Different inhibition of Gβγ-stimulated class IB phosphoinositide 3-kinase (PI3K) variants by a monoclonal antibody. Specific function of p101 as a Gβγ-dependent regulator of PI3Kγ enzymatic activity

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Class IB phosphoinositide 3-kinases γ (PI3Kγ) are second-messenger-generating enzymes downstream of signalling cascades triggered by G-protein-coupled receptors (GPCRs). PI3Kγ variants have one catalytic p110γ subunit that can form two different heterodimers by binding to one of a pair of non-catalytic subunits, p87 or p101. Growing experimental data argue for a different regulation of p87–p110γ and p101–p110γ allowing integration into distinct signalling pathways. Pharmacological tools enable distinguishing modulation of the two variants. Missing the ability of an anti-p110γ monoclonal antibody [mAb(A)p110γ] to block PI3Kγ enzymatic activity attracted us to characterize this tool in detail using purified proteins. In order to get insight into the antibody–p110γ interface, hydrogen–deuterium exchange coupled to MS (HDX-MS) measurements were performed demonstrating binding of the monoclonal antibody to the C2 domain in p110γ, which was accompanied by conformational changes in the helical domain harbouring the Gβγ-binding site. We then studied the modulation of phospholipid vesicles association of PI3Kγ by the antibody, p87–p110γ showed a significantly reduced Gβγ-mediated phospholipid recruitment as compared with p101–p110γ. Concomitantly, in the presence of mAb(A)p110γ, Gβγ did not bind to p87–p110γ. These data correlated with the ability of the antibody to block Gβγ-stimulated lipid kinase activity of p87–p110γ 30-fold more potently than p101–p110γ. Our data argue for differential regulatory functions of the non-catalytic subunits and a specific Gβγ-dependent regulation of p101 in PI3Kγ activation. In this scenario, we consider the antibody as a valuable tool to dissect the distinct roles of the two PI3Kγ variants downstream of GPCRs.

Key words: Gβγ, G-protein, p101, p87, phosphoinositide 3-kinase γ (PI3Kγ), signal transduction.

INTRODUCTION

Class I phosphoinositide 3-kinases (PI3Ks) are lipid kinases that transduce extracellular signals to trigger PtdIns(3,4,5)P_3 synthesis, an essential second messenger at the plasma membrane. PtdIns(3,4,5)P_3, together with its metabolites, PtdIns(3,4)P_2 and PtdIns(3,5)P_2, play fundamental roles in the regulation of basic cellular processes, such as proliferation, differentiation, growth and chemotaxis [1–8]. Class I PI3Ks are heterodimers composed of a catalytic (p110) and a non-catalytic subunit of the p85- or p101-type. Based on their interaction with non-catalytic subunits and their specific modes of regulation, class I PI3Ks can be further subdivided into class IA and class IB [2,3,9–12]. Class IA is characterized by heterodimers consisting of a catalytic p110κ, p110β or p110δ subunit associated with a p85-type non-catalytic subunit, which has dual roles acting as an adaptor and a regulator [11,13–16]. Although the p85-type subunit is indispensable for class IA PI3K stability and regulation, the p110 catalytic subunit determines the signalling specificity [17–24].

The class IB PI3Ks are represented by two enzymes consisting of one catalytic p110γ subunit associated with either a p101 or a p87 (also known as p87PRKAP or p84) non-catalytic subunit [25–29]. Both PI3Kγ variants, i.e. p87–p110γ and p101–p110γ, are stimulated by Gβγ heterodimers released upon G-protein-coupled receptor (GPCR) activation and by active Ras proteins [25–39]. The former view of p87 and p101 being redundant adapters in Gβγ-mediated recruitment of PI3Kγ variants to the membrane compartment [27–29] has been challenged by previous data showing a different contribution of Gβγ and Ras on the two PI3Kγ variants [38]. In particular, distinct Gβγ-binding affinities of the non-catalytic subunits for p110γ are intriguing [38,40,41]. These findings support data showing that PI3Kγ variants integrate into different and independent signalling cascades [39,42–44]. We have previously reported
specific features for p87 and p101, such as diverse spatial and temporal distribution in human tissues and a different regulatory impact on p110γ activity, which may contribute to the differential regulation of the PI3Kγ variants [40,41]. These findings, in combination with the fact that only a single class IB catalytic subunit is present in cells, led us to posulate that p87 and p101 serve as signal-discriminating regulatory subunits defining specific functions for both p87–p110γ and p101–p110γ variants [41]. However, the exact molecular mechanisms that maintain the specificity and selectivity of the two PI3Kγ variants are still unknown.

In the present study, we have identified and characterized a functional monoclonal anti-p110γ antibody that specifically inhibits the Gβγ-induced p87–p110γ enzymatic activity via contacting the C2 domain of p110γ. Our results point to a differential impact of the non-catalytic subunits thereby revealing a specific Gβγ-dependent regulatory role of p101 in PI3Kγ activation.

EXPERIMENTAL

Cell cultures and expression plasmids

Human embryonic kidney (HEK)-293 cells (German Resource Centre for Biological Materials) were cultured and transfected with expression plasmids encoding p101 and p110γ as described previously [27,37,38]. For preparation of whole cell lysates, cells were directly lysed by adding 1× Laemmli sample buffer [45].

Expression and purification of recombinant proteins

SF9 cells (fall armyworm ovary; Invitrogen) were cultured and infected as described previously [40]. Recombinant baculoviruses for expression of Gβγζ, PI3Kγ and PI3Kβ subunits as well as their expression in SF9 cells and purification of (His)6-tagged recombinant Gβγζ, (His)p110γ, p87–(His)p110α, p110γ, p101–(His)p110γ and p85–(His)p110β have been described elsewhere [38,40,41,48]. The pFastBac HTb baculovirus transfer vector (Invitrogen) was used to generate human full-length N-terminally (His)6-tagged H-Ras using BamHI/XhoI cloning site. H-Ras was produced in SF9 insect cells and isolated using the Triton X-114 partition method as described previously [48,49]. The post-translational processing and lipidation of the protein was verified by MS analysis. Purified proteins were quantified by Coomassie Brilliant Blue staining after SDS/PAGE (10% acrylamide) with BSA as the standard. The proteins were stored at −80°C.

Hydrogen–deuterium exchange coupled to MS measurements

Hydrogen–deuterium exchange coupled to MS (HDX-MS) analyses of PI3Kγ in the presence and absence of an anti-p110γ monoclonal antibody [mAb(A)p110γ] were performed following a similar protocol as described previously [21,48]. The rate of exchange of full-length p110γ(His)ζ alone and in the presence of a 3-fold molar excess of mAb(A)p110γ were compared. Reactions were initiated by mixing 10 μl of protein solution with 40 μl of deuterated buffer containing 20 mM Hepes, pH 7.2, 50 mM NaCl and 0.5 mM EGTA. Deuterium reactions were run for 3, 30, 300 and 3000 s on-exchange at 23°C, before being quenched by addition of 20 μl of a 2 M guanidinium chloride and 1.2% formic acid solution. The final deuterium concentration during the reaction was 78%. Every time point and state was a unique experiment and every HDX-MS experiment was repeated twice.

Samples were immediately frozen in liquid nitrogen and stored at −80°C for less than 1 week.

Analysis of the p110γ deuteration level was done as described previously [48], by sequentially digesting the protein with pepsin, separating the fragments on a C18 column and measuring the masses of peptides on a LTQ Orbitrap XL mass spectrometer. Manually selected peptides were then examined for deuterium incorporation by the HD-examiner software (Sierra Analytics). Results are presented as relative levels of deuteration with no correction for back exchange.

Gel electrophoresis, immunoblotting and antibodies

Generation and characterization of the anti-serum against the Gβγζ subunit are detailed elsewhere [31,50]. Specific antibodies against p87 and p101 were gifts from Michael Schaefer (Rudolf-Boehm-Institut für Pharmakologie und Toxikologie, Leipzig, Germany) and Len Stephens (Babraham Institute, Cambridge, U.K.) respectively. mAb(A)p110γ and mAb(B)p110γ were raised against full-length human p110γ using mouse hybridoma cells and were characterized earlier [37]. Large-scale preparations of mAb(A)p110γ were generated in co-operation with BioGenes. mAb(B)p110γ was as described earlier [31,40,41]. Generation and characterization of mAb(C)p110γ, raised against the N-terminal 210 amino acids of catalytic p110γ, was as detailed earlier [43]. Anti-Ras antibody was purchased from BD Biosciences. Anti-p110β antibody was purchased from Cell Signaling Technology. Proteins were fractionated by SDS/PAGE (10% acrylamide) and transferred onto nitrocellulose membranes (Hybond™C Extra, GE Healthcare). Visualization of specific antisera was performed using the ECL system (GE Healthcare) or the SuperSignal® West Pico Chemiluminescent Substrate (Pierce) according to the manufacturers’ instructions. Chemiluminescence signals were estimated using the VersaDoc™ 4000 MP imaging system (Bio-Rad Laboratories).

Immunoprecipitation of PI3K

Purified recombinant p110γ, p87–p110γ and p101–p110γ and p85α–p110β variants were subjected to immunoprecipitation (IP) using mAb(A)p110γ, mAb(B)p110γ or mAb(C)p110γ. IP experiments were performed as detailed previously [41] with some modifications. In brief, Protein A–Sepharose CL-4B beads (GE Healthcare) were pre-incubated with or without antibody, washed, incubated overnight with cleared cell lysates or purified proteins and washed again. Proteins bound to beads were either tested for their lipid kinase activity or eluted by adding 1× Laemmli sample buffer [45] and subjected to SDS/PAGE.

Analysis of PI3K enzymatic activity

The lipid kinase activity of PI3Kγ and analysis of Gβγζ, H-Ras and PI3Kγ association with phospholipid vesicles were performed as described previously [32,34,40,41,46].

Analytical ultracentrifugation analyses

Molecular mass and complex stability of purified p87–p110γ and p101–p110γ heterodimers were analysed by sedimentation equilibrium analysis using a Beckman Optima XL-1 centrifuge using the AN-60Ti rotor with the absorption optics set to 280 nm. Analyses were conducted in a buffer containing 20 mM Tris/HCl, pH 8.0, 150 mM NaCl, 2 mM DTT and 0.033% deca(ethylene...
In order to test the selectivity of the mAb(A)γ110γ antibody, we measured its effect on the activity of the class IA PI3Kβ, another Gβγ-sensitive PI3K. Recombinant and functionally active Gβγ-sensitive p85α–p110β was purified following heterologous expression in Sf9 cells (Figures 2A and 2B). IP experiments (Figure 2C) as well as analysis of the immunoprecipitates in the lipid kinase assays (Figure 2D) showed complete lack of interaction between mAb(A)γ110γ and p85α–p110β. Correspondingly, mAb(A)γ110γ did not inhibit lipid kinase activity of purified p85α–p110β (Figure 2E).

Mapping of p110γ regions affected by interaction with mAb(A)γ110γ.

Since mAb(A)γ110γ was generated by an immunization and selection protocol using full-length human catalytic p110γ subunit, the epitope of p110γ targeted by mAb(A)γ110γ was unknown. To determine the p110γ epitope recognized by mAb(A)γ110γ, we used HDX-MS. HDX-MS is a powerful technique that can map protein–protein and protein–lipid interactions, as well as provide useful information on the dynamics of proteins [53,54]. The technique is based on the differences in exchange rate of amide protons from a protein with solvent, a reaction that is influenced by secondary structure and solvent exposure.

To map the regions in p110γ that are affected by the interaction with mAb(A)γ110γ, we compared the HDX rates of p110γ in solution and when in a complex with mAb(A)γ110γ. A large proportion of the C2 domain shows a reduced HDX rate in the p110γ–mAb(A)γ110γ complex, suggesting that the antibody binds this region of p110γ (Figures 3A and 3B). More precisely, the most solvent-exposed part of the C2 domain, spanning residues 382–413, has a strongly reduced dynamics, probably stabilizing the β-strand underneath (residues 414–428). Interestingly, binding of mAb(A)γ110γ seems to induce allosteric changes in p110γ, as increased HDX rates are observed in two distinct domains of p110γ: the helical and kinase domains (Figure 3B). The increased dynamics in the p110γ helical domain (551–607) overlaps with the previously identified Gβγ-binding site (546–607) [48]. The two helices within the kinase domain that show increased dynamics (1035–1050) correspond to a region essential for inhibition of p110α by its regulatory subunit [55].

In summary, HDX-MS experiments revealed that mAb(A)γ110γ associates with the C2 domain of p110γ and induces conformational changes in the helical and kinase domains. Since both domains are important for PI3Kγ regulation, binding of mAb(A)γ110γ to p110γ might affect kinase enzymatic activity.

RESULTS

Inhibition of monomeric p110γ by mAb(A)γ110γ.

A monoclonal anti-p110γ antibody [mAb(A)γ110γ] raised against full-length human catalytic p110γ subunit used in earlier IP experiments [37] displayed interesting features attracting our attention. mAb(A)γ110γ failed to visualize p110γ in immunoblots (Figure 1A); however, it was able to interact with the intact protein in solution enabling IP experiments (Figure 1B). The feature of recognizing native p110γ made it worthwhile to test whether mAb(A)γ110γ interferes with p110γ activity. As shown in Figure 1(C), incubation with mAb(A)γ110γ led to a drastic reduction in p110γ lipid kinase activity stimulated by Gβγ, defining mAb(A)γ110γ as a putative PI3Kγ inhibitor.

Statistical analysis

Results (means±S.E.M.) were analysed using Student’s t test (*P < 0.05; **P < 0.01).

Effect of mAb(A)γ110γ on p87–p110γ and p101–p110γ heterodimer activity

Class IB PI3Kγ is present as two distinct functional p87–p110γ and p101–p110γ heterodimers in vivo [26,38,41,42]. We tested how mAb(A)γ110γ affects the enzymatic activities of these two PI3Kγ variants stimulated by Gβγ. Two additional monoclonal antibodies raised against full-length human catalytic p110γ subunit [mAb(B)γ110γ] and N-terminal amino acids 1–210 of p110γ [mAb(C)γ110γ] were also included in order to validate the specificity of interactions. As depicted in Figure 4(A), significant differences in the ability of the antibodies to affect lipid kinase activities of the two PI3Kγ variants became apparent. Although incubation of p87–p110γ with mAb(A)γ110γ resulted in drastic reduction in Gβγ-stimulated activity.
lipid kinase activity, inhibition of p101–p110γ activity by this antibody, at the concentrations tested, was weak. In contrast, mAb(B)p110β and mAb(C)p110β were ineffective in inhibiting enzymatic activity of either PI3Kγ variant under the identical experimental conditions (Figure 4A). The intriguing finding of the differential mAb(A)p110γ-mediated effect on the two PI3Kγ variants showing only weak inhibition of p101–p110γ as compared with strong inhibition of p87–p110γ prompted us to check whether mAb(A)p110γ was able to interact with p110γ when associated with p101. Comparable to monomeric p110γ (Figure 1A), immunoblotting (IB) analysis revealed that mAb(A)p110γ does not recognize denatured p101–p110γ complex (Figure 4B). In contrast, mAb(B)p110γ and mAb(C)p110γ recognize p110γ in immunoblots (Figure 4B). Nonetheless, the capability of mAb(A)p110γ to directly bind to p110γ when complexed with p101 could be verified by IP (Figure 4C).

Taken together, mAb(A)p110γ inhibits Gβγ-stimulated lipid kinase activity of p87–p110γ more potently than of p101–p110γ.

Interaction of p87–p110γ or p101–p110γ heterodimers with phospholipid vesicles

The HDX-MS data demonstrate binding of mAb(A)p110γ to the C2 domain of p110γ (Figure 3B). The C2 domain of p110γ, similarly to other C2 domains, is considered to mediate protein–lipid interactions [56–58]. This encouraged us to check whether mAb(A)p110γ interferes with Gβγ-mediated association of p87–p110γ or p101–p110γ to phospholipid vesicles in the absence and presence of another known PI3Kγ regulator, i.e. H-Ras. Strikingly, mAb(A)p110γ differently affected Gβγ-mediated phospholipid vesicle association of PI3Kγ variants. Whereas mAb(A)p110γ strongly reduced Gβγ-mediated vesicle association of p87–p110γ in a concentration-dependent manner, association of p101–p110γ remained unchanged (Figure 5A). mAb(A)p110γ did not change binding of p101–p110γ to phospholipid vesicles upon exposure to both regulators, Gβ1γ2 and H-Ras (Figure 5B). However, concomitant incubation with Gβ1γ2 and prenylated H-Ras partially rescued phospholipid vesicle association of p87–p110γ in the presence of mAb(A)p110γ. Nonetheless, membrane association was impaired by high concentrations of mAb(A)p110γ (Figure 5B). It should be pointed out that in these experiments p87, p101 and p110γ were found in ratios corresponding the starting condition suggesting that the stoichiometry of the PI3Kγ variants bound to phospholipid vesicles was not affected by mAb(A)p110γ (Figure 5, grey or white bars compared with black bars). Control experiments excluded that the association of Gβγ or H-Ras to phospholipid vesicles was significantly affected by mAb(A)p110γ (Table 1).

High complex stability was supported by equilibrium analytical ultracentrifugation showing Kd values of ≤0.2 μM for p87–p110γ and ≤0.1 μM for p101–p110γ (Figure 6).

The interference of mAb(A)p110γ with Gβγ-binding was tested by co-IP of p87–p110γ or p101–p110γ with Gβ1γ2 and H-Ras (Figure 7). In the case of p87–p110γ, a reduction in Gβ1γ2
Figure 3  Binding mAb(A)p110γ to the C2 domain of p110γ promotes allosteric changes in distinct domains

(A) Global HDX in p110γ was analysed for the following states: p110γ alone and p110γ associated with mAb(A)p110γ. The HDX percentage for each p110γ peptide is shown at 3, 30, 300 and 3000 s. The beginning and ending residues for each peptide are illustrated along with the charge state (CS), number of amide deuterons (#D) and retention time (RT). Peptides in p110γ showing reduced (blue) and increased (red) HDX rate after incubation with mAb(A)p110γ are indicated with brackets. (B) Mapping of the changes in deuteration levels between free p110γ and p110γ bound to mAb(A)p110γ are visualized on p110γ crystal structure (top, PDB ID 1E8X) and on a schematic representation of p110γ sequence (bottom). Peptides with significant changes are identified on the p110γ model according to the colour scheme shown (red and orange indicate increased exposure on binding and cyan and blue represent decreased exposure).

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mAb(B)p110

p110

mAb(C)p110

γ

and p101–p110

S.E.M. (n = 3). (B) mAb(A)p110, does not interact with the catalytic p110γ subunit of denatured p101–p110γ in immunoblots. Heterodimeric enzyme was expressed in and purified from Sf9 cells. Different amounts of the recombinant protein were subjected to SDS/PAGE (10 % acrylamide) followed by IB using p87–p110γ antibodies specific for G12 and their supernatants were subjected to SDS/PAGE (10 % acrylamide) followed by IB using p87–p110γ or p101–p110γ γ-unspecific antibody as described in the Experimental section. Duplicates of immunoprecipitates were separated by SDS/PAGE (10 % acrylamide) followed by IB with mAb(A)p110γ, mAb(B)p110γ, mAb(C)p110γ, or mAb(B)p110γ-unspecific antibody as described in the Experimental section. Duplicates of immunoprecipitates were separated by SDS/PAGE (10 % acrylamide) followed by IB with mAb(A)p110γ, mAb(B)p110γ, or mAb(C)p110γ.

Table 1 mAb(A)p110γ does not change the association of Gβ1γ2 and H-Ras with phospholipid vesicles

Recombinant purified Gβ1γ2 dimers (600 nM) and H-Ras (1000 nM) were mixed with 28 nM p87–p110γ or p101–p110γ and incubated with phospholipid vesicles in the absence or presence of 25 nM or 120 nM mAb(A)p110γ. Aliquots of sedimented phospholipid vesicles and their supernatants were subjected to SDS/PAGE (10 % acrylamide) followed by IB using antibodies specific for Gβ1γ2 and H-Ras proteins. Chemiluminescence signals were estimated with a VersaDoc™4000 MP imaging system (Bio-Rad Laboratories). For calculation of phospholipid vesicle-associated proteins, signal intensities in the sedimented phospholipid vesicles and their supernatants were added and considered as 100 %. Shown here are the mean values ± S.E.M. for at least three separate experiments.

<table>
<thead>
<tr>
<th>Incubation with PI3Kγ variants</th>
<th>mAb(A)p110γ (nM)</th>
<th>Gβ1γ2</th>
<th>H-Ras</th>
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<td>29.2 ± 5.3</td>
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<td>25</td>
<td>24.7 ± 5.2</td>
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<td>120</td>
<td>32.1 ± 7.9</td>
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Concentration-dependent inhibition of PI3Kγ variants by mAb(A)p110γ

We studied concentration-dependent inhibition of variously stimulated lipid kinase activities of p87–p110γ and p101–p110γ in the presence of increasing concentrations either of the pan-PI3K inhibitor wortmannin (Figures 8A–8D) or mAb(A)p110γ (Figures 8E–8H). Wortmannin, which blocks all class I PI3Ks by covalent binding to a lysine residue in the ATP-binding pocket of p110 isoforms [59], inhibited both PI3Kγ variants at similar IC50 concentrations under all conditions tested and failed to differentiate between the two PI3Kγ variants.

In the presence of mAb(A)p110γ, basal lipid kinase activities of the two PI3Kγ variants were inhibited in a concentration-dependent manner with IC50 values of 7.2 ± 1.3 nM and 17.8 ± 5.2 nM for p87–p110γ and p101–p110γ respectively (Figure 8E). Strikingly, the Gβ1γ2-stimulated activity of p87–p110γ was inhibited ∼30-fold more potently as compared with the p101–p110γ counterpart (IC50 of 1.6 ± 0.5 nM compared with 46.5 ± 12.6 nM; Figure 8F). In contrast, mAb(A)p110γ inhibition of H-Ras-stimulated variants was indistinguishable (Figure 8G). When the enzymes were co-stimulated by Gβ1γ2 and H-Ras, p87–p110γ was 10-fold more potently inhibited as compared with p101–p110γ by mAb(A)p110γ (IC50 of 4.3 ± 0.4 nM compared with 49.5 ± 4.9 nM; Figure 8H). Thus, mAb(A)p110γ not only represents a valuable experimental tool to understand the different regulation of PI3Kγ variants but also serves to selectively intervene into Gβγ-induced p87–p110γ lipid kinase activity.

Table 1 mAb(A)p110γ does not change the association of Gβ1γ2 and H-Ras with phospholipid vesicles

Associative properties of phospholipid vesicles are monitored by Gβγ-immunoreactivity as evident, whereas H-Ras levels remained unaffected (Figure 7). Taken together, the data show a mAb(A)p110γ-dependent inhibition of Gβ1γ2-induced recruitment of p87–p110γ to the lipid compartment. Next, we investigated the consequences for enzymatic activity.
The mAb(A)p110γ was tested for its ability to affect Gβγ-mediated association (600 nM Gβγγ) of purified recombinant PI3Kγ variants (28 nM) with phospholipid vesicles in the absence (A) or presence of 1000 nM H-Ras (B). Aliquots of supernatants and sedimented phospholipid vesicles were subjected to SDS/PAGE (10 % acrylamide). Association of each PI3Kγ or presence of 1000 nM H-Ras (B) was assayed using mAb(A)p110γ or antibodies specific against p87 or p101. Chemiluminescence signals were estimated with a VersaDoc™ 4000 MP imaging system (Bio-Rad Laboratories). For calculation of phospholipid vesicle-associated protein for immunization and selection procedure and, therefore, the exact antibody–p110γ interaction site was unknown [37,61]. HDX-MS, an approach that has provided insight into PI3Kγ regulation at the membrane and by regulatory partners [21,48,62], identified dynamic changes within three domains of p110γ upon association with mAb(A)p110γ. Residues 382–428 in the C2 domain of p110γ were protected from HDX, most probably due to binding of the antibody to this region. In addition, antibody–p110γ interaction induced increased dynamics in both the helical and the kinase domain of p110γ, probably as a result of allosteric modifications.

Generally, C2 domains have been associated with membrane interactions. The C2 domain of p110γ was also proposed to be involved in the interaction of p110γ with the plasma membrane [58]. However, recent data looking at lipid-binding sites of class I PI3Ks have identified the C-terminal helix of the kinase domain rather than the C2 domain to be involved in binding to lipids [21,48,63]. Our data obtained in phospholipid pull-down assays are in agreement with these recent data. The necessity of the C2 domain of p110γ to act as the membrane

Figure 5  Effect of mAb(A)p110γ on the association of PI3Kγ variants with phospholipid vesicles

Figure 6  Comparable complex stability of p87–p110γ and p101–p110γ measured by analytical sedimentation equilibrium

Three concentrations (0.5, 2.0 and 4.0 μM) of p87–p110γ (A) and p101–p110γ (B) were centrifuged and analysed to yield the equilibrium concentration distributions of the protein complexes (measured by their absorption at 280 nm) as a function of the radial distance from the centre of the rotor at 6000 (○) and 11000 (●) rev/min for each of the three sample channels. The unbroken lines depict the best non-linear least squares fit of the hetero-association model to each complex. The residuals of the fits are shown at the bottom of each channel along the dotted line at 0.0. Sedimentation equilibrium analysis yielded weight-average molecular masses (196.8 ± 7.8 kDa for p87–p110γ and 206.6 ± 10.2 kDa for p101–p110γ) which were slightly less than the values calculated from the sequences of the proteins (210.7 kDa for p87–p110γ and 223.8 kDa for p101–p110γ), assuming a 1:1 stoichiometry for the complexes. The Kd values determined from these data are presented in the Results section.
**Figure 7** mAb(A)p110γ affects binding of Gβ1γ2 to p87–p110γ

Purified recombinant p87–p110γ or p101–p110γ (0.375 μg of catalytic p110γ subunit) in the absence or presence of Gβ1γ2 (1.25 μg) and H-Ras (1.25 μg) were subjected to IP using mAb(A)p110γ as described in the Experimental section. Duplicates of immunoprecipitates were separated by SDS/PAGE (10% acrylamide) followed by IB with mAb(B)p110γ. Co-immunoprecipitated Gβ1γ2 and H-Ras were visualized using specific anti-Gβ1γ2 and anti-Ras antibodies. Weak unspecific chemiluminescence signals detected by anti-Ras antibody in PI3Kγ immunoprecipitates in the absence of Gβ1γ2 and H-Ras are caused by light chains of mAb(A)p110γ.

Our data argue for a different effect of mAb(A)p110γ on Gβγ-mediated stimulation of p87–p110γ and p101–p110γ. HDX-MS analyses indicate that binding of mAb(A)p110γ to the p110γ C2 domain induces allosteric changes in the helical domain. Since the helical domain is responsible for Gβγ binding [48], it is possible that the conformational changes directly affect the affinity of Gβγ for p110γ. Additionally, the different potencies by which mAb(A)p110γ inhibits Gβγ stimulation of PI3Kγ variants may be a consequence of a distinct effect of the two non-catalytic subunits, i.e. p87 and p101, on PI3Kγ activity (Figure 9). Alternatively, since the p110γ helical domain is stabilized by the associated p87 or p101 regulatory subunits [48,66], one possibility of discriminative inhibition of PI3Kγ variants is that p101 protects...

**Figure 8** Discriminative inhibition of heterodimeric PI3Kγ variants by mAb(A)p110γ.

The activities of p87–p110γ or p101–p110γ either in the basal condition or in the presence of Gβ1γ2, H-Ras and Gβ1γ2 together with H-Ras were measured in the presence of increasing concentrations of pan-PI3K inhibitor wortmannin (A–D) or mAb(A)p110γ (E–H). (A and E) The activities of PI3Kγ variants were measured under basal conditions with 7 nM (n=2) or 14 nM (n=1) kinase in the assay. (B and F) The activities of PI3Kγ variants (1.5 nM) were measured in the presence of ECo values of Gβ1γ2 (300 nM for p87–p110γ and 30 nM for p101–p110γ). (C and G) The activities of PI3Kγ enzymes (7 nM) were measured in the presence of ECo values of H-Ras (450 nM for p87–p110γ and 850 nM for p101–p110γ). (D and H) The activities of PI3Kγ variants (1.5 nM) were measured in the presence of ECo values of H-Ras (450 nM for p87–p110γ and 850 nM for p101–p110γ) and ECo values of Gβ1γ2 (300 nM for p87–p110γ and 30 nM for p101–p110γ). The data shown in graphs and in tables are the mean values ± S.E.M. for at least three separate experiments.

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from allosteric changes induced by mAb(A)\textsubscript{p110γ}, more than for p87 does. This would explain the reduced inhibitory effect of the antibody for the p101–p110γ heterodimer compared with p87–p110γ and to p110γ. Ample evidence suggests that p101 acts as a Gβγ adaptor [26,32,37,38]. Since p101 is able to rescue the stimulatory activity of p87γ, as was shown in the case of Ras stimulation [35], mAb(A)p110γ-induced structural change of the kinase domain may interfere and reduce the basal lipid kinase activities of p87γ–p110γ and p101–p110γ. Slight protection of p101–p110γ basal lipid kinase activity from the inhibitory effect of mAb(A)p110γ, is in line with the previous data showing stimulatory modulation of p110γ by p101 independently of its Gβγ adapter function [41].

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**AUTHOR CONTRIBUTION**

Aliaksei Shymanets, Christian Harteneck and Bernd Nürnberg designed the study. Aliaksei Shymanets, Prijwal, Oscar Vadas, Cornelia Czupalla, Jaclyn LoPiccolo, Alessandra Ghigo and Eberhard Krause performed the experiments. Aliaksei Shymanets, Oscar Vadas, Michael Brentowitz, Eberhard Krause, Emilio Hirsch, Reinhard Wetzel, Roger Williams, Christian Harteneck and Bernd Nürnberg analysed and interpreted the data and wrote the paper.

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**Figure 9** Schematic representation of putative molecular mechanisms induced by mAb(A)p110γ, resulting in discriminative inhibition of the PI3Kγ variants

(A) Effect of mAb(A)p110γ on the basal states of the PI3Kγ variants. Binding of mAb(A)p110γ to the C2 domain mediates allosteric modulation of residues 551–650 in the helical domain and residues 1035–1050 located in helices ku9 and ku10 of the C-terminal lobe of the kinase domain. These helices play an important role in allosteric activation of p110γ, as was shown in the case of Ras stimulation [35]. mAb(A)p110γ-induced structural change of the kinase domain may interfere and reduce the basal lipid kinase activities of p87γ–p110γ and p101–p110γ. Slight protection of p101–p110γ basal lipid kinase activity from the inhibitory effect of mAb(A)p110γ, is in line with the previous data showing stimulatory modulation of p110γ by p101 independently of its Gβγ adapter function [41].

(B) Effect of mAb(A)p110γ on the PI3Kγ variants stimulated by Gβγγ subunits. Binding of mAb(A)p110γ to the C2 domain of p110γ causes allosteric exposure of a region (residues 551–650) in the helical domain which also includes crucial amino acids involved in interaction with Gβγ, Arg552 and Lys553 [48]. This results in allosteric interference of mAb(A)p110γ with Gβγγ-binding to p110γ. p101 was shown to be also involved in interaction with Gβγ via putative Gβγ-binding domain (Gβγ-BD) located in the C-terminal region of p101 [48]. In contrast with p101, p87 contributed much lesser (if at all) to Gβγγ interaction [28,38,41,48]. In the scenario of discriminative inhibition, mAb(A)p110γ disrupts p110γ–Gβγγ interaction in a similar way for each PI3Kγ variant, whereas unaltered Gβγγ-binding capacity of p101 still allows effective translocation of p101–p110γ and regulatory activity. In contrast, p87–p110γ showed a reduced capability to interact with Gβγγ in the presence of mAb(A)p110γ, resulting in drastic reduction in enzymatic activity. Indicated are PtdIns(4,5)P2, PtdIns(3,4,5)P3, the Ras-binding domain (RBD, residues 220–311), the C2 domain (residues 357–522), the helical domain (residues 545–725), the kinase domain (residues 726–1092) of p110γ and Gβγ-BD of p101 [48].

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