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PKC β Phosphorylates PI3K γ to Activate It and Release It from GPCR Control

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Abstract

All class I phosphoinositide 3-kinases (PI3Ks) associate tightly with regulatory subunits through interactions that have been thought to be constitutive. PI3K γ is key to the regulation of immune cell responses activated by G protein-coupled receptors (GPCRs). Remarkably we find that PKC β phosphorylates Ser582 in the helical domain of the PI3K γ catalytic subunit p110 γ in response to clustering of the high-affinity IgE receptor (Fc ϵ RI) and/or store-operated Ca²⁺-influx in mast cells. Phosphorylation of p110 γ correlates with the release of the p84 PI3K γ adaptor subunit from the p84-p110 γ complex. Ser582 phospho-mimicking mutants show increased p110 γ activity and a reduced binding to the p84 adaptor subunit. As functional p84-p110 γ is key to GPCR-mediated p110 γ signaling, this suggests that PKC β -mediated p110 γ phosphorylation disconnects PI3K γ from its canonical inputs from trimeric G proteins, and enables p110 γ to operate downstream of Ca²⁺ and PKC β . Hydrogen deuterium exchange mass spectrometry shows that the p84 adaptor subunit interacts with the p110 γ helical domain, and reveals an unexpected mechanism of PI3K γ regulation. Our data show that the interaction of p110 γ with its adaptor subunit is vulnerable to phosphorylation, and outline a novel level of PI3K control.

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Abbreviations: A3AR, A3 adenosine receptor; ADA, adenosine deaminase; BAPTA-AM, 1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis(acetoxymethyl ester); BMDC, bone marrow-derived mast cell; CAMK, calmodulin-dependent kinase; Fc ϵ RI, high-affinity IgE receptor; GPCR, G protein-coupled receptor; GST, glutathione S-transferase; HDX-MS, hydrogen deuterium exchange mass spectrometry; MRM, multiple reaction monitoring; PAF, platelet activating factor; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PtdIns, phosphatidylinositol; PKB/Akt, protein kinase B; PTx, *B. pertussis* toxin; SCF, stem cell factor, c-kit ligand; SOCE, store-operated Ca²⁺ entry.

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Introduction

Class I phosphoinositide 3-kinases (PI3Ks) produce the lipid second messenger phosphatidylinositol(3,4,5)-trisphosphate [PtdIns(3,4,5)P₃] and consist of a p110 catalytic and a regulatory subunit. The class IA catalytic subunits, p110 α , β , and δ , are constitutively bound to p85-related regulatory proteins that link them to the activation by protein tyrosine kinase receptors. The only class IB PI3K member, p110 γ , is activated downstream of G protein-coupled receptors (GPCRs), and interacts with p101 or p84 (also known as p87^{PIKAP}) adaptor subunits [1–3]. A tight complex of the p110 γ catalytic subunit (PK3CG) with p101 was first discovered in neutrophils [1]. The p101 subunit (PI3R5) sensitizes PI3K γ for activation by G $\beta\gamma$ subunits of trimeric G proteins, and is essential for chemotaxis of neutrophils towards GPCR-ligands [1,4,5].

Mast cells do not express p101; however, they do express the homologous adaptor protein p84 ([PI3R6]) [6], which shares 30% sequence identity with p101. Both p101 and p84 potentiate the activation of p110 γ by G $\beta\gamma$, but the p110 γ -p101 complex is

significantly more sensitive towards G $\beta\gamma$, and displays an enhanced translocation to the plasma membrane as compared with p110 γ -p84 [7]. Although p84 is absolutely required to relay GPCR signals to protein kinase B (PKB/Akt) phosphorylation and degranulation [6], its role is not completely understood: contrary to p110 γ -p101, p110 γ -p84 requires additionally the presence of the small G protein Ras, and might operate in distinct membrane micro-domains [6,7].

Interestingly, genetic ablation of p110 γ blocks high-affinity IgE receptor (Fc ϵ RI)-dependent mast cell degranulation in vitro and in vivo [8]. In part this is due to the fact that initial IgE/antigen-mediated mast cell stimulation triggers the release of adenosine and other GPCR ligands to feed an autocrine/paracrine activation of PI3K γ , which then functions as an amplifier of mast cell degranulation. Interestingly, a substantial part of the observed PI3K γ -dependent histamine-containing granule release (ca. 40%) was found to be resistant to *Bordetella pertussis* toxin (PTx) pretreatment [9,10]. Furthermore, although adenosine activates PI3K γ via the A3 adenosine receptor (A₃AR; [ADORA3]), A₃AR

Author Summary

Phosphoinositide 3-kinases (PI3Ks) are involved in most essential cellular processes. Class I PI3Ks are heterodimers: class IA PI3Ks are made up of one of a group of regulatory p85-like subunits and one p110 α , p110 β , or p110 δ catalytic p110 subunit, and are activated via binding of their p85 subunit to phosphorylated tyrosine receptors or their substrates. The only, class IB PI3K member, PI3K γ , operates downstream of G protein-coupled receptors (GPCRs). Recent work suggested that PI3K γ also operates downstream of IgE-antigen complexes in mast cell activation, but no mechanism was provided. We show that clustering of the high-affinity IgE receptor Fc ϵ R1 triggers a massive calcium ion influx, which leads to PKC β activation. In turn, PKC β phosphorylates Ser582 of the PI3K γ catalytic p110 γ subunit's helical domain. Downstream of GPCRs, p110 γ requires a p84 adapter to be functional. Phosphomimicking mutations at Ser582 disrupt the p84-p110 γ interaction, and cellular Ser582 phosphorylation correlates with the loss of p84 from p110 γ . Thus our data suggest that PKC β phosphorylates and activates p110 γ downstream of calcium ion influx, while simultaneously disconnecting the phosphorylated p110 γ from GPCR signaling. Exploration of the p84-p110 γ interaction surface by hydrogen- deuterium exchange mass spectrometry confirmed that the p110 γ helical domain forms the main p84-p110 γ contact surface. Taken together, the results suggest an unprecedented mechanism of PI3K γ regulation.

null mice are still sensitive to passive systemic anaphylaxis, and degranulation in A $_3$ AR $^{-/-}$ bone marrow-derived mast cells (BMMCs) upon antigen stimulation remains functional [11,12]. This and the strong degranulation phenotype of PI3K γ $^{-/-}$ BMMCs suggest that GPCR signaling does not generate the full input to PI3K γ -dependent degranulation, but a GPCR-independent activation mechanism for PI3K γ has yet to be defined.

Here we identify a mechanism that activates PI3K γ independently of GPCRs: we demonstrate that (i) IgE/antigen complexes and extracellular Ca $^{2+}$ influx activate PI3K γ , (ii) PI3K γ is operationally linked to the Fc ϵ R1 specifically by PKC β (PRKCB), (iii) and that the phosphorylation of Ser582 located in the helical domain of p110 γ by PKC β leads to the dissociation of the p84 adapter to decouple phosphorylated p110 γ from GPCR inputs. Further we characterize the p110 γ -p84 interface, and delineate an activation process that seems to be conserved among class I PI3Ks.

Results

Thapsigargin-Induced Mast Cell Activation Needs PI3K γ

A committed step in mast cell activation is the influx of extracellular Ca $^{2+}$ by store-operated Ca $^{2+}$ entry (SOCE) [13]. Thapsigargin, which inhibits the sarco/endoplasmic reticulum Ca $^{2+}$ reuptake ATPase (SERCA), causes depletion of Ca $^{2+}$ stores, triggering SOCE. The latter achieves full-scale degranulation of BMMCs [14]. Surprisingly, BMMCs devoid of the p110 γ catalytic subunit of PI3K γ lost their responsiveness to thapsigargin and matched degranulation responses attained by wortmannin-pre-treated cells (Figure 1A). To investigate if thapsigargin-triggered, p110 γ -dependent degranulation involved release of adenosine, BMMCs were preincubated with adenosine deaminase (ADA) (Figure 1B) to convert adenosine to inosine, which has a very low affinity for adenosine receptors. ADA attenuated degranulation induced by IgE/antigen but did not affect thapsigargin-stimulated degranulation in wild type cells and did not further attenuate

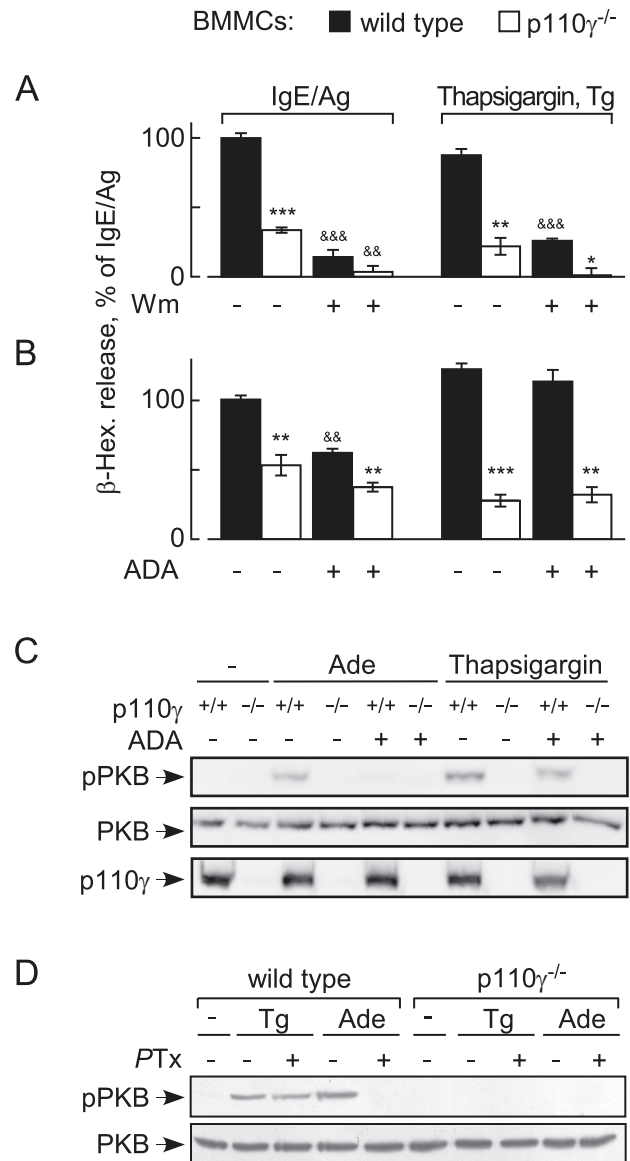


Figure 1. Thapsigargin-induced mast cell degranulation requires PI3K γ , but not GPCR signaling. (A) Granule release of wild type and p110 γ $^{-/-}$ BMMCs was determined detecting β -hexosaminidase (β -Hex) release into extracellular media. BMMC stimulation with IgE/antigen was initiated with the antigen (Ag, DNP-HSA at 10 ng/ml; 100 ng/ml IgE overnight). Alternatively, BMMCs were stimulated by the addition of thapsigargin (1 μ M). Where indicated, BMMCs were preincubated for 15 min with 100 nM wortmannin. Released β -Hex was quantified 20 min after stimulation, and is expressed as mean \pm standard error of the mean (SEM) ($n=3$; p -values in all figures are * or &: $p<0.05$, **: $p<0.005$; ***: $p<0.0005$; * depict here comparison with respective wild type control; & refer to comparison of untreated versus treated samples). (B) Granule release was assessed as above, but ADA (10 units/ml) was added to BMMC suspensions 1 min before stimulation where depicted. (C) Wild type or p110 γ $^{-/-}$ BMMCs were stimulated with adenosine (Ade; 1 μ M) or thapsigargin (1 μ M) for 2 min, and phosphorylation of PKB/Akt on Thr308 (pPKB), total PKB and p110 γ was analyzed by Western blotting. BMMCs were incubated in starving medium (2% FCS, without IL-3) for 3 h before stimulation, and pretreated with ADA where indicated. (D) Heterotrimeric G α_i proteins were inactivated by preincubation of wild type and p110 γ $^{-/-}$ BMMCs with 100 ng/ml PTx for 4 h, before thapsigargin (Tg) or adenosine was added as in (C).
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residual degranulation in p110 γ null BMMCs. Likewise, presence of ADA did not reduce the phosphorylation of PKB/Akt in response to thapsigargin via the p110 γ -dependent pathway (Figure 1C) but did reduce phosphorylation of PKB/Akt in response to adenosine—illustrating that the added ADA removes adenosine quantitatively. PTx treatment of BMMCs (Figure 1D) blocked adenosine—but not thapsigargin-stimulated PKB/Akt phosphorylation.

To exclude that autocrine/paracrine signaling to p110 γ occurred through PTx-insensitive G α q subunits to phospholipase β (PLC β [PLCB2, PLCB3]), and a subsequent Ras activation by the Ras guanine nucleotide exchange factor RasGRP4 as described earlier in neutrophils [15], we used platelet activating factor (PAF) to trigger cyclic AMP-responsive element-binding protein (CREB) phosphorylation. PAF was reported earlier to trigger a PTx-insensitive Ca²⁺ release from mast cells [9], and induced here a robust CREB phosphorylation comparable to adenosine and IgE/antigen. In contrast, PAF failed to trigger phosphorylation of PKB/Akt by itself, and did not enhance signaling of IgE/antigen to PKB/Akt (Figure S1).

Altogether, these results clearly illustrate that thapsigargin stimulates BMMCs via a PI3K γ -dependent activation pathway, which operates separately from adenosine-induced activation of G α i/o trimeric G proteins.

Thapsigargin-Induced PI3K γ Is Downstream of Extracellular Ca²⁺ Influx

PI3K γ activation has been linked to ligation of GPCRs [1,16], but not to elevated intracellular Ca²⁺ concentration ([Ca²⁺]_i). We therefore chelated extracellular Ca²⁺ using EDTA, and buffered intracellular Ca²⁺ with the cell permeable Ca²⁺ chelator 1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis(acetoxymethyl ester) (BAPTA-AM). Extracellular and intracellular Ca²⁺ chelation prevented phosphorylation of PKB/Akt induced by thapsigargin or the Ca²⁺ ionophore ionomycin, while IL-3 and adenosine signaling to PKB/Akt remained unperturbed (Figure 2A and 2B).

Interestingly, the concentration of Ca²⁺ required to trigger PI3K γ -dependent phosphorylation of PKB/Akt exceeded peak concentrations that are reached by GPCR stimulation: GPCR agonists release Ca²⁺ only from internal stores (maximum [Ca²⁺]_i < 300 nM), while thapsigargin (Figure 2C–2F) and IgE/antigen trigger SOCE and elevate [Ca²⁺]_i to μ M concentrations [9,14,17]. Moreover, the correlation of maximally achieved [Ca²⁺]_i after thapsigargin revealed a steep, switch-like activation of PKB/Akt. While Ca²⁺ release from internal stores triggered by G α i-coupled GPCRs (such as the A₃AR stimulated by N⁶-(3-iodobenzyl)-adenosine-5'-N-methylcarboxamide [IB-MECA]) is sensitive to PTx, thapsigargin-induced SOCE is not. Ca²⁺-induced activation of PI3K γ only occurs after SOCE, and is therefore clearly separated from the GPCR/trimeric G protein/PI3K γ axis.

PKC β Links Ca²⁺ Mobilization to PI3K γ Activation

Protein kinase C (PKC) inhibitors (Ro318425, G δ 6983, G δ 6976) targeting classical and atypical PKCs, and the inhibitor PKC412, which mainly inhibits classical PKCs, all substantially blocked PKB/Akt phosphorylation in response to thapsigargin and phorbol 12-myristate 13-acetate (PMA) (Figures 3A and S2A). Rottlerin, with a limited selectivity for PKC δ , had no effect on PKB/Akt activation. GPCR-dependent PI3K γ activation by adenosine was resistant to all tested PKC inhibitors (Figure S2B). The inhibitor profile suggested that a classical PKC activates PI3K γ . While PKB/Akt activation by PMA and thapsigargin was blocked in PKC β ^{-/-} BMMCs (Figure 3B), signaling in PKC α ^{-/-}

and PKC γ ^{-/-} BMMCs remained intact (Figure S2C). Deletion of PKC β eliminated phosphorylation of PKB/Akt on Thr308 and Ser473 completely, whereas a residual signal on Ser473 was observed after PI3K-inhibition by wortmannin. This may be explained by the observation that PKC β 2 can function as a Ser473 kinase [18]. Adenosine, IL-3, and stem cell factor (SCF)-induced PKB/Akt activation was not affected by elimination of PKC β (Figure 3C), demonstrating that PKC β does not relay adenosine signals to PI3K γ , and is not required in cytokine and growth factor receptor-dependent activation of class IA PI3Ks in mast cells.

The direct measurement of phosphoinositides in BMMCs confirmed that ablation of PKC β or its inhibition eliminated production of PtdIns(3,4,5)P₃ triggered by thapsigargin and PMA, but not adenosine (Figure 3D–3F). Interestingly, the link between PKC β and PI3K γ seems to be transient in nature, as PMA stimulation triggers short lived PtdIns(3,4,5)P₃ peaks (Figure 3F). Impaired Fc ϵ RI-triggered degranulation has been reported in both p110 γ ^{-/-} [9] and PKC β ^{-/-} BMMCs [19], and the sensitivity of degranulation to PKC inhibition fits the phospho-PKB/Akt output (Figure 3G). This, combined with the similarity of p110 γ and PKC β null phenotypes in IgE/antigen-induced degranulation (Figure 3H), suggests a direct link of PKC β and PI3K γ downstream of Fc ϵ RI.

PKC β Binds and Phosphorylates PI3K γ

Co-expression of p110 γ with tagged full length or truncated PKC β 2 (Figure 4A) revealed that only the catalytic domain fragment and a pseudo-substrate deletion mutant of PKC β 2 formed complexes with p110 γ (Figure 4B), suggesting that the presence of the pseudo-substrate in PKC β results in a closed conformation that is unable to interact with p110 γ . An in vitro protein kinase assay with recombinant PKC β 2 and glutathione S-transferase (GST)-tagged wild type p110 γ or catalytically inactive p110 γ (KR; Lys833Arg mutant) as substrate, showed that PKC β robustly phosphorylated p110 γ (Figure 4C). The capability of p110 γ to auto-phosphorylate [20] was not required in the process.

Analysis of phosphorylated, catalytically inactive p110 γ by liquid chromatography tandem mass spectrometry (LC-MS/MS) identified Ser582 as a target residue of PKC β (YES^P[582]LKHPK; spectra in Figure S3). Mass spectrometric multiple reaction monitoring (MRM) (Figure S3C) showed that Ser582 phosphorylation was absent in assays lacking PKC β , or when PKC-inhibitor was added (Figure 4D). Ser582 phosphorylation was also detected by MRM in PMA and IgE/antigen stimulated BMMCs (Figure 4D, lower panel).

Phospho-Ser582 site-specific antibodies (see Figure S4) revealed p110 γ Ser582 phosphorylation in PMA-, thapsigargin-, and IgE/antigen-stimulated BMMCs, but not in mast cells exposed to adenosine or IgE alone (Figure 5A). Consistent with the requirement of Ca²⁺ mobilization, IgE/DNP-induced phosphorylation of p110 γ on Ser582 was blocked by extracellular (EDTA, EGTA) and intracellular (BAPTA/AM) Ca²⁺ chelation (Figure 5B).

Although the extended peptide around Ser582 scores as a PKC substrate site, the core Arg-X-X-Ser582 sequence is a putative recognition site for several protein kinases (scores are PKC > protein kinase A > calcium/calmodulin-dependent kinases [CAMK]). As mast cell activation is accompanied by a massive influx of extracellular Ca²⁺, we assessed if CAMK could phosphorylate p110 γ directly. In the presence of ³²P- γ -ATP, recombinant CAMKII (CAMK2) incorporated equal amounts of phosphate into free and p84-bound p110 γ . In the same experiment, PKC β preferentially phosphorylated free p110 γ , illustrating a preference

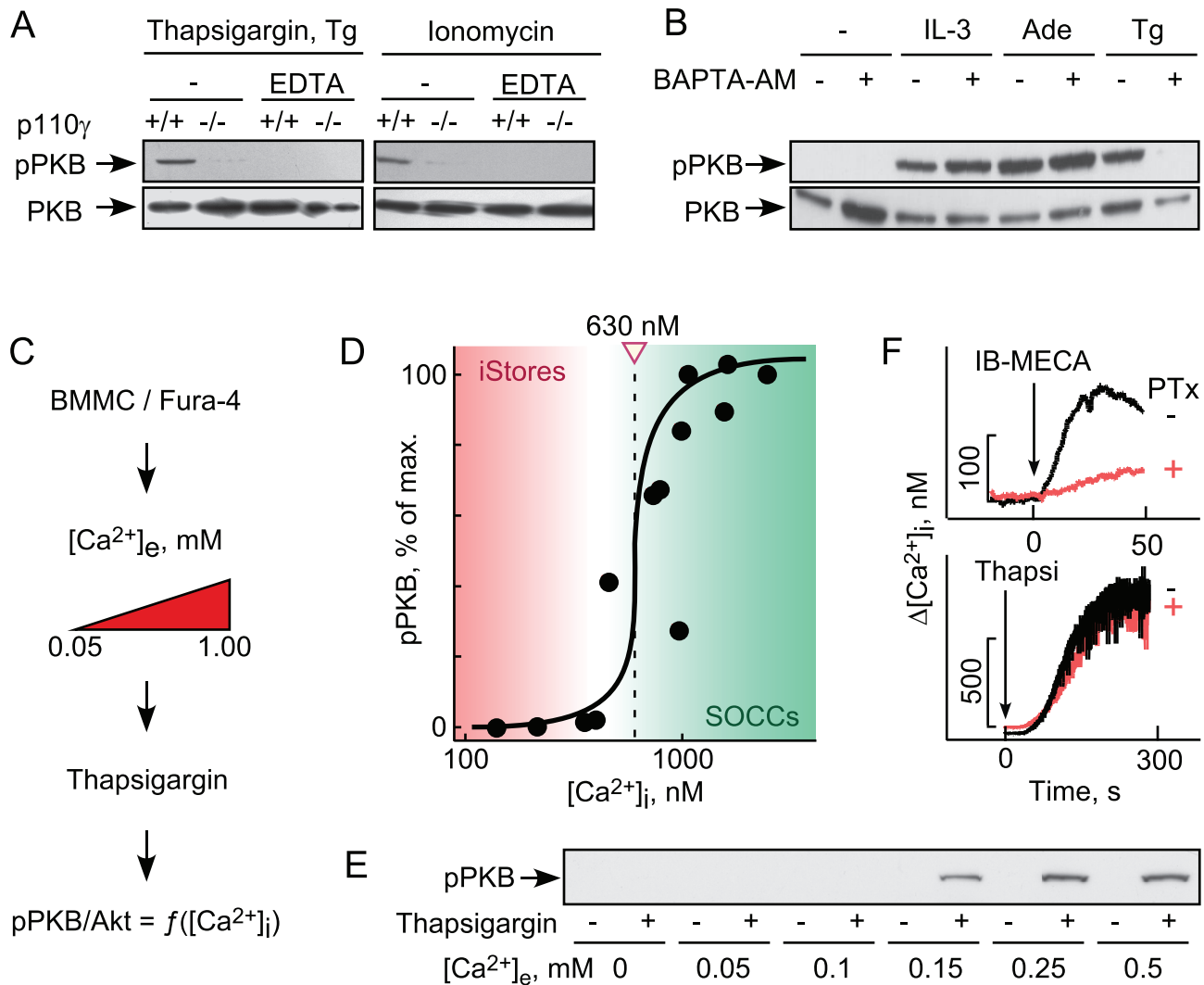


Figure 2. Thapsigargin-triggered PI3K γ activation requires influx of extracellular Ca $^{2+}$. (A) Where indicated, IL-3 starved BMMCs were incubated with EDTA (5 mM) for 5 min, before cells were stimulated with thapsigargin (1 μ M) or ionomycin (1 μ M). Cells were lysed 5 min after stimulation, and phosphorylation of PKB/Akt on Ser473 was analyzed. (B) BMMCs as in (A) were pretreated for 10 min with the cell-permeable Ca $^{2+}$ -chelator BAPTA/AM (10 μ M) and stimulated either with IL-3 (10 ng/ml), adenosine (1 μ M), or thapsigargin (1 μ M). (C, D) BMMCs were loaded with the ratiometric low affinity Ca $^{2+}$ probe Fura-4F/AM for 10 min in physiologic HEPES buffer at 1 mM Ca $^{2+}$ (for details see Text S1). After the loading, washed cells were resuspended in the presence of increasing Ca $^{2+}$ concentrations (extracellular Ca $^{2+}$, [Ca $^{2+}$]_e) to modulate maximal stimulation-induced intracellular Ca $^{2+}$ levels ([Ca $^{2+}$]_i). Cells were then stimulated with 0.5 μ M thapsigargin, and maximal [Ca $^{2+}$]_i increase and phosphorylation of PKB/Akt were measured. pPKB S473 levels are displayed as a function of the individually determined [Ca $^{2+}$]_i. Data points come from two independently performed experiments. (E) Representative anti-phospho-PKB/Akt immunoblot as used to determine pPKB/Akt levels in (D). (F) Intracellular Ca $^{2+}$ concentrations were measured in wild type BMMCs following stimulation with the adenosine 3A receptor-selective agonist *N*⁶-(3-iodobenzyl)-adenosine-5'-*N*-methylcarbox-amide (IB-MECA) (10 nM) or thapsigargin (1 μ M). *B. Pertussis* toxin (100 ng/ml) was added 4 h before stimulation where marked. doi:10.1371/journal.pbio.1001587.g002

of PKC β for p110 γ surfaces obscured in the p84-p110 γ complex. CAMK also substantially phosphorylated p84, which was borderline in *in vitro* assays with PKC (12 versus 3 mol %) (Figure S5A). Probing the phosphorylation of Ser582 *in vitro* demonstrated that access to this site is blocked when p84 is bound to p110 γ (Figure S5B).

In cellular assays stimulating BMMCs with thapsigargin, CAMK activation could be monitored using anti-phospho-CAMKII antibodies. While PKC inhibitors left CAMKII phosphorylation >50% intact, Ser582 and PKB/Akt phosphorylation were both reduced to background levels (Figure S6), even in a context favoring Ca $^{2+}$ -triggered responses. The above, and the fact

that PKC $\beta^{-/-}$ BMMCs showed a major reduction in phospho-Ser582 after PMA or IgE/antigen stimulation (Figure 5C and 5D), illustrate that phosphorylation of p110 γ is mainly mediated by PKC β , and that other PKC isoforms (see also Figure S2) and Ca $^{2+}$ -dependent kinases attribute to less than 18% of the observed overall signal.

Phosphorylation of Ser582 Positively Regulates p110 γ 's Activity and Displaces p84

To evaluate if the Ser582 phosphorylation affected the intrinsic activity of p110 γ , a phosphorylation-mimicking mutant (Ser582-Glu) was produced. The activity of the p110 γ Ser582Glu mutant

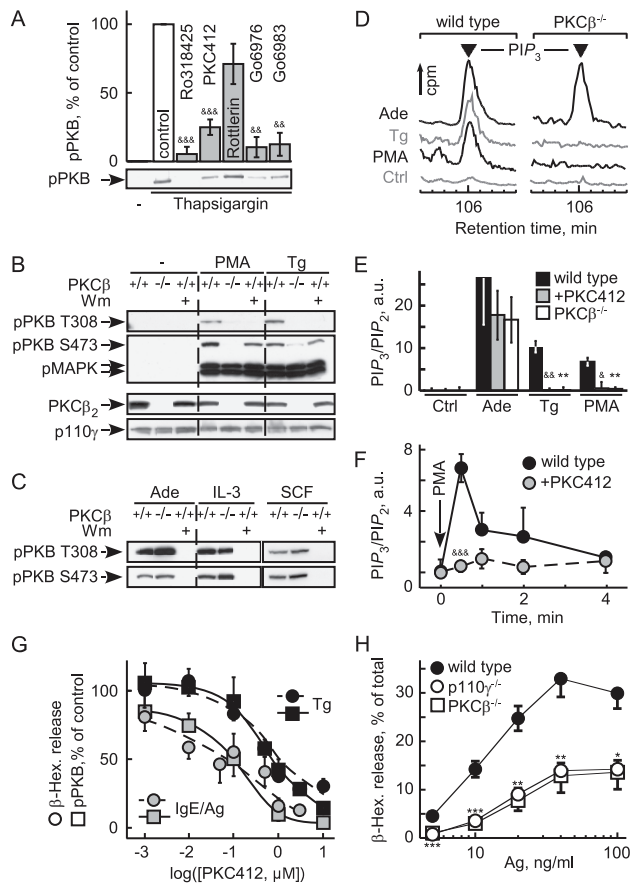


Figure 3. PKC β relays thapsigargin-induced PI3K γ activation.

(A) Effect of PKC inhibitors on thapsigargin-induced PKB phosphorylation on Ser473 (S473). IL-3 starved BMMCs were preincubated with the indicated compounds for 20 min before stimulation (pan-PKC: Ro318425, Gö6983; classical PKC: PKC412 (CPG41251); classical and atypical PKC: Gö6976; Rotterlin: broad band inhibitor; see Text S1; & refers to comparison with untreated control; *p*-values see Figure 1). (B) PKB/Akt activation in response to 100 nM PMA or 1 μ M thapsigargin was analyzed in wild type and PKC $\beta^{-/-}$ BMMCs. Cells were IL-3 deprived as in (A), and were pretreated with wortmannin (Wm, 100 nM) for 15 min before stimulation where indicated. Cells were lysed 2 min after stimulation, and analyzed for phosphorylation of PKB/Akt (T308 and S473) and MAPK (T183/Y185). (C) Wild type and PKC $\beta^{-/-}$ BMMCs were stimulated with 1 μ M adenosine, 10 ng/ml IL3, or 10 ng/ml SCF, and processed as in (B). (D–F) PtdIns(3,4,5) P_3 (PIP $_3$) levels were determined in untreated (Ctrl) and classical PKC-inhibitor (PKC412)-treated wild type BMMCs and PKC $\beta^{-/-}$ BMMCs after stimulation with 0.5 μ M thapsigargin, 200 ng/ml PMA, or 5 μ M adenosine (30 s). BMMCs were metabolically labeled with [32 P]-orthophosphate, lipids were extracted, deacylated, and applied to high-pressure liquid chromatography (HPLC). (D) shows representative elution peaks of PIP $_3$ of the HPLC chromatograms. (E) Levels of PIP $_3$ in relation to PtdIns(4,5) P_2 (PIP $_2$) were quantified by integration of the peak areas of PIP $_3$ and PIP $_2$ and expressed as ratio of PIP $_3$ /PIP $_2$ (data shown as mean \pm standard error of the mean [SEM], *n* = 4–6). (F) Cellular PIP $_3$ production was measured over time in wild type BMMCs in response to PMA (200 nM) stimulation in the presence or absence of the classical PKC inhibitor PKC412 (mean \pm SEM, *n* = 3). (G) Granule release and PKB activation (S473) in response to thapsigargin (1 μ M) or IgE/antigen (100 ng/ml IgE overnight, 10 ng/ml DNP) was measured in the presence of increasing concentrations of the classical PKC inhibitor PKC412. Cells starved as in (A) were stimulated with IgE/antigen (IgE/Ag) or thapsigargin (Tg), and PKB phosphorylation and β -hexosaminidase release assays were performed in parallel (mean \pm SEM, *n* = 3). (H) β -hexosaminidase release determined in wild type, PKC $\beta^{-/-}$, and p110 $\gamma^{-/-}$ BMMCs incubated with IgE, and stimulated with the indicated antigen (Ag) concentrations (mean \pm SEM, *n* = 5; * refer to comparison with wild

type control. Only the higher *p*-values of the overlapping data points are indicated).

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was enhanced approximately 2-fold independently of the substrate used (PtdIns(4,5) P_2 , PtdIns, or auto-phosphorylation) (Figure 6A–6C). Ser582 is localized in the helical domain of p110 γ , and it is interesting to note that helical domain mutants of p110 α found in tumors display a similar increase in enzyme activity [21–25].

As mutations in the helical domain of p110 α attenuate contacts with the p85 regulatory subunit [21], we examined binding of p110 γ mutants to the PI3K γ adaptor subunit p84: the substitution of Ser582 with Glu and Asp, abrogated p110 γ -p84 interactions in HEK293 cells and BMMCs, while the Ser582Ala replacement favored p110 γ -p84 complex formation (Figure 6D–6F). In line with this, PMA-induced phosphorylation of Ser582 in BMMCs was suppressed by the overexpression of p84 (Figure 6G), which fits the very limited access of PKC β II to in vitro phosphorylate Ser582 in the p110 γ -p84 complex (reduced to 20% of phosphorylation of free p110 γ) (Figure S5B).

Most importantly, the correlation of phosphorylation of Ser582 on p110 γ and the release of p84 could also be established in wild type BMMCs: when stimulated with PMA, the amount of p84 that could be co-precipitated with p110 γ was reduced significantly, and was linked to Ser582 phosphorylation of p110 γ . In the inverse co-immunoprecipitation, anti-p84-associated p110 γ was reduced, and phosphorylation was below detection levels in the remaining p84-associated p110 γ (Figure S7). The collected results are in agreement with a mechanism in which PKC β II-mediated phosphorylation of Ser582 and the interaction of p84 and p110 γ are exclusive events, and in which PKC β action displaces p84 from p110 γ .

The Helical Domain of p110 γ Binds and Is Stabilized by p84

In order to understand how p84 could mask Ser582 phosphorylation, and to map the p110 γ -p84 contact interface, hydrogen deuterium exchange mass spectrometry (HDX-MS) was used. HDX-MS elucidated contacts of class IA p110 δ with its p85 regulatory subunit, and the mechanism of action of cancer-linked mutations in p110 α [25,26]. HDX-MS relies on amide hydrogen exchange with solvent at a rate dependent on their involvement in secondary structure and solvent accessibility. Following proteolysis, location and extent of deuterium uptake are analyzed by peptide mass determination. The primary sequence of p110 γ was covered >90% by 202 peptide fragments (Figure S8; Table S1).

Deuterium (2 H) incorporation into free and p84-complexed p110 γ was analyzed at seven time points (3 to 3,000 s). Differences in 2 H-exchange of free and complexed p110 γ were mapped onto the crystal structure of p110 γ lacking the N-terminal domain (PDB ID:2CHX, residues 144–1,093), to visualize conformational changes induced by p84 (Figures 7A, S9, and S10). Peptides with highest decrease in 2 H incorporation (>1.0 Da) clustered to the RBD-C2 linker, the C2-helical domain linker, and the helical domain. The 2 H-incorporation in the presence of p84 is visualized as integrated average difference in exchange at all seven time points in Figure 7B, illustrating that the helical domain provides the dominant interface with p84. Due to difficulties in producing free p84, the contacts on p84 with p110 γ could not be mapped. Interestingly, in the absence of the p84 subunit, the majority of peptides in the helical domain exhibited broad isotopic profiles (HDX of peptide 623–630, which is representative of peptides in the helical domain, is shown in Figures 7C and S11). This type of profile known as type 1 exchange (EX1) kinetics is indicative of

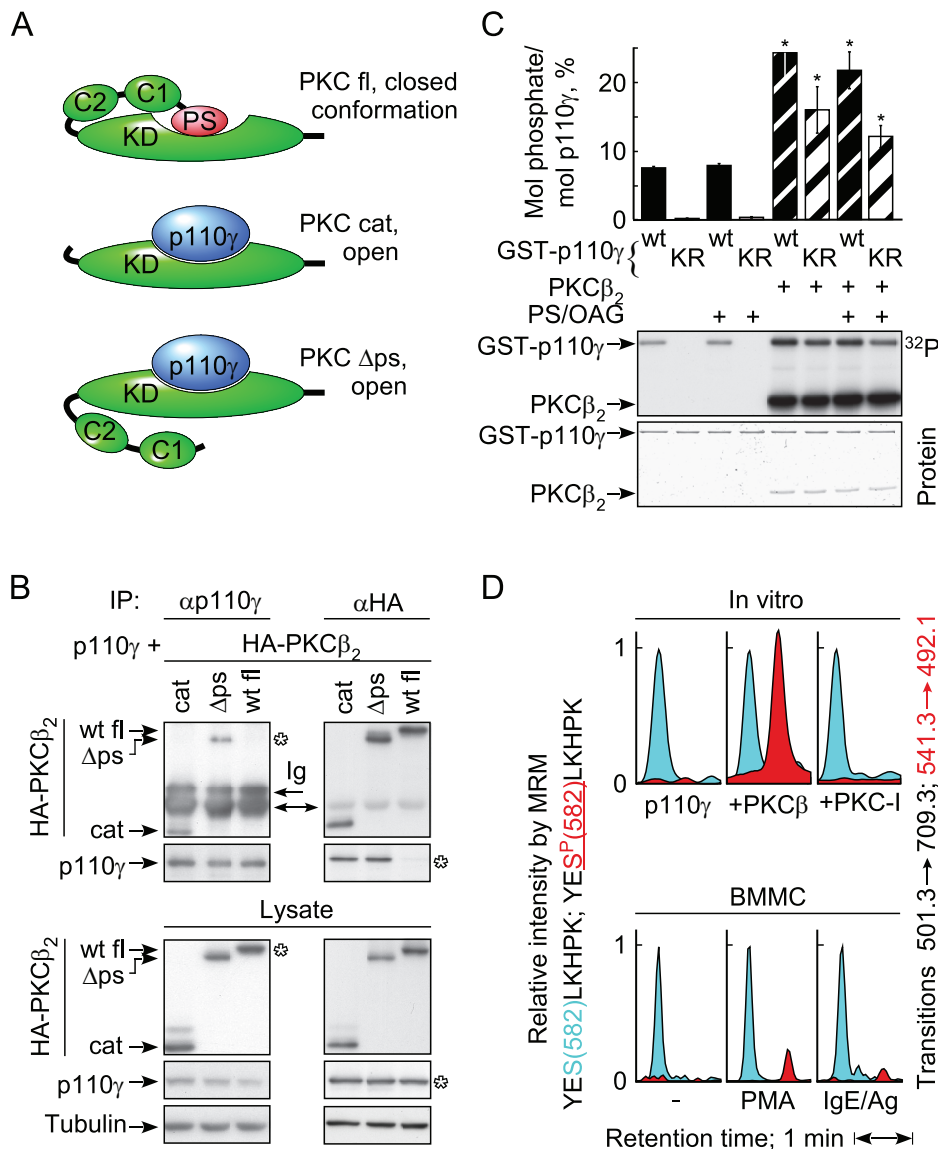


Figure 4. PKC β interacts with and phosphorylates the catalytic subunit of PI3K γ . (A) Schematic representation of the PKC β -p110 γ interaction: full-length (fl) PKC β is in a closed conformation due to the interaction of the pseudo-substrate domain with the catalytic pocket of PKC β , while the truncated catalytic domain (cat; amino acids 302–673) and pseudo-substrate deletion mutant (Δ ps; deletion of aa 19–31) give access to p110 γ . (B) HEK293 cells were co-transfected with p110 γ and HA-tagged PKC β_2 constructs. Protein complexes were immunoprecipitated with anti-p110 γ or anti-HA antibodies, before HA-PKC β_2 and p110 γ was detected by immunoblotting. Ig: immunoglobulin heavy chain signals of mouse anti-p110 γ and anti-HA antibodies. (C) Recombinant GST-p110 γ wild type (wt) or a catalytically inactive p110 γ mutant (KR, Lys833Arg mutant) were incubated with recombinant PKC β_2 and [γ ³²P]-ATP in kinase buffer for 30 min, before proteins were denatured and separated by SDS-PAGE. Phosphatidylserine (PS) lipid vesicles containing 1-oleoyl-2-acetyl-sn-glycerol (OAG) were present during the reaction where marked. Protein-bound ³²P was determined by radioisotope imaging, and recombinant proteins were stained with Coomassie blue (mean \pm standard error of the mean [SEM], $n = 3$; * point to comparison with respective sample without PKC). (D) In vitro and in vivo phosphorylation of PI3K γ on S582, analyzed by LC-MRM. S582 non-phospho- and phospho-peptides were detected in the MRM mode, quantifying the transition 501.1 to 709.3 for the non-modified peptide (blue) and 541.3 to 492.1 for the phospho-peptide (red). Data were normalized to the transition of the non-modified peptide, which was set to 1. Upper part: recombinant catalytically inactive human GST-PI3K γ (2 μ g) was incubated alone, together with PKC β_2 or with PKC β_2 and PKC-inhibitor (Ro318425, 2 μ M) as in (C). After SDS-PAGE and Coomassie staining, PI3K γ was excised from the gel and prepared for LC-MRM. Lower part: wild type BMMCs (300 M cells/stimulation) were starved for 4 h in IL-3 free medium/2% FCS, and were left unstimulated or were treated for 2 min with 50 nM PMA or for 4 min with 10 ng/ml antigen (cells preloaded with 100 ng/ml IgE overnight). Endogenous PI3K γ was immunoprecipitated from cell lysates, resolved by SDS-PAGE and analyzed with LC-MRM. doi:10.1371/journal.pbio.1001587.g004

concerted dynamic motions of a substructure in a protein, rather than the local fluctuations characteristic of EX2 kinetics [27].

The N-terminus of p110 γ was shown to stabilize the p110 γ -p101 heterodimer [28]. Expression of p110 γ mutants lacking the first 130 amino acids (Δ 130-p110 γ) seemed to support this view, as

association with p84 was lost (Figure 7D). However, when truncated p110 γ was N-terminally tagged with GST (GST- Δ 130-p110 γ), binding of p84 was restored. Although we detected a small decrease in the ²H-incorporation in two N-terminal p110 γ peptides (59–70 and 107–113) in the presence of p84, this

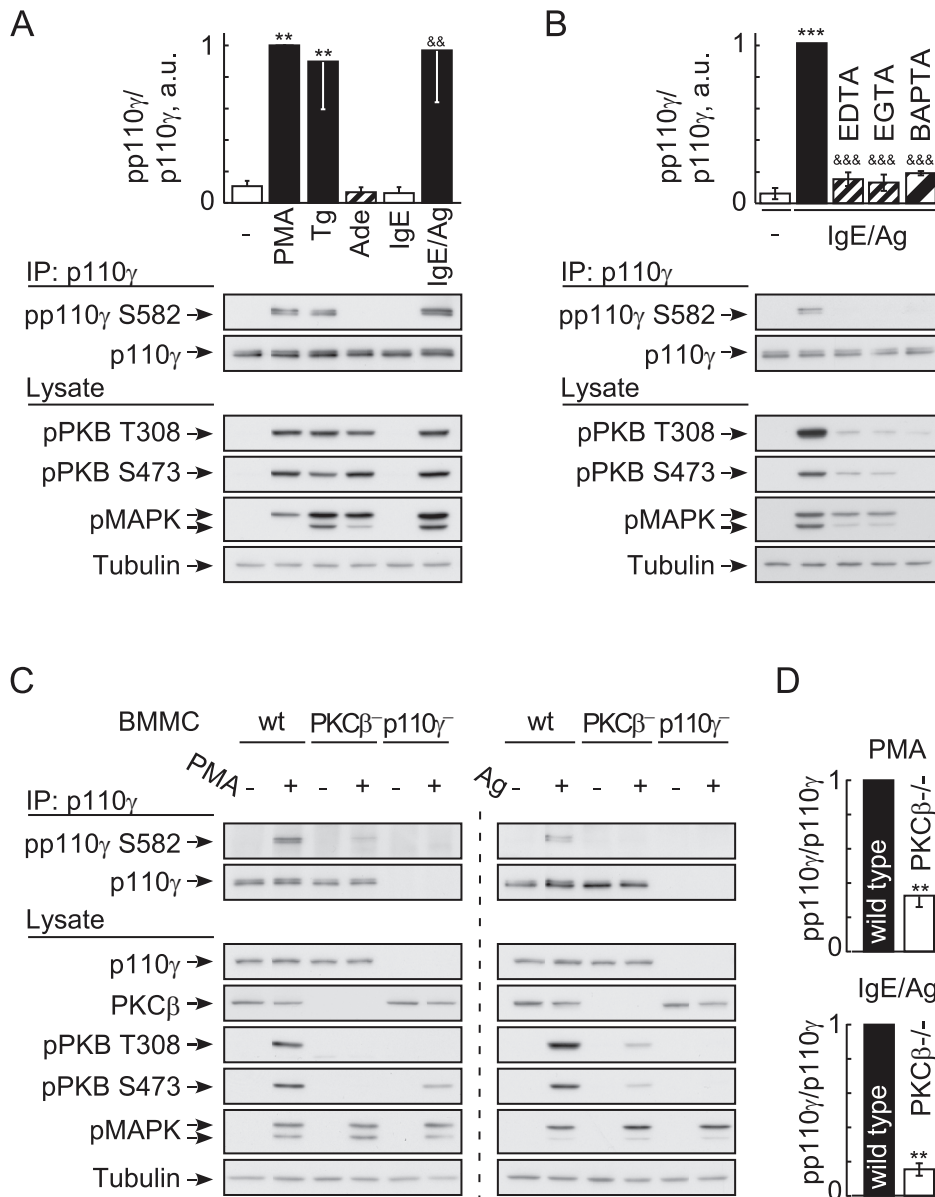


Figure 5. Phosphorylation of PI3K γ requires Ca²⁺ and is PKC β -dependent. (A) Stimulus-induced phosphorylation of endogenous p110 γ on Ser582 in wild type BMMCs. IL-3 deprived cells were stimulated with 100 nM PMA, 1 μ M thapsigargin, 1 μ M adenosine, or 20 ng/ml DNP for 2 min. Where indicated (IgE), BMMCs were loaded with IgE (100 ng/ml) overnight. PI3K γ was immunoprecipitated from cell lysates with an anti-PI3K γ antibody, before precipitated protein was probed for phosphorylated p110 γ (pp110 γ) with a phospho-specific anti-Ser582 antibody (validation of antibody see Figure S4). PI3K γ phosphorylation is shown normalized to total p110 γ levels (mean \pm standard error of the mean [SEM], $n=3$; * depict analysis using unstimulated control. & reference point is IgE only). (B) IgE/antigen-induced Ser582 phosphorylation of p110 γ requires Ca²⁺ influx. Cells were stimulated as in (A), but exposed to EDTA, EGTA, or loaded with BAPTA/AM where indicated (see Figure 2). Phosphorylated p110 γ was detected as in (A); mean \pm SEM, $n=3$; * comparison with unstimulated control; & analysis of stimulated versus chelator treated). (C) Phosphorylation of p110 γ in wild type and PKC β ^{-/-} BMMCs. Experimental settings were as in (A), and (D) depicts quantification of pp110 γ in relation to total p110 γ protein (mean \pm SEM; PMA $n=4$, antigen $n=3$). Cells devoid of p110 γ were included as negative control. doi:10.1371/journal.pbio.1001587.g005

interaction seems to be dispensable. The N-terminus of p110 γ instead has a role in stabilizing the intact catalytic subunit. The helical domain is the main location of interaction with p84; however, it appears that this interaction is vulnerable and easily broken, as a single phosphorylation at Ser582 is able to disrupt the contact.

Discussion

The activation of PI3K γ has been tightly linked to GPCRs-triggered dissociation of trimeric G α i proteins, and has been

shown to require the interaction of G β γ subunits with p110 γ and the PI3K γ adaptor subunits p101 and p84 [1,2,4,16,29,30]. Moreover, GPCRs generate PI3K signals typically through PI3K γ , thereby controlling extravasation of hematopoietic cells [31,32], cardiovascular parameters [33,34], and metabolic output [35,36].

Non-GPCR-mediated activation of PI3K γ has not been reported so far, but it has been shown that phorbol esters and Ca²⁺ ionophores can modulate phosphoinositide levels in a variety of cells, including platelets [37], adipocytes [38], fibroblasts [39], and hematopoietic cells [40]. The proposed mechanisms have

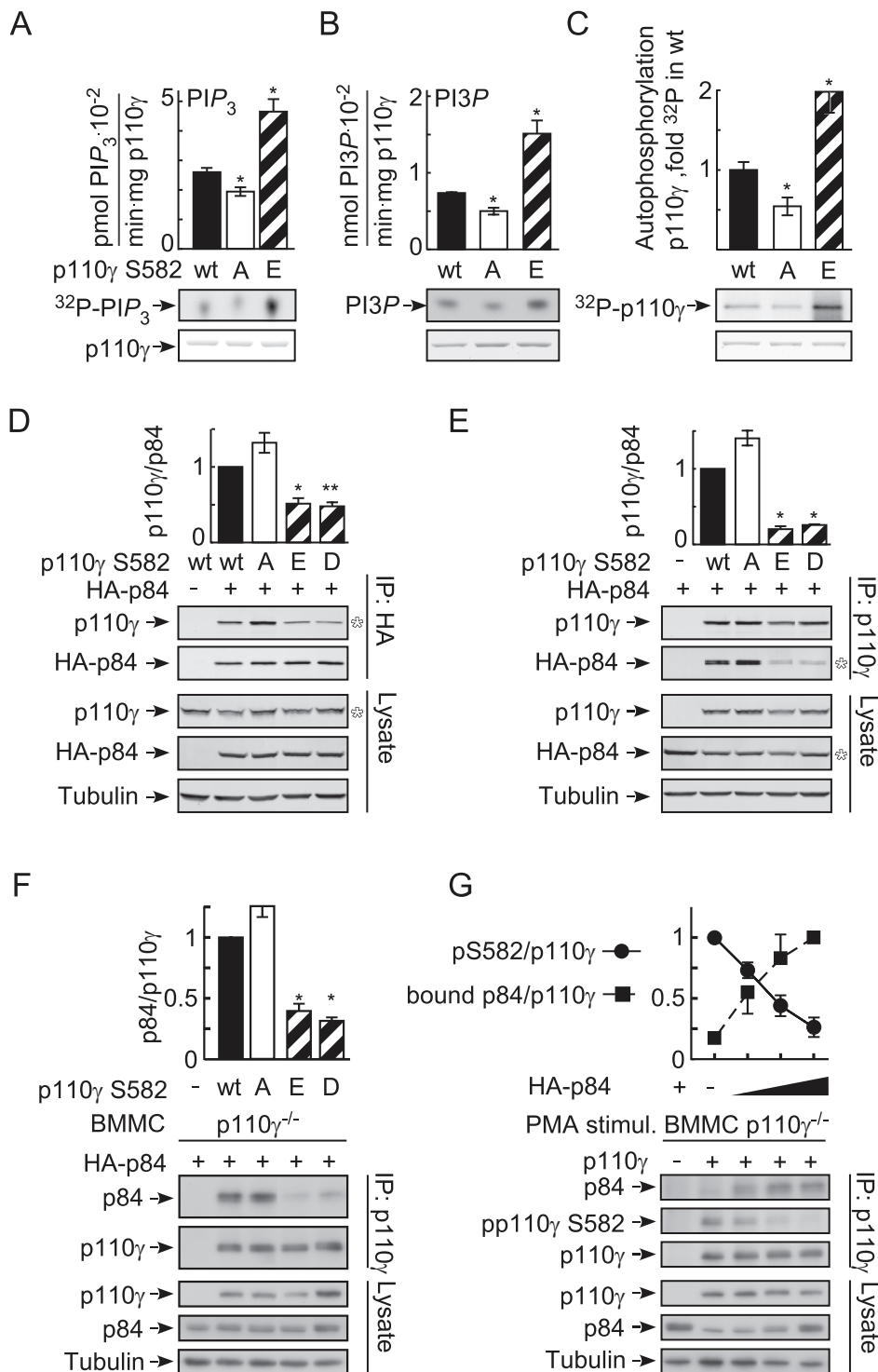


Figure 6. Ser582 phosphorylation increases p110 γ activity and displaces p84. (A–C) Lipid and protein kinase activities of recombinant p110 γ wild type (wt) and phosphorylation site mutants (Ser582Ala, [A]; Ser582Glu, [E]). (A) Mixed phospholipid vesicles containing PtdIns(4,5)P₂ were used to measure PtdIns(3,4,5)P₃ (PIP₃) production, while in (B) PtdIns was used as a substrate. Protein kinase activity of p110 γ was determined by auto-phosphorylation in (C). Bars display quantifications of incorporated ³²P as mean \pm standard error of the mean [SEM] ($n=3$; * comparison with wt protein), and representative thin layer chromatograms [³²P] and loaded p110 γ protein (Coomassie blue) are shown below. (D, E) Wild type p110 γ and Ser582 mutants (Ser582Asp, [D]) were expressed together with HA-tagged p84 in HEK293 cells. In (D), the PI3K γ complex was immunoprecipitated with anti-HA antibodies directed against HA-p84 ($n=4$). In (E) PI3K γ was immunoprecipitated using anti-p110 γ antibodies ($n=2$). (F) Wild type p110 γ and Ser582 mutants were co-expressed with p84 in p110 γ ^{-/-} BMMCs. PI3K γ was immunoprecipitated from cell lysates as in E (mean \pm SEM, $n=3$). (G) The p84 adapter competes with PKC β for access to p110 γ . Increasing amounts of HA-p84 expression plasmid were transfected into p110 γ ^{-/-} BMMCs, before the cells were stimulated with PMA for 30 s. PI3K γ was subsequently immunoprecipitated with an anti-p110 γ antibody, and bound p84 and Ser582 phosphorylation were quantified. The values are normalized to total p110 γ protein (mean \pm SEM, $n=2$).
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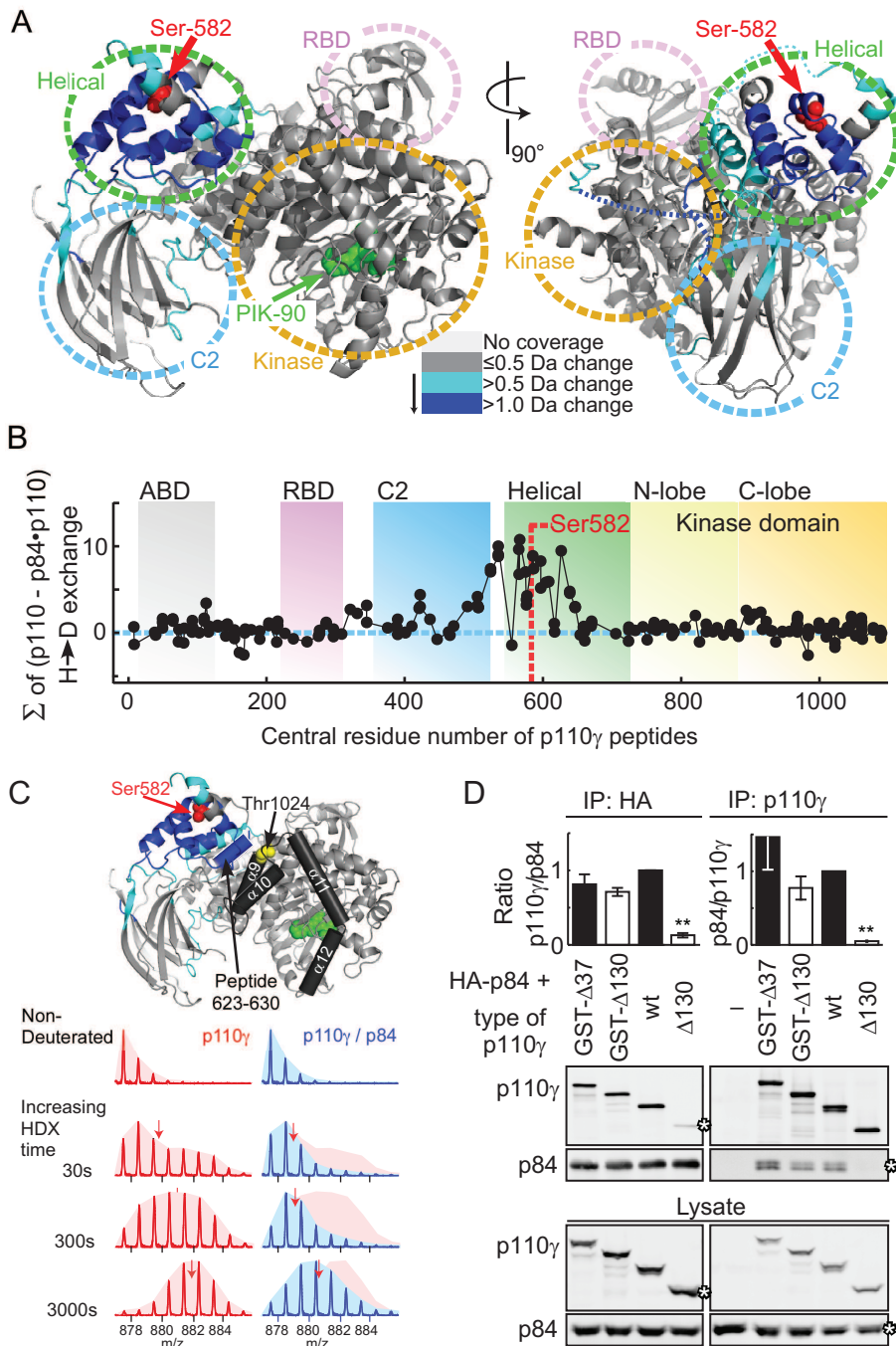


Figure 7. p84 interacts with the helical domain of p110 γ . (A) Changes in deuteration levels between free and p84-bound PI3K γ are mapped onto the crystal structure of PI3K γ (PDB ID: 2CHX). Regions that are covered by peptides of PI3K γ (labeled A–R) that showed greater than 0.5 or 1.0 Da changes in deuteration are colored light or dark blue, respectively. The greatest difference in exchange observed at any time was used for the mapping. S582 is labeled red. The ATP competitive inhibitor PIK-90 in the crystal structure is shown in green as a reference point for the kinase domain. The linker regions between the RBD and the C2 domain and the C2 and the helical domain are shown as dotted lines (right part). (B) The percent deuterium exchange differences between free and p84-bound PI3K γ were summed up over all seven time points for every identified peptide (y-axis), which were graphed according to their central residue number (x-axis). (C) A selected peptide (623–630) from the helical domain is shown at four time points of H/D on-exchange \pm the p84 subunit. In the absence of the p84 adaptor the majority of peptides in the helical domain showed broadening of the isotopic profiles indicative of EX1 kinetics (see 30, or 300 s in free p110 γ). The helix A3 (624–631) selected is located at the interface of the helical domain with the C-lobe. Ser582 and Thr1024 have been highlighted as a reference. (D) p84 was coexpressed with GST-tagged or untagged PI3K γ constructs in HEK293 cells. N-terminal deletions of 37 or 130 amino acids are denoted Δ 37 or Δ 130, respectively. HA-p84 (left) or PI3K γ (right) was immunoprecipitated from cell lysates with anti-HA or anti-PI3K γ antibodies and protein G beads. PI3K γ -p84 interactions were analyzed by Western blotting, quantified with Odyssey Imager software and expressed as fold of untagged, full-length p110 γ -p84 association (mean \pm standard error of the mean [SEM], left: $n = 4, 6, 6, 6$; right: $n = 2, 4, 4, 4$). doi:10.1371/journal.pbio.1001587.g007

been diverse and involved protein tyrosine kinases and GPCR signaling. A recent finding that protein kinase D (PKD) can phosphorylate two distinct sites on the p85 regulatory subunit to control class IA PI3K activity [41] is an indication that PI3K control is more complex than anticipated.

PI3K γ has been shown to be a key element in enhancing IgE/antigen output by the release of adenosine. This process involves signaling downstream of G α i-coupled A $_3$ AR, and is sensitive to PTx and ADA [9]. The resistance of thapsigargin-induced degranulation to ADA shown here, and the fact that PTx did not diminish the PI3K γ -dependent, thapsigargin-induced phosphorylation of PKB/Akt, points to a novel mechanism of PI3K γ activation, which is clearly distinct from GPCR action. This Ca $^{2+}$ -mediated PI3K γ activation requires SOCE and [Ca $^{2+}$] $_i$ >600 nM. In contrast, GPCRs yield phosphorylation of PKB/Akt in mast cells even in the absence of a change in [Ca $^{2+}$] $_i$. Furthermore, increases in [Ca $^{2+}$] $_i$ triggered via GPCRs remain at levels incapable of engaging a Ca $^{2+}$ -dependent activation of PI3K γ .

Thapsigargin bypasses the signaling chain from IgE/antigen-clustered Fc ϵ RI to the activation of phospholipase C γ (PLC γ) and inositol(1,4,5)-trisphosphate (Ins(1,4,5)P $_3$) production, and triggers SOCE by the depletion of Ca $^{2+}$ stores. That thapsigargin requires functional PI3K activity to induce mast cell degranulation was first demonstrated using the PI3K inhibitors wortmannin and LY294002 [14], but no link between [Ca $^{2+}$] $_i$ rise and PI3K γ activity was established previously. The fact that inhibitors for classical PKCs only prevented the thapsigargin- and PMA-induced phosphorylation of PKB/Akt, while the GPCR-mediated phosphorylation of PKB/Akt remained intact, points to a link between classical PKCs and PI3K γ . Experiments on BMMCs lacking PKC α , PKC β , and PKC γ , showed that only the PKC β null cells lost the ability to activate PKB/Akt in response to thapsigargin or PMA stimulation. As the PMA- and thapsigargin-induced phosphorylation of PKB/Akt on Ser473 showed a partial resistance to wortmannin, and as it has been reported that PKC β can directly phosphorylate Ser473 in the hydrophobic motif of PKB/Akt [18], the effect of genetic and pharmacological targeting of PKC β was also validated measuring PtdIns(3,4,5)P $_3$ production directly. The lack of PtdIns(3,4,5)P $_3$ production in thapsigargin or PMA-stimulated BMMCs treated with the PKC inhibitor PKC412, and in cells devoid of PKC β , is in agreement with a requirement of PKC β upstream of PI3K γ . That signaling from PKC to PI3K plays a role in mast cell degranulation is further supported by the close correlation of PKC inhibitor sensitivity of phosphorylated PKB/Akt and degranulation responses. Moreover, the loss of PKC β or PI3K γ results in a similar reduction of degranulation over a wide range of IgE/antigen concentrations. The results obtained here are in agreement with previous findings that mast cells and mice devoid of p85 α /p55 α /p50 α [42,43] and p85 β [44] remain fully responsive to IgE/antigen complexes. A previous report showed a biphasic activation of PI3K with PI3K γ having an early role and PI3K δ a later role downstream of Fc ϵ RI in murine mast cells [45,46].

A mechanistic link between PKC β and the catalytic subunit of PI3K γ was initially difficult to establish, as the direct PtdIns(3,4,5)P $_3$ response to PMA-stimulation was transient (main peak half life <1 min), and the two full-length enzymes interacted only weakly. The observation that truncated, activated forms of PKC β formed stable complexes with p110 γ , suggested that PKC β must attain an open conformation to interact with p110 γ , and that PKC β binds to p110 γ via its catalytic domain. This contact resulted in phosphorylation of Ser582 on p110 γ , which could be detected both in vitro and in PMA or IgE/antigen-stimulated

BMMCs by mass spectrometry. The PKC β -mediated phosphorylation of p110 γ was confirmed using site-specific anti-phospho-Ser582 antibodies. Stimuli like PMA, thapsigargin, and IgE/antigen complexes all required PKC to signal to PKB/Akt, which correlated with the phosphorylation of Ser582 on p110 γ . Moreover, the phosphorylation of Ser582 on p110 γ was sensitive to removal of extracellular Ca $^{2+}$, buffering of [Ca $^{2+}$] $_i$, and the genetic deletion of PKC β . Adenosine stimulates PI3K γ via GPCRs and PTx-sensitive trimeric G proteins [9] in a Ca $^{2+}$ -independent process, and did not yield a detectable phosphorylation of Ser582.

The increased turnover of PtdIns and PtdIns(4,5)P $_2$, and the increased rate of auto-phosphorylation displayed by p110 γ with a phosphate-mimicking mutation (Ser582Glu), suggests that a structural change in the helical domain of p110 γ is sufficient to increase the catalytic activity independent of the presence of the p84 subunit. Previous work examining the activation of the class IA p110 α , p110 β , and p110 δ catalytic subunits has shown that part of the activation mechanism occurs through a conformational change from a closed cytosolic form to an open form on membranes [25,26]. The helical domain of p110 γ is exquisitely well placed to propagate conformational changes due to the fact that it is in contact with every other domain in p110 γ . In the crystal structure of N-terminally truncated (Δ 144) p110 γ , the side chain of Ser582 points inward [47,48], and has to rotate to accommodate a phosphate. Our HDX-MS results showed a dynamic “breathing” motion in the helical domain in the free p110 γ catalytic subunit that may allow for temporary exposure of Ser582, enabling modification by PKC. HDX results showed that the p84 subunit slowed or prevented this dynamic motion, and this correlated with a decreased efficiency of phosphorylation by PKC in cells in the presence of p84.

Although Ser582 is not in a direct contact with the kinase domain, it is structurally linked to it: the heat repeat HA1/HB1 housing Ser582, and the connecting intra-helical loop (residues 560 to 570), along with helix A3 (624–631) are in contact with helices κ 9 and κ 10 in the C-lobe of the kinase domain (known as the regulatory arch [49,50]) and could transduce a conformational change to the catalytic center of p110 γ (Figure 7C). The phosphorylated Ser582 and phosphorylation-mimicking mutants may activate lipid kinase activity by causing a conformational shift at this interface. It has been shown recently that this region of the p110 γ kinase domain is critical in regulating lipid kinase activity, as phosphorylation of Thr1024 in the κ 9 by protein kinase A (PKA) negatively regulates p110 γ activity in vitro and in cardiomyocytes (Figure 7C) [51].

In contrast to the p110 α -p85 heterodimer, stabilized by the N-terminal adaptor-binding domain (ABD)/inter SH2 domain interaction, the association of the p110 γ subunit with its adaptor subunit is quite vulnerable, and the Ser582Glu mutant, but not Ser582Ala, abrogated the formation of a p110 γ -p84 complex. HDX-MS revealed that the helical domain of p110 γ was stabilized by p84. Ser582 is located in the center of the p110 γ -p84 contact surface, which explains how a change in charge (Ser582Glu) breaks the interaction with p84, either by direct contact or by destabilization of the helical domain. Overexpression of p84 shields p110 γ from a PMA-induced phosphorylation, and suggests that binding of p84 to p110 γ , and Ser582 phosphorylation by PKC β are mutually exclusive. This implies that the two activation modes of PI3K γ —by GPCRs or PKC β —are completely separated. At low [Ca $^{2+}$] $_i$, PI3K γ is exclusively activated by G β γ subunits. It has been demonstrated that a PI3K γ adapter protein is absolutely needed for functional GPCR inputs to p110 γ [6]. If the interaction of p84 with p110 γ is blocked by the phosphorylation of

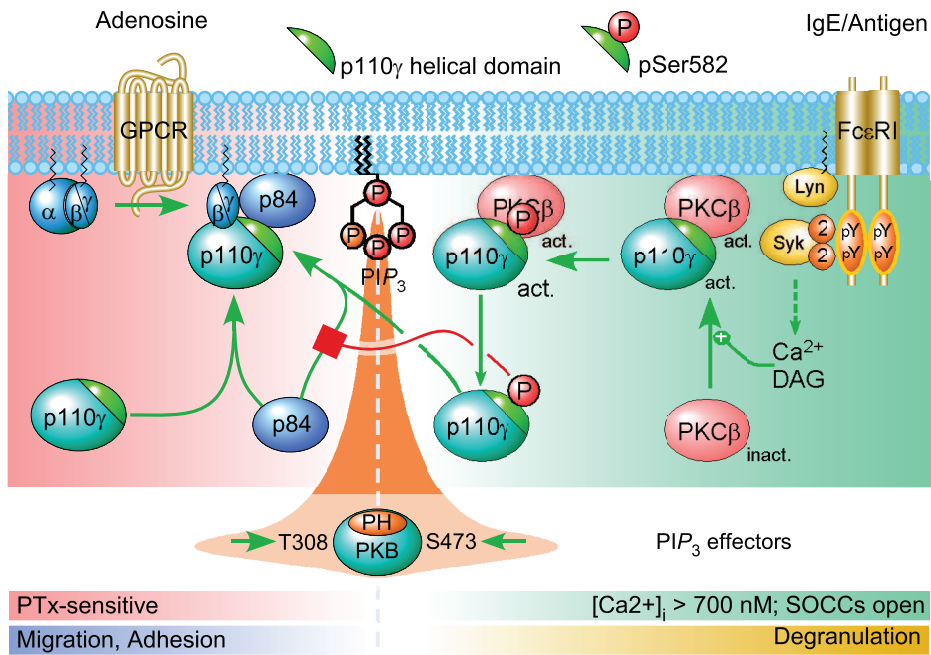


Figure 8. Phosphorylation of Ser582—loss of GPCR coupling of p110 γ . In a resting mast cell, the PI3K γ complex is responsive to GPCR-mediated dissociation of trimeric G proteins. An adapter protein (here p84) is required for a productive relay of the GPCR signal to PI3K γ . When Fc ϵ RI receptors are clustered via IgE/antigen complexes, a signaling cascade is initiated, which triggers the depletion of intracellular Ca²⁺ stores and the opening of store-operated Ca²⁺ channels. The resulting increase in [Ca²⁺]_i and PLC γ -derived diacylglycerol activate PKC β , which binds to p110 γ and subsequently phosphorylates Ser582 (\rightarrow pp110 γ). Phosphorylated p110 γ cannot interact with p84, and is therefore unresponsive to GPCR inputs. GPCR input to PI3K γ coincides with migration and adhesion, while Ca²⁺/PKC β activation of p110 γ occurs when mast cells degranulate. The phosphorylation of PKB/Akt occurs downstream of PtdIns(3,4,5)P₃, which originates from G protein-activated p84-p110 γ complex or PKC β -activated pp110 γ . The phosphorylated residues Thr308 and Ser473 of PKB/Akt are used to monitor PI3K activation. More detailed effector signaling event schemes can be found in [52].

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Ser582, p110 γ is decoupled from its GPCR input (for a schematic view of the process see Figure 8). The PKC β -mediated activation and phosphorylation of p110 γ constitutes therefore an unprecedented PI3K molecular switch, which enables the operation of p110 γ downstream of Fc ϵ RI signaling, and will elucidate cell type-specific activation processes in allergy and chronic inflammation.

Materials and Methods

Cells and Mice

BMMCs were derived from bone marrow of 8–12-wk-old C57BL/6J wild type, p110 γ ^{-/-}, PKC α ^{-/-}, PKC β ^{-/-}, and PKC γ ^{-/-} mice, and cultured and characterized as described in [9]. Animal experiments were carried out in accordance with institutional guidelines and national legislation. Human embryonic kidney 293 (*HEK293*) cells were grown in DMEM supplemented with 10% HI-FCS, 2 mM L-glutamine, 100 units/ml penicillin, 100 μ g/ml streptomycin. *Sf9* cells were cultivated in IPL-41 medium (Genaxxon Bioscience) supplemented with 10% HI-FCS, 2% yeastolate, 1% lipid concentrate, 50 μ g/ml gentamicin (Invitrogen), and 2.5 μ g/ml amphotericin B (Genaxxon Bioscience). Detailed descriptions and references are available in Text S1.

Cellular PtdIns(3,4,5)P₃ Measurements

PtdIns(3,4,5)P₃ levels have been measured as described in [9] with some modifications. BMMCs were cultured for 2 h in phosphate-free RPMI medium/2% FCS at 37°C and 5% CO₂, followed by labelling with 1 mCi/ml [³²P]-orthophosphate for 4 h. Cells were washed, stimulated, and lysed by the addition of

chloroform/methanol (1:2, v/v, with butylated hydroxytoluene and carrier phosphoinositides). Lipids were extracted, deacylated, and separated by high-pressure liquid chromatography (HPLC).

In Vitro Lipid Kinase Assay

PI3K γ -His₆ was incubated with PtdIns(4,5)P₂-containing lipid vesicles (PE/PS/PC/SM/PIP₂ = 30/20/10/4.5/1.2; PIP₂ final 5 μ M), 10 μ M ATP, and 4 μ Ci of [³²P]-ATP in lipid kinase buffer (40 mM HEPES [pH 7.4], 150 mM NaCl, 4 mM MgCl₂, 1 mM DTT [1,4-Dithio-DL-threitol], 0.1 mg/ml fatty-acid free BSA) for 10 min at 30°C. Alternatively, PtdIns/PS vesicles (~200 μ M each) were used. Reactions were terminated by addition of 1 M HCl and CHCl₃/MeOH. Lipids were isolated by chloroform extraction, separated by TLC and quantified on a Typhoon 9400.

Statistical Analysis

Numeric results were tested for significance using a two-tailed Student's *t* test, (paired or unpaired, as imposed by datasets). * or &, ** or &&, and *** or &&& refer to *p*-values *p* < 0.05, *p* < 0.005, and *p* < 0.0005. * and & were used for comparison of different genotypes, stimuli and conditions as indicated. Calculations were carried out using Graph Pad Prism, Microsoft Excel, or Kaleidagraph software.

Deuterium Exchange Reactions

Protein stock solutions (5 μ l; Hsp110 γ -C-His₆: 30 μ M; Hsp110 γ -C-His₆/Mmp84-C-His₆: 35 μ M) were prepared in 20 mM Tris [pH 7.5], 100 mM NaCl, 1 mM ammonium sulfate,

and 5 mM DTT. Exchange reactions were initiated by addition of 25 μ l of a 98% D₂O solution containing 10 mM HEPES (pH 7.2), 50 mM NaCl, and 2 mM DTT, giving a final concentration of 82% D₂O. Deuterium exchange reactions were allowed to carry on for seven time periods, 3, 10, 30, 100, 300, 1,000, and 3,000 s of on-exchange at 23°C, before addition of quench buffer. On-exchange was stopped by the addition of 40 μ l of a quench buffer containing 1.2% formic acid and 0.833 M guanidine-HCl, which lowered the pH to 2.6. Samples were then immediately frozen in liquid nitrogen until mass analysis. The full HDX-MS protocol can be found in Text S1.

Supporting Information

Figure S1 PAF-mediated signaling does not activate PI3K, and does not synergize with Fc ϵ RI co-stimulation (related to Figure 1). IgE-sensitized (100 ng/ml IgE, overnight) or non-sensitized wild type BMMCs were IL-3 depleted for 3 h and stimulated with either 1 μ M adenosine (Ade), 1 μ M PAF, or 5 ng/ml antigen (post IgE-sensitization) \pm PAF for 2 min. Subsequently, cell lysates were subjected to SDS-PAGE. Phosphorylation of Ser473 in PKB/Akt (A), (B) Ser133 in cyclic AMP-responsive element-binding protein (CREB) and Ser660 in PKC β II was monitored by immunodetection with phosphosite-specific antibodies ($n = 3$, *: $p < 0.05$; * refers to unstimulated control). (EPS)

Figure S2 Effect of PKC-inhibitors on PMA- or adenosine-induced PKB phosphorylation (S473) (related to Figure 3). (A/B) Wild type BMMCs were starved for 3 h in IL-3 free medium supplemented with 2% FCS, and preincubated with the inhibitors for 20 min before stimulation (pan-PKC: Ro318425, G66983; classical PKC: PKC412 [CPG41251]; classical and atypical PKC: G66976; Rotterlin: broad band inhibitor; all 1 μ M; & refers to comparison with untreated, stimulated control). (C) PKB activation in response to 100 nM PMA or 1 μ M Thapsigargin (2 min) was analyzed in wild type, PKC $\alpha^{-/-}$ and PKC $\gamma^{-/-}$ BMMCs. Cells were deprived of IL-3 as in (A/B). PKB (S473) and MAPK (T183/Y185) phosphorylation was determined by Western blotting. (D) Wild type, PKC $\beta^{-/-}$ and PI3K $\gamma^{-/-}$ BMMCs were stimulated with different concentrations of thapsigargin, before degranulation was quantified by the measurement of β -hexosaminidase release into cell supernatants (data are the average of three independent experiments \pm standard error of the mean [SEM]; * comparison to wild type (both genotypes); & only the p110 $\gamma^{-/-}$ dataset reached significance). (EPS)

Figure S3 Identification of PI3K γ phosphorylation sites by MS (related to Figure 4). (A/B) Recombinant, catalytically inactive GST-PI3K γ (K833R mutant; GST fused to p110 γ amino acids 38–1,102) was phosphorylated *in vitro* by recombinant PKC β in the presence of 100 μ M ATP/[γ -³²P]-ATP. Proteins were separated by SDS-PAGE and trypsin-digested PI3K γ was analyzed by LC-MS/MS. (A) Enhanced product ion spectra of the tryptic phospho-S582-peptide of PI3K γ . The γ - and β -fragments detected are indicated in the sequence. Fragments showing a H₃PO₄ loss are marked with an asterisk. The β_2 , γ_6 , and γ_7 fragments allow assignment of the phosphorylation to serine 3 in the peptide. (B) Enhanced product ion spectra of the non-phosphorylated form of this peptide. (C) Relevant information for the MRM analysis of the peptides containing Ser582. The amino acid numbering is as in Swiss-Prot entry P48736. (D) Sequence alignment of the beginning of the helical domain of class I PI3Ks.

Alignments were done by inspection of the crystal structures of PI3K γ (1E8Y), PI3K α (3HHM), PI3K β (2Y3A), and PI3K δ (2WXR). Secondary structure elements are labeled as indicated in the legend. S582 is colored red, while cancer-associated PI3K α mutations are marked as blue. (TIF)

Figure S4 Anti-phospho-Ser582 antibody validation (related to Figure 5). (A) Wild type BMMCs were transfected with empty vector, expression plasmid for GFP-PI3K γ wild type or the GFP-PI3K γ S582A mutant. On the next day, cells were stimulated with 200 nM PMA for 45 s, and PI3K γ was immunoprecipitated from cell lysates. Specificity of the anti-S582 antibodies was validated by Western blotting. (EPS)

Figure S5 Global and site-specific *in vitro* phosphorylation of monomeric p110 γ and p110 γ -p84 complexes by PKC β and CamKII (related to Figure 5). (A) Equal amounts of purified recombinant p110 γ -His₆ (2.5 pmole) or p110 γ -His₆/EE-p84 complexes were incubated with 20 pmole wortmannin to eliminate auto-phosphorylation signals. Free or complexed p110 γ (with p84 protein) was incubated with 10 μ M ATP and 5 μ Ci of [³²P]- γ -ATP, and equal specific activity of recombinant PKC β II and CamKII (Life Technologies assays: 30 pmol phosphate incorporation/min.) for 30 min at 30°C. Subsequently, proteins were denatured and separated by SDS-PAGE followed by Coomassie staining (lower panel). ³²P-incorporation was visualized by autoradiography and quantified on a phospho-imager (Typhoon 9400, middle panel). Band intensities were quantified with ImageQuant TL Software (Amersham Biosciences, top panel; $n = 4$, * $p < 0.05$). Insert: quantification of p84 phosphorylation from the reactions shown in (A), (open bars, $n = 4$, * $p < 0.05$). Phospho-PKC β II levels were subtracted from phospho-p84 signals (due to identical apparent Mr on SDS-PAGE). The filled bar represents phosphorylated p84-His₆ after Ni²⁺-NTA pull-down to minimize contaminating PKC β II autophosphorylation signals ($n = 3$). (B) Equal amounts of purified recombinant p110 γ -His₆ (2.5 pmole) or p110 γ -His₆/EE-p84 complexes were incubated as indicated with ATP (100 μ M), recombinant PKC β II and CamKII for 30 min at 30°C. Proteins were denatured, separated by SDS-PAGE followed by immunological detection of p110 γ and p84 protein, as well as site-specific phosphorylation of residue Ser582 (pp110 γ S582) in p110 γ . PKC β II and CamKII input was adjusted to equal protein kinase activity (30 pmol of transferred phosphate/min). For quantification, Ser582 phosphorylation levels were normalized ($n = 4$, *: $p < 0.05$, ***: $p < 0.0005$). (EPS)

Figure S6 Phosphorylation of p110 γ requires active PKC β (related to Figure 5). (A) Effect of PKC inhibitors on thapsigargin induced p110 γ Ser582 phosphorylation. IL-3 deprived BMMCs were preincubated with PKC inhibitors (1 μ M Ro318425 or 1 μ M AEB071) for 20 min before stimulation with 1 μ M thapsigargin for 2 min. PI3K γ was immunoprecipitated from cell lysates with anti-p110 γ antibody (see Methods). Precipitated protein was then probed for phosphorylated p110 γ (pp110 γ) using phospho-specific anti-pSer582 antibodies. PI3K γ phosphorylation is shown normalized to total p110 γ levels (mean \pm standard error of the mean [SEM], $n = 3$; * depict comparison with stimulated control). (B) Cells were stimulated as in (A). PKB (T308) and CamKII (T286) phosphorylation was determined by Western blotting. Data are the average of three independent experiments \pm SEM. (EPS)

Figure S7 Ser582 phosphorylation releases p84 from p110 γ (related to Figure 6). IL-3 deprived BMDCs were stimulated with 100 nM PMA for 2 min. PI3K γ complex was co-immunoprecipitated from cell lysates using either (A) anti-p110 γ or (B) anti-p84 antibodies. The amount of p84 co-immunoprecipitated with p110 γ (A) or p110 γ co-immunoprecipitated with p84 (B) was normalized to the total amount of p110 γ or p84, respectively. Data are the average of five independent experiments \pm standard error of the mean [SEM]. (EPS)

Figure S8 PI3K γ domain order and peptide coverage after pepsin digestion (related to Figure 7). Identified and analyzed peptides are shown under the primary sequence of PI3K γ , which has been colored according to the domain boundaries shown above. (EPS)

Figure S9 Changes in deuteration levels of PI3K γ in the presence of p84 (related to Figure 7). Peptides spanning PI3K γ (labeled A–Q) that showed greater than 0.5 Da changes in deuteration in the presence and absence of p84 were mapped onto the PI3K γ structure (PDB ID: 2CHX, residues 144–1,093). The greatest difference in exchange observed at any time was used for the mapping. S582 is shown as red balls. The ATP competitive inhibitor PIK-90 is shown in green as a reference point for the kinase domain. The linker regions between the RBD and the C2 domain and the C2 and helical domain are shown as dotted lines. (EPS)

Figure S10 Changes in deuteration levels of PI3K γ peptides in the presence of p84 (related to Figure 7). The graphs showing the number of incorporated deuterium atoms in the presence (o) and absence (•) of p84 at seven time points in peptides that showed a greater than 0.5 Da H/D exchange difference. Data represent mean \pm standard deviation (SD) of two independent experiments. (EPS)

Figure S11 Deuteration levels in free and p84-bound p110 γ (related to Figure 7). Changes in deuteration levels were mapped onto the crystal structure of PI3K γ (PDB ID: 2CHX) as in Figure 7. The isotopic profiles of two selected peptides (579–592, 623–630) from the helical domain are shown at three or

four time points of H/D on exchange \pm the p84 subunit. In the absence of the p84 adaptor the majority of peptides in the helical domain showed broadening of the isotopic profiles indicative of EX1 kinetics (see 30 s of HDX in free p110 γ). The helices HB1, HA2 (579–592), and HA3 (624–631) selected are all structurally linked, with HA3 located at the interface of the helical domain with the C-lobe. Ser582 (red) and Thr1024 (yellow) have been highlighted as a reference.

(TIF)

Table S1 Deuterium exchange data of all analyzed peptides of PI3K γ in the absence or presence of p84 are summarized in tabular form (related to Figure 7).

Percent hydrogen deuterium exchange was calculated for each of the seven time points and colored according to the legend. Data show the mean of two independent experiments. The charge state (Z), maximal number of exchangeable amides (#D), starting residue number (S), and ending residue number (E) are displayed for every peptide.

(XLSX)

Text S1 Extended experimental procedures, reference to animals and plasmids. Detailed description of experimental procedures, materials, and further reference to the origin of genetically modified mice used here, and a primer to the determination of deuterium incorporation (HDX_MS).

(DOCX)

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Author Contributions

The author(s) have made the following declarations about their contributions: Conceived and designed the experiments: RW JEB EG TB RLW MPW. Performed the experiments: RW JEB EG TB XZ DH PK MuL MPW. Analyzed the data: RW JEB EG TB DH RLW MPW. Contributed reagents/materials/analysis tools: MiL EH. Wrote the paper: RW JEB EG TB RLW MPW.

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