Design and purification of active truncated phosphoinositide 3-kinase gamma protein constructs for structural studies

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Abbreviations and symbols

PI3K	phosphoinositide 3-kinase
PtdIns(4,5) P_2	phosphatidylinositol (4,5)-bisphosphate
PtdIns(3,4,5) P_3	phosphatidylinositol (3,4,5)-triphosphate
HDX-MS	Hydrogen/Deuterium exchange – mass spectrometry

Keywords

phosphoinositide 3-kinase, PI3K, signaling, purification, baculovirus, Sf9, HDX-MS, crystallization

Abstract

Phosphoinositide 3-kinase gamma (PI3K γ) is a lipid kinase that plays a crucial role in cell migration, chemotaxis, oxidative burst and myocardial contractility. It is activated downstream of G protein-coupled receptors (GPCRs) and small GTPases of Ras superfamily. PI3K γ is a heterodimer composed of a catalytic and a regulatory subunit that is expressed mostly in hematopoietic cells and in the heart. Although it has attracted a lot of attention because of its link with tumor inflammation and heart diseases, its regulation is still not fully understood. This can be attributed to the absence of high-resolution structural details of the PI3K γ heterodimer. Here we describe the design and purification of PI3K γ constructs where flexible loops in the regulatory subunit have been removed based on structural information obtained by hydrogen/deuterium exchange – mass spectrometry (HDX-MS). The soluble constructs retain both basal activity and sensitivity to GPCR stimulation, and are thus an optimal tool to further explore their regulation using a structure-based approach.

Introduction

Phosphoinositide 3-kinase gamma (PI3K γ) is a lipid kinase and a signal transducer enzyme. Upon activation by G-protein coupled receptors (GPCRs) and small GTPases of Ras superfamily, PI3K γ phosphorylates plasma membrane lipid phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5) P_2) to form phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5) P_3), a lipid second messenger molecule. PtdIns(3,4,5) P_3 recruits many downstream effector proteins, such as Ser/Thr kinase AKT, *via* binding of its pleckstrin homology (PH) domain, initiating PI3K/Akt/mTOR signaling cascade [1]. The PI3K γ /Akt/mTOR pathway regulates cell migration, oxidative burst,

resolution of inflammation, myocardial contractility and its deregulation is implicated in cancers, cardiovascular disease, chronic inflammation, allergy and autoimmune diseases [2-4]. PI3K γ -deficient mice are viable, fertile and with a normal life span but show impaired leukocyte recruitment [5] and myocardial contractility [6]. In line with its role in inflammation and immune surveillance, PI3K γ shows highest expression levels in myeloid cells [1, 7]. Recently, PI3K γ blockade was shown to be a valuable strategy to overcome tumor-mediated immunosuppression, promoting tumor regression without targeting cancer cells directly [8, 9]. Besides its catalytic function, cardiac PI3K γ has a kinase-independent role in modulating cardiac contractility [6, 10].

Because of its vast implication in diseases, PI3K γ has attracted ample attention from academia and industries, and there are currently several ATP-competitive drugs targeting this enzyme in clinical trials [11]. However, the regulatory mechanisms that control enzyme activity are still not fully understood, and such information would serve as a basis to develop novel strategies to modulate PI3K γ activity.

PI3K γ is one of the eight PI3K isoforms found in mammals. PI3Ks are divided into three classes based on structural similarity and substrate specificity to: class I (PI3K α , β , γ , δ), class II (PI3K ϵ , ζ) and class III (Vps34). All four class I PI3K isoforms catalyze transfer of ATP γ -phosphate to inositol 3-hydroxyl head group of PtdIns(4,5) P_2 to form PtdIns(3,4,5) P_3 . Despite catalyzing the same reaction, class I PI3Ks have non-redundant cellular functions. These differences relate to their structural differences as well as different expression patterns and spatiotemporal distribution within cells [12]. Class I PI3Ks are heterodimers, formed of catalytic (p110) and regulatory subunits and are further divided into class IA and class IB depending on their regulatory subunit. PI3K γ is the only member of class IB in which catalytic p110 γ subunit

associates with either p84 or p101 regulatory subunit. In contrast, class IA PI3Ks catalytic subunits p110 α , β and δ can associate with one of five p85-like regulatory subunits that mediate activation downstream of receptor tyrosine kinases [12]. The molecular details that determine functional specificity for the different class I PI3K isoforms are not yet fully understood.

The catalytic subunit p110 γ is a soluble and active protein *in vitro*, but it requires association with either p84 or p101 for cellular activity [13, 14]. Association of p110 γ with a regulatory subunit stimulates its basal activity and sensitizes the enzyme to G-protein betagamma (G $\beta\gamma$) heterodimer, released by activated GPCR, *via* an unknown mechanism [15-17]. *In vitro*, the regulatory subunits minimally contribute to membrane association, whereas membrane-resident G $\beta\gamma$ increases membrane affinity of p110 γ , especially when in complex with the p101 regulatory subunit [15, 18]. The p84 regulatory subunit is essential for activation by the GTPase Ras, and the molecular details of how Ras stimulates p110 γ are known from a crystal structure of a p110 γ -Ras complex [14, 19]. How both p101 and p84 modulate p110 γ activity is still unclear and whether they contribute to enhanced membrane residence time, allosteric modifications or a combination of both is still a matter of debate.

To elucidate the molecular details of how p110 γ activity is controlled by each of its regulatory subunits and *via* interaction with G $\beta\gamma$ heterodimers downstream of GPCRs, high-resolution structures of the different complexes are greatly needed. These structures would not only shed light on the mechanism of enzyme regulation, but also provide novel options to modulate PI3K γ activity that differ from ATP-competitive inhibitors and thus limit off-target toxic effects.

With the ultimate goal to obtain an active and stable p110 γ /p101 construct amenable to X-ray crystallography, we set out to produce pure and active p110 γ in complex with p101 deletion

mutants. Design of p101 deletion mutants was guided by HDX-MS data of the p110 γ /p101 complex [20]. Using this approach, three pure and homogeneous p110 γ /p101 deletion mutant complexes were produced in insect cells. These deletion mutants all retained basal activity and G $\beta\gamma$ sensitivity, serving as an ideal basis for protein crystallography experiments.

Materials and methods

Cloning of bicistronic vectors encoding p110 γ with p101 mutants

The baculovirus transfer vectors pACEBac1 was modified to express non-tagged human p110 γ followed by insertion of a second cassette containing the polyhedrin promoter, porcine p101 and a polyA tail. The *p101* gene was preceded by a Strep-tag II tag, a His-10 tag and a TEV protease recognition site ('SHT'-tag, MASWSHPQFEKAGHMHHHHHHHHHHHHGSGGSENLYFQG). Insertion of the cassette into pACEBac1 was done using the *Bst*XI restriction site to generate a bicistronic vector [21]. Insertion of a *Kpn*I restriction site after TEV recognition site and of a *Xba*I restriction site on the 3' end of *p101* gene allowed for insertion of truncated *p101* genes initially cloned into monocistronic vectors. Generation of p101 truncations was done by inserting restrictions sites at the site of deletion by PCR. Amino acid boundaries for the p101 deletion mutants and the primers used for cloning are listed in Tables 1 and 2. All constructs were verified by sequencing. The pACEBac1 modified bicistronic vectors were then transformed into EMBacY competent cells [21] carrying baculovirus shuttle vector (bacmid) that can recombine with the donor vector by Tn7 transposition to generate recombinant bacmid, which is then used for transfection of Sf9 insect cells.

p110 γ /p101 constructs expression and purification

One litre of Sf9 insect cells at a density of 1.5×10^6 cells/ml were infected with a single virus encoding both p110 γ and p101 genes. Cells were grown for 70 h at 27 °C before harvesting (2000 g for 15 min). Pellets were washed with 50 ml ice cold phosphate-buffered saline supplemented with 100 μ l protease inhibitor cocktail from Sigma-Aldrich (P8340) prior to flash freezing in liquid nitrogen. Pellet from 1 l of Sf9 cell culture (5 g) were thawed and resuspended in 50 ml lysis buffer L1 (50 mM Tris-HCl pH 8.5, 300 mM NaCl, 2 mM β -mercaptoethanol, 100 μ l protease inhibitor cocktail from Sigma-Aldrich, P8340), sonicated for 20 seconds, 20 % cycle, 40 % power (Bandelin, sonoplus) and passed once through a French press (ThermoFisher Scientific) for cell lysis. After centrifugation at 43 000 g for 45 min at 4 °C, supernatant was filtered through a 5 μ m filter (Millex-SV, Merck Millipore) and loaded on a 5-ml prepacked Strep-Tactin superflow high capacity column (IBA GmbH, Germany) pre-equilibrated in a wash buffer (50 mM Tris-HCl, pH 8.5, 150 mM NaCl, 2 mM DTT). Column was washed with 20 column volumes of the wash buffer prior to incubation with 6 ml of the wash buffer containing 3 μ M (100 μ g/ml) of TEV protease for an overnight cleavage at 4 °C. To recover the protein of interest, the column was washed with 15 ml of wash buffer and concentrated to 600 μ l using Amicon-15 concentrators (Merck Millipore) with 100 kDa membrane cut-off. Concentrated protein was loaded on a Superdex 200 10/300 GL (GE Healthcare) column at 4 °C equilibrated with gel filtration buffer (20 mM Hepes-NaOH pH 7.5, 150 mM NaCl and 2 mM DTT). Protein fractions were concentrated to 4-11 mg/ml using Amicon-4 concentrators of 100 kDa membrane cut-off (Merck Millipore). Protein yield for all the constructs was about 1.5 mg of pure protein for 1 l of culture.

Expression and purification of G β ₁ γ ₂ expressed in Sf9 insect cells

Recombinant $G\beta_1\gamma_2$ heterodimer of human N-terminally tagged His₆- $G\beta_1$ and bovine $G\gamma_2$ subunit was done as previously described [20]. Briefly, three litres of Sf9 cells at 1×10^6 cells/ml were infected with 10ml of a bicistronic vector encoding $G\beta_1$ and $G\gamma_2$. After 48h infection, cells were harvested and the pellet resuspended in 30 ml of lysis Buffer L2 (50 mM Hepes pH 8, 100 mM NaCl, 3 mM MgCl₂, 100 mM EDTA, 10 mM β -mercaptoethanol, 10 μ M GDP) supplemented with 200 μ l of protease inhibitor Sigma-Aldrich, P8340. Cells were lysed by passage through French Press and the solution was spun for 10 min at 800 g. The pellet was discarded and the membranes were pelleted by centrifugation for 30 min at 80,000 g, 4°C using a Ti-45 rotor (Beckman). Supernatant was discarded and the membranes were resuspended using a Potter homogenizer in 50 ml buffer B (20 mM Hepes pH 8, 100 mM NaCl, 3 mM MgCl₂, 10 mM β -mercaptoethanol, 1 % Na-cholate. After 1 hour incubation at 4°C, solution was spun at 100,000 g for 1h at 4°C. Pellet was discarded and the supernatant was complemented to a final 15 mM imidazole. Five ml of Ni-Sepharose (IBA) beads were added and incubated under mild agitation for 1 hour at 4 °C. Resin was washed and excess of $G\alpha$ was removed by washing the resin with 20 ml of buffer C (20 mM Hepes pH 8, 100 mM NaCl, 50 mM MgCl₂, 0.9 % Na-cholate, 10 μ M GDP, 30 μ M AlCl₃, 10 mM β -mercaptoethanol. Solubilized $G\beta_1\gamma_2$ was then eluted from the resin with 15 ml of buffer D (20 mM Hepes pH 8, 150 mM imidazole, 50 mM NaCl, 0.8 % Na-cholate, 10 mM β -mercaptoethanol). Protein was concentrated to 0.5 ml using Amicon centrifugal units (10 kDa cutoff) and loaded on a 10/300 Superdex 200 size-exclusion column equilibrated with buffer E (20 mM Hepes pH 8, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.8 % Na-cholate). Pure protein complex had a retention volume of 15.2 ml. Pure fractions were pooled and concentrated to 2.5 mg/ml using Amicon centrifugal units before freezing in liquid nitrogen. Protein yield was about 100 μ g of pure protein for 1 l of culture.

Thermal shift assays

Protein was diluted to 400 nM concentration in gel filtration buffer. SYPRO® Orange (Sigma) dye was diluted 2500-fold in the same buffer. Samples were prepared by mixing 10 µl of protein solution with 10 µl of dye solution in MicroAmp Fast Optical 96-well plate (Applied Biosystems). After 5 min incubation, plate was centrifuged at 500 x g for 1 min and fluorescence emission was monitored at 568 nm (470 nm excitation) on a StepOnePlus RT-PCR instrument (Applied Biosystems) while applying a temperature gradient from 20 °C to 75 °C in 40 min. Melting temperature values (T_m) correspond to the temperature at which derivative of the fluorescence reaches a maxima.

Lipid kinase activity assays

Measurement of both basal and $G\beta\gamma$ -stimulated PI3K γ activity was done using ADP-Glo™ Kinase Assay (Promega) that correlates ATP consumption with luminescence signal. Lipid vesicles were prepared as previously described, with the following composition: 5 % Brain PI(4,5)P2 (PIP2), 20 % DOPS, 45 % DOPE, 15 % DOPC, 10 % cholesterol, 5 % egg-sphingomyelin [20]. Percentages are weight percentages and all lipids are from Avanti. Kinase reactions were done in 384-well low-volume black plates (Corning 3820) at room temperature (RT). For basal activity assays, 5 µl of p110 γ /p101 constructs were pipetted at 2-fold final concentration in 2x kinase buffer (20 mM Hepes pH 7.5, 1 mM EGTA, 0.2 mM EDTA, 5 mM MgCl₂, 150 mM NaCl, 2 mM DTT, 0.1 % BSA). Serial dilutions of PI3K γ constructs ranging from 1000 to 12 nM were prepared. Reactions were initiated by addition of 5 µl of lipid-substrate mix containing lipids at 2 mg/ml and ATP at 200 µM. Kinase activity assays looking at $G\beta\gamma$ sensitivity were done by preparing PI3K γ stock solutions at 100 nM in 2x kinase buffer. Lipid-substrate solutions contained vesicles at 2 mg/ml loaded with lipid-anchored $G\beta\gamma$ at

concentrations ranging from 2000 nM to 0 nM and 200 μ M ATP. Reactions were started by adding 5 μ l of the G $\beta\gamma$ -containing lipid-substrate solution to 5 μ l of PI3K γ stock solution. Concentration of detergent coming from G $\beta\gamma$ was kept constant in all reactions including in absence of G $\beta\gamma$. All reactions were run for 15 min before quenching by transferring 5 μ l of the reaction mix with 5 μ l of ADP-Glo reagent (Promega). After 45 min incubation at RT with shaking, 10 μ l of Kinase Detection Reagent were added to each well and incubated for 30 min at RT with shaking. Luminescence was read using a CLARIOstar plate reader (BMG Labtech). All reactions were performed in triplicate. Statistical analysis was done using unpaired t-test in Prism and p values are indicated in the figure legends.

Results

Design of p101 regulatory subunit truncation constructs

PI3K γ plays essential role in cell migration and invasion, and association of the p110 γ catalytic subunit with the p101 regulatory subunit is required for stimulation downstream of GPCRs [14, 20]. The p110 γ subunit has a multidomain organization similar to other class I PI3Ks [12]. In contrast, p101 only shares sequence similarity with p84 and no high-resolution structural information is available for those proteins. Primary sequence alignment of p101 from various species shows that N- and C-terminal residues (33-183 and 650-841) make conserved clusters, whereas the central region is less conserved [22]. A series of experiments with truncated p101 constructs as well as studies on full-length p110 γ /p101 using hydrogen/deuterium exchange mass spectrometry (HDX-MS) technology identified N-terminal residues 25-175 to be involved in p110 γ binding and C-terminal residues 769-780 and 813-828 to mediate direct contacts with G $\beta\gamma$ (Fig. 1) [20, 22]. HDX-MS is a powerful technology that provides dynamic information on intra-

and inter-molecular protein contacts based on the rate of exchange of amide protons with solvent [23-25]. It is highly used to study protein folding, protein-protein interactions, to map protein-lipid interfaces and to identify allosteric modifications associated with binding events [26-28]. In addition, HDX-MS technology is a valuable tool for rational design of deletion constructs amenable to X-ray crystallography [29, 30]. Study of p101 dynamics by HDX-MS when in complex with p110 γ identified several highly flexible regions (Figure 1.A) [20]. Most of these flexible regions are distant from both the p110 γ or from the G $\beta\gamma$ binding sites and thus represent attractive targets to be removed to increase protein homogeneity. With the aim to generate p110 γ /p101 constructs with increased stability, we selected four flexible regions in p101 and tested the influence of deleting them on protein expression and purification. Region 1 (Δ 1, 165-174) is a short sequence following the putative p110 γ binding site (Fig. 1). Regions 2 (Δ 2, 272-296) and 3 (Δ 3, 352-414) are longer stretches that lie at the center of p101 sequence. Finally, region 4 (Δ 4, 836-877) corresponds to the last 40 residues of p101 and is close to the G $\beta\gamma$ binding site. All combinations of single, double, triple and quadruple deletions constructs were cloned to be tested for their expression in insect cells (Figure 1.B).

Deletion of flexible regions 3 and 4 in p101 yields soluble homogeneous proteins

Heterodimers of p110 γ associated with deletion constructs of p101 were expressed in Sf9 insect cells and purified using Strep-tag affinity chromatography. Of the 16 PI3K γ constructs expressed, only WT p110 γ /p101 and three p110 γ /p101 deletion constructs yielded soluble complexes. Deletion of N-terminal regions 1 or 2 yielded insoluble protein, showing that these flexible loops are necessary for proper protein folding. Interestingly, deletion of regions 3 and region 4, or a combination of both, yielded soluble protein that could be purified to homogeneity (Figure 2.A). Soluble protein obtained after deletion of region 3 that spans 63 residues (352-414) is a

confirmation that large flexible loops can be excised from p101 without affecting protein folding. Size-exclusion chromatography chromatograms showed that WT and truncated p110 γ /p101 constructs eluted at the expected volume forming a symmetrical peak, indicating that they were properly folded. Deletion of loops in p101 induced a shift in the retention volume indicating that loop deletions in p101 reduce the size of the PI3K γ heterodimer (Figure 2.B). These results validate the use of HDX-MS as a tool to identify flexible regions in proteins that can be excised without affecting folding and homogeneity.

Deletions in p101 differentially affect overall protein stability

The removal of intra-molecular regions within a protein can promote rearrangements that lead to either protein stabilization or destabilization, depending on the importance of that loop for the overall three-dimensional structure. To test the influence of truncating p101 on p110 γ /p101 stability, we performed thermal shift assays comparing WT and p110 γ /p101- Δ 3, - Δ 4 and - Δ 3+4 protein deletion constructs [31]. As seen in Figure 3, excision of region 3 does not affect stability, whereas truncation of the last 42 residues of p101 (Δ 4) significantly reduces stability. Seeing no difference in stability between WT and p110 γ /p101- Δ 3 suggests that the large 352-414 loop in p101 is not involved in intra-molecular interactions within p101/p110 γ complex, and that it has no destabilizing effect on the protein. The decrease in T_M observed for both p110 γ /p101- Δ 4 and - Δ 3+4 constructs suggests that the p101 extreme C-terminus (836-877), besides mediating G $\beta\gamma$ interactions (769-780 and 813-828) is also contributing to protein stability [20]. These results identified a 63 amino acid loop (residues 352-414) within p101 that can be excised without affecting PI3K γ thermal stability.

All PI3K γ constructs show kinase activity

To test the effect of p101 deletions on p110 γ /p101 activity, we performed lipid kinase activity assays with substrate vesicles that mimic plasma membrane composition. All soluble PI3K γ constructs retained basal activity, with each of the p101 truncations having distinct effect on activity. The central deletion in p110 γ /p101- Δ 3 showed a slight but significant reduction in basal activity compared to the WT enzyme, whereas the less stable p110 γ /p101- Δ 4 heterodimer showed increased activity (Figure 4.A). Thus, although p101- Δ 3 deletion did not affect stability, this loop is necessary for PI3K γ maximal basal activity. The p101 353-414 loop could contribute to basal enzymatic activity in several ways i) either *via* stabilization of neighboring structure elements, ii) by contributing directly to membrane association or iii) by affecting structural elements involved in p110 γ catalytic cycle. The effect of p101- Δ 3 truncation is dominant over p101- Δ 4, as the p110 γ /p101- Δ 3+4 has an activity similar to the p110 γ /p101- Δ 3 mutant.

In cells, PI3K γ is activated downstream of GPCRs *by* direct interaction with G $\beta\gamma$ heterodimers, released upon receptor activation. Lipid anchored G $\beta\gamma$ directly contact both subunits of PI3K γ , in the linker region between the C2 and helical domain of p110 γ and in the C-terminus of p101 [20]. Activity of isolated p110 γ is stimulated by G $\beta\gamma$, but it requires association with p101 for maximal activation [16]. To test the influence of p101 truncations on PI3K γ stimulation downstream of GPCRs, we performed lipid kinase activity assays in the presence of increasing concentrations of lipid-anchored G $\beta\gamma$. In contrast to basal activity, the central p101- Δ 3 deletion significantly increased G $\beta\gamma$ sensitivity, whereas the C-terminal p101- Δ 4 truncation had the opposite effect (Figure 4.B). The increased G $\beta\gamma$ sensitivity of the p110 γ /p101- Δ 3 mutant compared to WT p110 γ /p101 suggests that the central flexible loop (352-414) in p101 may mask a G $\beta\gamma$ interaction site on PI3K γ . Deletion of that loop would thus facilitate PI3K γ association with G $\beta\gamma$ and likely

increase affinity between the two proteins. On the other hand, the reduced sensitivity of the p110 γ /p101- Δ 4 for G $\beta\gamma$ compared to WT PI3K γ shows that an intact p101 C-terminus is necessary to maintain an optimal conformation of the G $\beta\gamma$ interaction site on p101. The combination of p101- Δ 3 and - Δ 4 truncations in PI3K γ - Δ 3,4 restores G $\beta\gamma$ sensitivity similar to WT PI3K γ , demonstrating that each truncation affects PI3K γ activity *via* independent mechanisms. Altogether, activity assays show that p101 truncations can both increase and decrease basal or G $\beta\gamma$ -stimulated p110 γ /p101 activity.

Conclusion

Here we present the design and biochemical characterization of PI3K γ truncated constructs, including identification of a p101 regulatory subunit deletion mutant that sensitizes the enzyme for stimulation by G protein- $\beta\gamma$ heterodimers downstream of GPCRs. Pharmacological inhibition of PI3K γ combined with immune checkpoint blockade has recently shown great promise in the treatment of cancers, confirming the critical implication of PI3K γ in immune and inflammatory disorders [8, 9]. However, how the activity of PI3K γ in immune cells is controlled and especially what is the contribution of the p101 regulatory subunit in that process remains unclear. The crystal structures of p110 γ bound to ATP-competitive inhibitors or in complex with the GTPase H-Ras made major advances in understanding PI3K γ catalysis and its regulation by Ras [19, 32]. HDX-MS studies also revealed direct contacts of both p110 γ and p101 with G $\beta\gamma$, showing that this interaction increases PI3K γ membrane affinity [20]. To fully understand PI3K γ regulation, high-resolution structures of p110 γ /p101 heterodimer and in complex with membrane-anchored G $\beta\gamma$ is highly desirable. The identification of a homogeneous, stable and active p110 γ /p101 construct will facilitate structural characterization of the p110 γ /p101 complex. In addition,

PI3K γ - Δ 3 construct is a promising target for crystal structure determination of the transient p110 γ /p101-G β γ complex, whose determination will reveal the full details of PI3K γ regulation.

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Tables

Table 1:

No.	Construct name	Amino acid boundaries	Ligated PCR fragments
1	p101Δ1	1-164, 175-877	I, II
2	p101Δ2	1-271, 297-877	III, IV
3	p101Δ3	1-351, 415-877	V, VI
4	p101Δ4	1-835	VII
5	p101Δ1+4	1-164, 175-835	I, VIII
6	p101Δ2+4	1-271, 297-835	III, IX
7	p101Δ3+4	1-351, 415-835	V, X
8	p101Δ1+2	1-164, 175-217, 297-877	I, XI, IX
9	p101Δ2+3	1-271, 297-351, 415-877	III, XIII, X
10	p101Δ1+3	1-164, 175-351, 415-877	I, XII, X
11	p101Δ1+2+4	1-164, 175-271, 297-835	I, XI, IX
12	p101Δ2+3+4	1-271, 297-351, 415-835	III, XIII, X
13	p101Δ1+3+4	1-164, 175-351, 415-835	I, XII, X
14	p101Δ1+2+3	1-164, 175-271, 297-351, 415-877	I, XI, XIII, VI
15	p101Δ1+2+3+4	1-164, 175-271, 297-351, 415-835	I, XI, XIII, X

Table 1. List of *porcine* p101 deletion mutants and corresponding amino acid boundaries.

Table 2:

PCR fragment	p101 fragments	Forward primer	Reverse primer	Enzyme digestion
I	p101(1-164)	TATGggtaccATGCAGCCA GGGGCCAC	CAtGGAAtTCGAGCACC GTGACGGTGGAG	<i>KpnI/ EcoRI</i>
II	p101(175-877)	TGCTGAaTTCCTTGACGT GGCCGACAAG	cgacaagcttgtcgagactgcag	<i>EcoRI/ XbaI</i>
III	p101(1-271)	TATGggtaccATGCAGCCA GGGGCCAC	AGGTcTCgAgGACACCA GGGAAGCCGGC	<i>KpnI/ XhoI</i>
IV	p101(297-877)	TGTCcTcGAgAGCTTCGA CATCCTGCAGGAAATC	cgacaagcttgtcgagactgcag	<i>XhoI/ XbaI</i>
V	p101(1-351)	TATGggtaccATGCAGCCA GGGGCCAC	GTGGGcGGCCGCCGAGC CGG	<i>KpnI/ NotI</i>
VI	p101(415-877)	CTCGGCGGCCgCCGGGC AGAAGTTCAACAGGATC	cgacaagcttgtcgagactgcag	<i>NotI/ XbaI</i>
VII	p101(1-835)	TATGggtaccATGCAGCCA GGGGCCAC	aggctctagactaCTAGCAGG GCGAGACCTCGC	<i>KpnI/ XbaI</i>
VIII	p101(175-835)	TGCTGAaTTCCTTGACGT GGCCGACAAG	aggctctagactaCTAGCAGG GCGAGACCTCGC	<i>EcoRI/ XbaI</i>
IX	p101(297-835)	TGTCcTcGAgAGCTTCGA CATCCTGCAGGAAATC	aggctctagactaCTAGCAGG GCGAGACCTCGC	<i>XhoI/ XbaI</i>
X	p101(415-835)	CTCGGCGGCCgCCGGGC AGAAGTTCAACAGGATC	aggctctagactaCTAGCAGG GCGAGACCTCGC	<i>NotI/ XbaI</i>
XI	p101(174-272)	TGCTGAaTTCCTTGACGT GGCCGACAAG	AGGTcTCgAgGACACCA GGGAAGCCGGC	<i>EcoRI/ XhoI</i>

XII	p101(175-351)	TGTCcTcGAgAGCTTCGA CATCCTGCAGGAAATC	GTGGGcGGCCGCCGAGC CGG	<i>XhoI/ NotI</i>
XIII	p101(297-351)	TGCTGAaTTCCTTGACGT GGCCGACAAG	GTGGGcGGCCGCCGAGC CGG	<i>EcoRI/ NotI</i>

Table 2. PCR primers used for cloning of porcine *p101* deletion mutants.

Figures

Figure 1:

Fig. 1. Design of p101 deletions based on protein dynamics information obtained from HDX-MS

A. Schematic presentation of p101 dynamics based on HDX-MS measurements. Each bar represents a peptide that spans p101 sequence, with its relative deuterium level expressed on the y axis. The coloured band below the graph schematically represents p101 dynamics. Red indicates highly flexible, whereas blue shows rigid regions. No colouring is indicative of absence of protein coverage. Positions of the p101 amino acid deletions are shown as $\Delta 1(165-174)$, $\Delta 2(272-296)$, $\Delta 3(352-414)$ and $\Delta 4(836-877)$. Regions in p101 previously assigned to a specific biological function are shown at the bottom. **B.** Schematic representation of the four regions deleted in p101, with all the protein constructs tested for expression drawn below. Dotted lines indicate deleted regions.

Figure 2:

Fig. 2. Purification and homogeneity of soluble p110 γ /p101 deletion mutants expressed in Sf9 insect cells **A.** Coomassie stained gel showing purity of the wild type p110 γ /p101 (WT) and three soluble p110 γ /p101 deletion mutants: p110 γ /p101- Δ 3 (Δ 3), p110 γ /p101- Δ 4 (Δ 4), p110 γ /p101- Δ 3+4 (Δ 3+ Δ 4) **B.** Superposed size exclusion chromatograms of the WT and p110 γ /p101- Δ 3+4 deletion mutant are shown. Samples were loaded on Superdex 200 10/300 column run with 20 mM Hepes pH 7.5, 150 mM NaCl and 2 mM DTT. Truncation of two regions Δ 3 and Δ 4 of 50 and 40 residues, respectively, increases retention volume by about 0.5 ml.

Figure 3:

Fig. 3. Deletion of central 50 amino acids (p101 $\Delta 3$) does not affect p110 γ /p101 stability, whereas removal of the last 40 amino acids of p101 (p101 $\Delta 4$) reduces thermal stability. Thermal shift assays (thermofluor) were done using Sypro-ORANGE dye, whose fluorescence is sensitive to protein exposure of hydrophobic regions.

Figure 4:

Fig. 4. Effect of p101 truncations on basal and G $\beta\gamma$ -stimulated activity of the p110 γ /p101 heterodimers. *In vitro* lipid kinase activity assays were done using substrate vesicles mimicking plasma membrane composition containing 5 % of PI(4,5)P₂. **A.** Measure of basal activity of three p110 γ /p101 deletion constructs tested at five different PI3K γ concentrations. **B.** Stimulation of p110 γ /p101 mutants (50 nM) by lipid-anchored G-protein $\beta\gamma$ heterodimers (G $\beta\gamma$). Data are represented as fold activation over basal activity for each construct. All experiments were done in triplicate. Measures that are statistically different compared to WT p110 γ /p101 are indicated with stars above or below the value. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.