial contractility, giving rise to a function for PI3K\(\gamma\) in the cardiovascular system (17).

Activation of PI3K\(\gamma\) through GPCR is mediated by G\(\beta\gamma\) subunits. Both subunits of PI3K\(\gamma\) have been shown to bind G\(\beta\gamma\) in vitro, albeit p101 does so with at least 5-fold higher affinity (9). On the other hand, lipid kinase activity of p110\(\gamma\) can be stimulated by G\(\beta\gamma\) in the absence of p101 (18). Therefore, the role of the p101 subunit in the activation by G\(\beta\gamma\) was controversial. However, we could recently show that, in heterologous expression systems, the p101 subunit is essential for G\(\beta\gamma\)-mediated activation of PI3K\(\gamma\) in living cells. p101 binds to G\(\beta\gamma\) released from heterotrimeric G proteins and, thereby, recruits the bound p110\(\gamma\) catalytic subunit to the plasma membrane, which is a prerequisite for GPCR-induced PI3K\(\gamma\) activity in living cells (19).

In contrast to the well established domain structure of the p85/p55 class IA regulatory subunits and despite its important role in the activation of PI3K\(\gamma\), the structural organization of p101 still remains largely elusive. Therefore, we asked whether a domain structure analogous to the p85/p55 subunits can be established for p101. We generated p101 deletion mutants and assayed their ability to bind to p110\(\gamma\) and G\(\beta\gamma\) in living cells by recruitment assays as well as fluorescence resonance energy transfer (FRET) and coimmunoprecipitation (co-IP) studies.

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\^ The on-line version of this article (available at http://www.jbc.org) contains supplemental Table 1 and supplemental Figs. 1 and 2.

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1. The abbreviations used are: PI3K, phosphoinositide 3-kinase; aa, amino acid(s); co-IP, coimmunoprecipitation; fMLP, formyl-methionyl-leucyl-phenylalanine; FRET, fluorescence resonance energy transfer; GPCR, G-protein-coupled receptor; GRP1, general receptor for phosphoinositides-1; HEK, human embryonic kidney; PH, pleckstrin homology; SH, Src homology; PtdIns, phosphatidylinositol; GFP, green fluorescent protein; CFP, cyan fluorescent protein; YFP, yellow fluorescent protein; EGF, epidermal growth factor; EST, expressed sequence tag.
We show here that distinct regions within the p101 primary structure are responsible for interaction with p110 and Gβγ.

**EXPERIMENTAL PROCEDURES**

**Generation of Expression Plasmids—**cDNAs coding for deletion mutants of p101 were generated by PCR (PCR system Expand HF; Roche Applied Science) and subcloned into pcDNA3.1/V5/HIS/TOPO (Invitrogen). Porcine p101 cDNA was used as template. Primers used for the generation of p101 deletion mutants are listed in Table 1 of the supplemental material. These primers introduce optimized ribosomal binding sites, appropriate stop codons, and, in some cases, restriction sites to allow for the fusion of deletion mutants to CFP. All PCR fragments were verified by sequencing (DYEnamic ET kit, Amersham Biosciences; ABI-377, PerkinElmer Life Sciences). N- or C-terminal fusion to CFP was achieved by subcloning into the custom-made vectors pcDNA3-NCFP or pcDNA3-CFP (20), respectively. pcDNA3-NCFP was generated by subcloning the open reading frame of CFP into the HindIII site of pcDNA3 via BsmBI and HindIII.

p110-FLAG was generated by subcloning of human p110 cDNA via HindIII and XbaI into a custom-made pcDNA3 vector encoding the acid sequence DYKDDDDK downstream of the XbaI site. YFP-GRP1 was excised from the plasmid encoding GFP-GRP1-PH (21) via HincII and BsrGI into pcDNA3 (BD Biosciences). Construction of expression plasmids coding for p101, CFP-p101, p110-FLAG, YFP-p110, p110-FLAG, Gβγ, and the human MLP receptor has been described elsewhere (19).

**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 (MEM) supplemented with 10% FCS, 2 mM glutamine, 100 μg/ml streptomycin, and 100 units/ml penicillin.

**Immunoprecipitation and Immunoblot—**Immunoprecipitation experiments were performed 24–48 h after transfection 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.

**RESULTS**

**Characterization of p101 Deletion Mutants—**To establish a basic functional map of p101, a series of deletion mutants was generated and fused to CFP (Fig. 1C). Expression of these constructs in HEK293 cells yielded diverse localization patterns (Fig. 1A, upper panels). If expressed without p110, full-length p101 located mainly to the cell nucleus. A similar distribution was observed for CFP-p1011–613 and p101249–877-CFP. A construct comprising amino acids (aa) 249–613 was localized exclusively within the nucleus, whereas expression of CFP-p1011–333 or of p101517–877-CFP resulted in a predominant cytosolic fluorescence with nuclear accumulation without nuclear exclusion (Fig. 1A). Analysis of the p101 primary structure using PSORT II (24) revealed three nuclear localization signals located at positions 400–403, 499–502, and 558–565. Of these, only that at 499–502 is conserved among various species (see supplemental Fig. 1).

Upon coexpression with p110, which localizes to the cytosol in HEK293 cells, CFP-p101 is mostly excluded from the nucleus and localizes to the cytosol, indicating an interaction between the two proteins (19). A similar p110-γ-mediated redistribution was also observed for the C-terminally truncated construct CFP-p1011–613—whereas the nuclear accumulation of p101249–877-CFP and CFP-p101249–613 remained unchanged in the presence of p110 (Fig. 1A, upper and middle panels). These data indicate that the N terminus of p101 is crucial for interaction of p101 with p110, whereas the C terminus and middle parts of p101 appear to be not sufficient to bind to p110·γ.

To further validate these findings and to assess the immediate proximity between p101 deletion mutants and p110 more directly, FRET experiments were performed (Fig. 1B). As shown previously, FRET efficiencies of about 9% are measured between coexpressed CFP- and YFP-tagged p101 and p110, provided that the fluorescent tags are fused to the same termini in both proteins (19). Comparable FRET efficiencies were determined between YFP-p110 and CFP-p101249–877 as well as YFP-p110 and CFP-p1011–333. Deletion of the p101 N terminus (p101249–877-CFP), however, led to a complete loss of binding to p110-YFP, indicated by FRET efficiencies comparable with those determined between free CFP and p110-YFP. Likewise, p101517–877-CFP and CFP-p101249–613 did not exhibit FRET if coexpressed with p110-YFP or YFP-p110. Thus, the N terminus of p101 is both necessary and sufficient to bind p110, whereas C-terminal elements appear to be neither sufficient nor necessary for this interaction.

Coexpression of CFP-p101 and Gαiiγ, led to a redistribution of CFP-p101 from the nucleus to the cytosol and plasma membrane (Fig. 1A, upper and lower panels), indicative of an interaction between mainly membrane-localized Gβγ complexes and CFP-p101 (19). This redistribution was also observed for
deletion mutants containing the C terminus of p101, i.e. p101249–877-CFP and p101517–877-CFP. All constructs with C-terminal deletions lacked noticeable differences in their subcellular distribution upon coexpression with Gα1 or Gγ2. These data point to a crucial role of the p101 C terminus in the interaction with Gα1/Gγ2.

Accordingly, both N and C termini of p101 were necessary for activation of PI3Kγ signaling in an heterologous system (Fig. 2). In HEK293 cells expressing the fMLP receptor, p110γ, full-length p101 and the YFP-fused PtdIns-3,4,5-P3-binding PH domain of GRP1 (21), a pronounced translocation of YFP-GRP1-PH was observed upon stimulation with 1 μM fMLP (Fig. 2, upper panels). This translocation was completely abrogated if p101249–877 was expressed instead of full-length p101 (Fig. 2, middle panels), presumably because p110γ was unable to bind to the truncated p101 lacking the N terminus of full-length p101 (see Fig. 1, A and B) and was therefore not co-translocated to the plasma membrane. Likewise, translocation of YFP-GRP1-PH upon fMLP stimulation was abolished if p1011–613 was expressed instead of full-length p101 (Fig. 2, lower panels), presumably because the truncated p101 lacking the C terminus complexed with p110γ but failed to bind to Gγ2 (see Fig. 1A), which is a prerequisite to membrane translocation of the PI3Kγ complex.

The p110γ Binding Site of p101—Constructs spanning different areas of the p101 N terminus were generated to define the borders of the p110γ interaction site in steps of about 25 aa (Fig. 3C). Upon fusion to CFP and coexpression with YFP,
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p101, these constructs were used for a FRET-based monitoring of the protein-protein interaction. Since the protein fragments were tagged and sequentially shortened at opposite termini, we expect similar FRET efficiencies until the binding interface is affected. With the exception of CFP-p1011-200, which showed only a minor but statistically significant reduction of FRET efficiencies, C-terminal deletions up to aa 175 did not result in significant reduction of FRET efficiencies between YFP-p110γ and CFP-fused p101 deletion mutants as compared with CFP-p101 (Fig. 3A). A significant decrease in FRET efficiencies was observed for CFP-p1011-150 and CFP-p1011-131, and the value obtained for YFP-p110γ and CFP-p1011-100 was close to that of free CFP and YFP-p110γ, indicating a loss of interaction. A construct lacking the 25 N-terminal aa (p10135-333-CFP) still yielded FRET with YFP-p110γ, whereas CFP-p10150-333 and CFP-p10175-333 did not exhibit FRET upon coexpression with YFP-p110γ.

To corroborate these results based on a novel application of FRET to map protein interaction domains in the context of living cells, co-IP experiments were performed. In agreement with the FRET-based approach, CFP-p101 and CFP-tagged p101 deletion mutants with C-terminal deletions up to aa 131 coimmunoprecipitated with p110γ-FLAG from lysates of transfected HEK293 cells (Fig. 3B). CFP-p1011-100 and deletion mutants lacking more than 25 N-terminal aa could not be detected in the respective immunoprecipitates. Likewise, no binding was detected for the N-terminally truncated p101149-877-CFP (see above) as well as for a CFP-YFP-fusion protein used as a control for unspecific binding of CFP. Thus, the minimum binding site on p101 for p110γ consists of aa 25–131. However, a FRET efficiency comparable with that of full-length p101 requires at least a common portion comprising aa 25–175 of p101.

To control for a possible generation of an artificial binding site due to truncation of p101 or fusion with the fluorescent protein, FRET-based competition assays were performed. p101-CFP was displaced from binding to p110γ-YFP when coexpressed with the same amount or a 4-fold excess of non-fluorescent wild-type p101, resulting in a reduction of the FRET efficiency (Fig. 3D, left panel). A displacement by non-fluorescent wild-type p101 was also observed for FRET between CFP-p1011-131 and YFP-p110γ (Fig. 3D, right panel). The reduction in FRET efficiency brought about by coexpression of untagged p101 is comparable for p101-CFP and CFP-p1011-131, indicating that both proteins can be similarly displaced by wild-type p101 from a common binding site on p110γ.

The Gβγ Binding Site of p101—Based on the finding that a C-terminal fragment of p101 comprising aa 517–877 was sufficient to bind to Gβγ (see Fig. 1C), further constructs were generated to map the Gβγ binding site of p101 within these boundaries (Fig. 4A). Upon coexpression with Gβγ, p101430-877-CFP was recruited to the plasma membrane, whereas CFP-p101517-711 was not (Fig. 4B). If p101430-877 was further shortened in steps of ~25 aa, membrane recruitment upon coexpression with Gβγ was retained in p101450-877-CFP and CFP-p101430-850 but was lost in p101450-877-CFP, p101700-877-CFP, CFP-p101620-825, and CFP-p101620-800 (Fig. 4C and data not shown for CFP-p101700-877 and CFP-p101620-800). Taken together, binding to Gβγ was retained in p101 containing a common portion comprising aa 650–850 of p101 (Fig. 4D), whereas further deletions completely abolished binding to Gβγ. Indeed, the CFP-fused binding domain (CFP-p101450-850) was expressed as a soluble protein in the cytosol but recruited to the plasma membrane upon coexpression with Gβγ. Thus, the Gβγ binding domain of p101 is confined to aa 650–850. Since CFP-p10150-850 displaced a coexpressed full-length p101 from binding to Gβγ (Fig. 4E), CFP-p101450-850 and full-length p101 utilize the same binding site on Gβγ. Interestingly, the efficient competition and the more pro-
nounced membrane localization of CFP-p101650–850 point to an even higher G\textsubscript{\textgamma}\textsubscript{2} binding affinity of the isolated domain compared with full-length p101. We conclude that the G\textsubscript{\textgamma}\textsubscript{2} binding site on p101 is relatively conserved among species, but the N-terminal aa 25–175 of porcine p101 mediate binding to G\textsubscript{\textgamma}. Functional Domains of p101 Are Conserved among Various Species and a Putative p101 Homologue—Taken together, the N-terminal aa 25–175 of porcine p101 mediate binding to G\textsubscript{\textgamma}y (thus termed heterodimerization domain), whereas aa 650–850 within the C terminus are responsible for binding to G\textsubscript{\textgamma}y (thus termed G\textsubscript{\textgamma}y binding domain; Fig. 6A). Comparison of the primary sequence of porcine p101 with those of various species ranging from Xenopus to men corroborates the results derived from the experimental data. Depending on the phylogenetic distance of the species compared, the overall pairwise similarities between these sequences range from 40–99%. Based on the alignments, one may estimate the borders of the interaction domains. For the heterodimerization domain, aa 33 marks the beginning of a sequence stretch showing no clusters of non-similar aa and no gaps until aa 183 (supplemental Fig. 1). Likewise, aa 650–841 constitute a conserved cluster and may, thus, mark the borders of the G\textsubscript{\textgamma}y binding domain. A slightly higher degree of conservation is observed within the

CFP-p101650–850 (Fig. 5C), which presumably competes with PLC\beta\textsubscript{2} for released G\textsubscript{\textgamma}y.

**Functional Domains of p101 Are Conserved among Various Species and a Putative p101 Homologue**—Taken together, the N-terminal aa 25–175 of porcine p101 mediate binding to G\textsubscript{\textgamma}y (thus termed heterodimerization domain), whereas aa 650–850 within the C terminus are responsible for binding to G\textsubscript{\textgamma}y (thus termed G\textsubscript{\textgamma}y binding domain; Fig. 6A). Comparison of the primary sequence of porcine p101 with those of various species ranging from Xenopus to men corroborates the results derived from the experimental data. Depending on the phylogenetic distance of the species compared, the overall pairwise similarities between these sequences range from 40–99%. Based on the alignments, one may estimate the borders of the interaction domains. For the heterodimerization domain, aa 33 marks the beginning of a sequence stretch showing no clusters of non-similar aa and no gaps until aa 183 (supplemental Fig. 1). Likewise, aa 650–841 constitute a conserved cluster and may, thus, mark the borders of the G\textsubscript{\textgamma}y binding domain. A slightly higher degree of conservation is observed within the
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**FIG. 6.** Domain structure of p101 and dot-plot comparison with a putative p101 homologue. A, scheme representing the functional organization of the p101 primary structure derived from the data presented here. B, dot-plot comparison of p101 with a putative homologue. The dot-plot was generated using the MEGALIGN software (DNS-TAR). Parameters were set to a minimum match of 28% over a window of 45 aa. The predicted interaction domains are shaded. Murine p101 (GenBank™ accession number NM_177320) and the predicted protein encoded by GenBank™ accession number BC028998 mRNA were compared. For an alignment see supplemental Fig. 2.

**Discussion**

In this report we show that the p101 subunit of PI3Kγ features a modular architecture typically observed for signalizing proteins. An N-terminal domain mediates heterodimerization with the p110γ subunit, whereas binding to Gβγ complexes is achieved through a C-terminal interaction domain. Both interaction domains are required for the GPCR-induced activation of PI3Kγ, since deletion of either interaction domain led to complete abrogation of PI3Kγ activation. So far, it has been assumed that p101 lacks such a domain structure, mainly because of previous findings implying large areas of p101 as being responsible for the interaction with p110γ (25). In contrast to our data, all truncated versions of p101 could be coimmunoprecipitated with p110γ to some extent in that study (25). Furthermore, lipid kinase activities of coimmunoprecipitated heterodimers of truncated p101 and p110γ were found to depend on the presence of both N- and C-terminal parts of p101 (25), which was interpreted in terms of disruption of interaction sites on p101 for p110γ and not for Gβγ. Alternatively, it was considered that binding sites on p101 for p110γ and Gβγ are simultaneously disrupted by deletions in p101. However, from the data presented here it becomes clear that both interactions are largely independent of each other.

The various methods employed here allowed a separate mapping of the binding sites involved and showed conclusively the existence of discrete and independent binding sites on p101 for both p110γ and Gβγ in living cells. The mapping procedure presented here for the heterodimerization domain of p101 is, to our knowledge, the first application of FRET in the mapping of an interaction domain. Being suitable for measurements in living cells, it should prove valuable also for the characterization of other protein-protein interaction domains within their native context.

For the Gβγ binding domain, an abrupt loss of binding to Gβγ was observed upon deletion of residues pertaining to this domain. Thus, the whole domain appears to be necessary for binding to Gβγ, i.e. no elements exist within this domain which are only of accessory nature. The importance of certain residues at the domain periphery may be either due to a domain fold depending on certain elements at the domain borders or due to a bipartite sequence motif mediating binding to Gβγ. Structural analysis may resolve these issues in future work. A similar behavior was observed at the N-terminal border of the heterodimerization domain, where binding to p110γ was completely lost upon deletion of the N-terminal 50 aa. At the C-terminal border, however, a gradual decrease in FRET efficiencies from values comparable with that measured with the full-length p101 protein down to negligible values was observed within a range of ~75 aa. On the one hand, this may be due to complex properties of the CFP fusion proteins. To establish an orientation of the fluorescent proteins similar to that in the complex of CFP-p101 and YFP-p110γ, certain structural elements next to the p110γ binding domain itself may be necessary in the p101 deletion mutants, although they do not contribute to the binding event itself. However, since the peptide fused to CFP remains unchanged within at least 100 aa, one may argue that CFP remains in a comparable orientation and distance to the YFP-fused p110γ for all assayed deletion mutants with C-terminal deletions. In that case, p101 may indeed feature a p110γ binding domain consisting of a core region sufficient for binding flanked by elements that additionally increase affinity for p110γ by contributing to the binding interface.

Comparison of p101 orthologues shows that both interaction domains represent the most highly conserved parts of the protein within a broad range of species, pointing to their functional relevance and to a similar mode of PI3Kγ activation among various species. The function of the less conserved intervening sequence, which includes a conserved sequence stretch (aa
500–561), remains elusive. It may function as a scaffold for the interaction domains or may perhaps be involved in the interaction with other, not yet identified interaction partners. A homologue to p101 may exist, whose identity we plan to investigate. It features clusters of conserved residues within the interaction domains, indicating a conservation of function similar to that observed for the p101 orthologues. It may play a role in tissues reported to express p110γ and to perform Gβγ-dependent PI3K signaling events but with no reports of p101 expression and function. Both domains may represent novel protein-protein interaction modules. However, except for the expression and function. Both domains may represent novel PI3K signaling events but with no reports of p101 analogy.

The data obtained here improve our understanding of the role of p101 in PI3K signaling by gaining insight into the architecture and the structure-function relationship of this protein. In addition, knowledge about the interaction domains of p101 may help to generate novel targets for an isotype-specific expression of PI3K, based on interference with protein-protein interactions rather than on inhibition of lipid kinase activity, which is hampered by the difficulties of generating specific ATP effectors may be identified.

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