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Different inhibition of Gβγ-stimulated class I_B 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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Abstract

Class I_B phosphoinositide 3-kinases (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 enabling distinct modulation of the two variants are missing. The

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AUTHOR CONTRIBUTION

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

ability of an anti-p110γ monoclonal antibody (mAb(A)$_{p110\gamma}$) 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 mass spectrometry 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)$_{p110\gamma}$ 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 times 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.

Keywords

Gβγ; G-protein; p101; p87; PI3Kγ; signal transduction

INTRODUCTION

Class I phosphoinositide 3-kinases (PI3Ks) are lipid kinases that transduce extracellular signals to trigger phosphatidylinositol 3,4,5-trisphosphate (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 I_A and class I_B [2,3,9–12]. Class I_A is characterized by heterodimers consisting of a catalytic p110α, 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 I_A PI3K stability and regulation, the p110 catalytic subunit determines the signalling specificity [17–24].

The class I_B PI3Ks are represented by two enzymes consisting of one catalytic p110γ subunit associated with either a p101 or a p87 (also known as p87^{PIKAP} or p84) non-catalytic subunit [25–29]. Both PI3Kγ variants, *i.e.* p87-p110γ and p101-p110γ, are stimulated by Gβγ-heterodimers (Gβγ) released upon G-protein-coupled receptor 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 recent 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 recently 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 I_B catalytic subunit is present in cells led us to postulate 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

HEK293 cells (German Resource Centre for Biological Materials) were cultured and transfected with expression plasmids encoding p101 and p110γ as described previously [27,37,38]. HL-60 cells were grown in RPMI-1640 supplemented with 10% fetal calf serum, 1% non-essential amino acids, 2 mM L-glutamine, 40 μg/ml folic acid and antibiotics, 100 U/ml penicillin, and 100 μg/ml streptomycin. 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 $G\beta_1\gamma_2$, PI3Kγ and PI3Kβ subunits as well as their expression in Sf9 cells and purification of hexahistidine $(His)_6$-tagged recombinant $G\beta_1(His)_6\gamma_2$, $(His)_6$p110γ, p87-$(His)_6$p110γ, p101-$(His)_6$p110γ, and p85-$(His)_6$p110β have been described elsewhere [38,40,41,46–48]. 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 posttranslational 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 mass spectrometry measurements

Hydrogen-deuterium exchange coupled to mass spectrometry (HDX-MS) analyses of PI3Kγ in the presence and absence of $mAb(A)_{p110_\gamma}$ were performed following a similar protocol as described previously [21,48]. The rate of exchange of full length p110γ$(His)_6$ alone and in the presence of a 3-fold molar excess of $mAb(A)_{p110_\gamma}$ 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. Deuteration reactions were run for 3,

30, 300 and 3000 s of on-exchange at 23 °C, before being quenched by addition of 20 μl of a 2 M guanidine-HCl and 1.2% formic acid solution. Final deuterium concentration during the reaction was of 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 a week.

Analysis of 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 antiserum against the $G\beta_1$ subunit are detailed elsewhere [31,50]. Specific antibodies against p87 and p101 were generous gifts from Michael Schaefer (Leipzig, Germany) and Len Stephens (Cambridge, U.K.), respectively. Monoclonal anti-p110γ antibody, $mAb(A)_{p110_\gamma}$ and $mAb(B)_{p110_\gamma}$, were raised against full-length human p110γ using mouse hybridome cells and characterized earlier [37]. Large scale preparations of $mAb(A)_{p110_\gamma}$ were generated in cooperation with BioGenes, Berlin, Germany. $mAb(B)_{p110_\gamma}$ was described earlier [31,40,41]. Generation and characterization of monoclonal anti-p110γ antibody, $mAb(C)_{p110_\gamma}$, raised against the N-terminal 210 amino acids of catalytic p110γ was detailed earlier [43]. Anti-Ras antibody was purchased from BD Biosciences (#610002). Anti-p110β antibody was purchased from Cell Signaling (#3011S). Proteins were fractionated by SDS/PAGE (10% acrylamide) and transferred to nitrocellulose membranes (Hybond™-C Extra, GE Healthcare). Visualization of specific antisera was performed using the ECL chemiluminescence 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).

Immunoprecipitation of PI3K

Purified recombinant p110γ, p87-p110γ, and p101-p110γ, and p85α-p110β variants were subjected to immunoprecipitation (IP) using monoclonal anti-p110γ antibodies, $mAb(A)_{p110_\gamma}$, $mAb(B)_{p110_\gamma}$ or $mAb(C)_{p110_\gamma}$. IP experiments were performed as detailed previously [41] with some modifications. In brief, Protein A-Sepharose CL-4B beads (GE Healthcare) were preincubated 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 $G\beta_1\gamma_2$, H-Ras, and PI3Kγ association with phospholipid vesicles were performed as described previously [32,34,40,41,46].

Analytical ultracentrifugation analyses

Molecular weight and complex stability of purified p87-p110γ and p110-p110γ heterodimers were analyzed by sedimentation equilibrium analysis using a Beckman Optima XL-I 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% polyoxyethylene-10-lauryl ether ($C_{12}E_{10}$) at 10 °C. Sample and buffer (120 μl each) were loaded into six-channel cell assemblies. Replicate scans were taken following a 24 h equilibration at 6,000 rpm and then following a second 24 h equilibration at 11,000 rpm. Scans were also taken at 22 h at each speed so that equilibration could be confirmed. The equilibrium protein concentration distributions were globally analyzed using the program HeteroAnalysis ver. 1.1.58 [51,52]. Sednterp version 20120828 Beta (http://sednterp.unh.edu) was used to calculate the partial specific volume of the proteins from their sequence and the density of the buffer from its composition neglecting the contribution of the detergent. The sedimentation parameters were corrected to standard conditions (20, w) using these values. The 280 nm extinction coefficients calculated from each protein's sequence were used to calculate the concentrations of the protein complexes (http://web.expasy.org/protparam/).

Statistical analysis

Results (mean ± S.E.) were analyzed using Student's *t* test (*, $p \leq 0.05$; **, $p \leq 0.01$).

RESULTS

Inhibition of monomeric p110γ by a monoclonal anti-p110γ antibody, mAb(A)$_{p110\gamma}$

A monoclonal anti-p110γ antibody (mAb(A)$_{p110\gamma}$) raised against full-length human catalytic p110γ subunit used earlier in immunoprecipitation (IP) experiments [37] displayed interesting features attracting our attention. mAb(A)$_{p110\gamma}$ 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)$_{p110\gamma}$ interferes with p110γ activity. As shown in Figure 1C, incubation with mAb(A)$_{p110\gamma}$ led to a drastic reduction of p110γ lipid kinase activity stimulated by $G\beta_1\gamma_2$, defining mAb(A)$_{p110\gamma}$ as a putative PI3Kγ inhibitor.

In order to test the selectivity of the mAb(A)$_{p110\gamma}$ antibody, we measured its effect on the activity of the class I_A 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)$_{p110\gamma}$ and p85α-p110β. Correspondingly, mAb(A)$_{p110\gamma}$ did not inhibit lipid kinase activity of purified p85α-p110β (Figure 2E).

Mapping of p110γ regions affected by interaction with mAb(A)$_{p110\gamma}$

Since mAb(A)$_{p110\gamma}$ was generated by an immunization and selection protocol using full-length human catalytic p110γ subunit, the epitope of p110γ targeted by mAb(A)$_{p110\gamma}$ was unknown. To determine the p110γ epitope recognized by mAb(A)$_{p110\gamma}$, we used hydrogen-

deuterium exchange (HDX) coupled to mass spectrometry (MS). HDX-MS is a powerful technique that can map protein-protein and protein-lipid interaction, 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 solvents, a reaction that is influenced by secondary structure and solvent exposure.

To map the regions in p110γ that are affected by the interaction with $mAb(A)_{p110\gamma}$, we compared the HDX rates of p110γ in solution and when in a complex with the $mAb(A)_{p110\gamma}$. A large proportion of the C2 domain shows reduced HDX rate in the p110γ-$mAb(A)_{p110\gamma}$ 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 beta-strand underneath (residues 414–428). Interestingly, binding of $mAb(A)_{p110\gamma}$ 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, 622–630, 636–650) 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 activity by its regulatory subunit [55].

In summary, HDX-MS experiments revealed that $mAb(A)_{p110\gamma}$ 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 $mAb(A)_{p110\gamma}$ to p110γ might affect kinase enzymatic activity.

Effect of $mAb(A)_{p110\gamma}$ on p87-p110γ and p101-p110γ heterodimers activity

Class I_B PI3Kγ is present as two distinct functional p87-p110γ and p101-p110γ heterodimers *in vivo* [26,38,41,42]. We tested how $mAb(A)_{p110\gamma}$ affects enzymatic activities of these two PI3Kγ variants stimulated by $G\beta_1\gamma_2$. Two additional monoclonal antibodies raised against full-length human catalytic p110γ subunit ($mAb(B)_{p110\gamma}$) and N-terminal amino acids 1–210 of p110γ ($mAb(C)_{p110\gamma}$) were also included in order to validate the specificity of interactions. As depicted in Figure 4A significant differences in the ability of the antibodies to affect lipid kinase activities of the two PI3Kγ variants became apparent. While incubation of p87-p110γ with $mAb(A)_{p110\gamma}$ resulted in drastic reduction of $G\beta_1\gamma_2$-stimulated lipid kinase activity, inhibition of p101-p110γ activity by this antibody, in the concentrations tested, was weak. In contrast, $mAb(B)_{p110\gamma}$ and $mAb(C)_{p110\gamma}$ were ineffective in inhibiting enzymatic activity of either PI3Kγ variant under the identical experimental conditions (Figure 4A). The intriguing finding of differential $mAb(A)_{p110\gamma}$-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 $mAb(A)_{p110\gamma}$ was able to interact with p110γ when associated with p101. Comparable to monomeric p110γ (Figure 1A), immunoblotting analysis revealed that $mAb(A)_{p110\gamma}$ does not recognize denatured p101-p110γ complex (Figure 4B). In contrast, $mAb(B)_{p110\gamma}$ and $mAb(C)_{p110\gamma}$ recognize p110γ in immunoblots (Figure 4B). Nonetheless, the capability of $mAb(A)_{p110\gamma}$ to directly bind to p110γ when complexed to p101 could be verified by IP (Figure 4C).

Taken together, mAb(A)$_{p110_\gamma}$ 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)$_{p110_\gamma}$ 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)$_{p110_\gamma}$ interferes with G$\beta_1\gamma_2$-mediated association of p87-p110γ or p101-p110γ to phosholipid vesicles, in the absence and presence of another known PI3Kγ regulator, *i.e.* H-Ras. Strikingly, mAb(A)$_{p110_\gamma}$ differently affected G$\beta_1\gamma_2$-mediated phospholipid vesicle association of PI3Kγ variants. Whereas mAb(A)$_{p110_\gamma}$ strongly reduced G$\beta_1\gamma_2$-mediated vesicle association of p87-p110γ in a concentration-dependent manner, association of p101-p110γ remained unchanged (Figure 5A). mAb(A)$_{p110_\gamma}$ did not change binding of p101-p110γ to phospholipid vesicles upon exposure to both regulators, G$\beta_1\gamma_2$ and H-Ras (Figure 5B). However, concomitant incubation with G$\beta_1\gamma_2$ and prenylated H-Ras partially rescued phospholipid vesicle association of p87-p110γ in the presence of mAb(A)$_{p110_\gamma}$. Nonetheless, membrane association was impaired by high concentrations of mAb(A)$_{p110_\gamma}$ (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)$_{p110_\gamma}$ (Figure 5, grey or white bars vs. black bars). Control experiments excluded that the association of Gβγ or H-Ras to phospholipid vesicles was significantly affected by mAb(A)$_{p110_\gamma}$ (Table 1). High complex stability was supported by equilibrium analytical ultracentrifugation showing K_d values of ≤ 0.2 μM for p87-p110γ and ≤ 0.1 μM for p101-p110γ (Figure 6).

The interference of mAb(A)$_{p110_\gamma}$ with Gβγ-binding was tested by co-immunoprecipitation of p87-p110γ or p101-p110γ with G$\beta_1\gamma_2$ and H-Ras (Figure 7). In the case of p87-p110γ a reduction of G$\beta_1\gamma_2$ monitored by Gβ_1-immunoreactivity was evident whereas H-Ras-levels remained unaffected (Figure 7). Taken together the data show a mAb(A)$_{p110_\gamma}$-dependent inhibition of G$\beta_1\gamma_2$-induced recruitment of p87-p110γ to the lipid compartment. Next, we asked for consequences on enzymatic activity.

Concentration-dependent inhibition of PI3Kγ variants by mAb(A)$_{p110_\gamma}$

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)$_{p110_\gamma}$ (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 IC_{50} concentrations under all conditions tested and failed to differentiate between the two PI3Kγ variants.

In the presence of mAb(A)$_{p110_\gamma}$, basal lipid kinase activities of the two PI3Kγ variants were inhibited in a concentration-dependent manner with IC_{50} values of 7.2 ± 1.3 nM and 17.8 ± 5.2 nM for p87-p110γ and p101-p110γ, respectively (Figure 8E). Strikingly, the G$\beta_1\gamma_2$-stimulated activity of p87-p110γ was inhibited about 30 times more potently as compared to

the p101-p110γ counterpart (IC_{50}: 1.6 ± 0.5 nM *vs.* IC_{50}: 46.5 ± 12.6 nM; Figure 8F). In contrast, $mAb(A)_{p110_\gamma}$ inhibition of H-Ras-stimulated variants was indistinguishable (Figure 8G). When the enzymes were co-stimulated by $G\beta_1\gamma_2$ and H-Ras, p87-p110γ was 10 times more potently inhibited as compared to p101-p110γ by $mAb(A)_{p110_\gamma}$ (IC_{50}: 4.3 ± 0.4 nM *vs.* IC_{50}: 49.5 ± 4.9 nM; Figure 8H). Thus, $mAb(A)_{p110_\gamma}$ represents not only 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.

DISCUSSION

We recently described p87-p110γ as a constitutively and ubiquitously expressed class I_B PI3Kγ variant [41]. In contrast, p101-p110γ appeared as an inducible counterpart which is up-regulated upon activation and expressed in various tissues side-by side with p87-p110γ. In line with this view, growing experimental evidence indicate a divergent function and regulation of the two class I_B PI3Kγ variants [38,39,42–44]. Unfortunately, pharmacological tools discriminating between the two variants are not available [60]. Here, we identified a monoclonal antibody $mAb(A)_{p110_\gamma}$ as a potent inhibitor of PI3Kγ isoforms acting at low nanomolar concentrations. $mAb(A)_{p110_\gamma}$ blocked basal lipid kinase activities of either p87-p110γ or p101-p110γ with potencies comparable to that of wortmannin, an inhibitor acting at the ATP-binding site. Interestingly, enzymatic activities were differentially inhibited with a significant preference for p87-p110γ following stimulation by Gβγ. This preferential inhibition of p87-p110γ activity by $mAb(A)_{p110_\gamma}$ persisted even in experiments stimulating the PI3Kγ variants simultaneously with Ras and Gβγ.

The monoclonal antibody $mAb(A)_{p110_\gamma}$ was generated using full-length human p110γ protein for immunization und selection procedure and, therefore, the exact antibody-p110γ-interaction site was unknown [37,61]. Hydrogen-deuterium exchange coupled to mass spectrometry (HDX-MS), an approach that has provided inside into PI3K regulation at the membrane and by regulatory partners [21,48,62], identified dynamic changes within three domains of p110γ upon association with $mAb(A)_{p110_\gamma}$. Residues 382–428 in the C2 domain of p110γ were protected from HDX-MS exchange, most likely 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 interaction module in the regulation of PI3Kγ was not hitherto experimentally validated. Although Kirsch et al. [64] have shown that the phospholipid binding of a p110γ fragment comprising amino acids 740–1068 was significantly lower than the binding of full-length p110γ, this truncation construct lacked more than just the C2 domain (comprising residues 357–522). In addition to phospholipid-binding, C2 domains have been reported to

exhibit additional functions. In p110α, the C2 domain seems to be crucial for the inhibitory function of p85 on p110, whereas the C2 domain of p110β harbours a nuclear localization signal motif mediating translocation into the nucleus [11,15,65].

Our data argue for a different impact of $mAb(A)_{p110\gamma}$ on Gβγ-mediated stimulation of p87-p110γ and p101-p110γ. HDX-MS analyses indicate that binding of $mAb(A)_{p110\gamma}$ to 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 $mAb(A)_{p110\gamma}$ inhibits Gβγ stimulation of PI3Kγ variants may be a consequence of a distinct impact of the two non-catalytic subunits, *i.e.* p87 and p101, on PI3Kγ activity (Figure 9).

Ample evidence suggests that p101 acts as a Gβγ adaptor [26,32,37,38]. Since p101 is able to rescue the stimulatory effect of $G\beta_1$ mutants deficient in stimulating p110γ [40] and enhances Gβγ-induced stimulation of lipid-associated p110γ [41] we characterize p101 as a Gβγ-dependent regulator of PI3Kγ enzymatic activity. HDX-MS analysis on the p101-p110γ complex has identified two regions within the C-terminal part of p101 to mediate PI3Kγ activation by Gβγ [48]. In contrast, whether p87 functionally interacts with Gβγ remains an open question. Although p87 exhibits significant degree of homology with p101 at the C-terminal region [27–29], up to now we could not find any evidence that it displays a Gβγ-adapter function or serves as a Gβγ-dependent regulator [38,40,41]. Therefore, we suppose that in the presence of Gβγ, $mAb(A)_{p110\gamma}$ induces structural alterations in the helical domain that result in more drastic consequences for p87-p110γ than for p101-p110γ on phospholipid vesicle recruitment and enzymatic activation. An alternative mechanism of discriminative inhibition of PI3Kγ variants would be that $mAb(A)_{p110\gamma}$ binding induces the allosteric effect in monomeric p110γ and p87-p110γ, while p101 prevents the binding-induced change in p110γ. However, the molecular dynamics of deuterium exchange from p110γ were indistinguishable when complexed with p87 or p101 [48,66].

Taken together, we have characterized the inhibitory action of the monoclonal anti-p110γ antibody, $mAb(A)_{p110\gamma}$, mapped the antibody-p110γ interface and present new structure-functions insights of PI3Kγ activity. Specific features of $mAb(A)_{p110\gamma}$ to differentially block Gβγ-mediated association of p87-p110γ and p101-p110γ and, hence, their enzymatic activities provide the basis for a selective inhibition of Gβγ-initiated hormonal pathways of PI3Kγ variants and argues for a specific Gβγ-dependent regulatory role of p101 in PI3Kγ activation. This supports the idea of a differential regulatory impact of p87 and p101 on PI3Kγ activation.

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ABBREVIATIONS

$C_{12}E_{10}$	polyoxyethylene-(10)-lauryl ether
Gβγ	βγ dimer of the heterotrimeric G-protein
GPCR	G-protein-coupled receptor
HDX-MS	hydrogen-deuterium exchange coupled to mass spectrometry
His_6	hexahistidine tag
IB	immunoblotting
IP	immunoprecipitation
PI3K	phosphatidylinositol 3-kinase
PtdIns(3,4,5)P_3	phosphatidylinositol 3,4,5-trisphosphate

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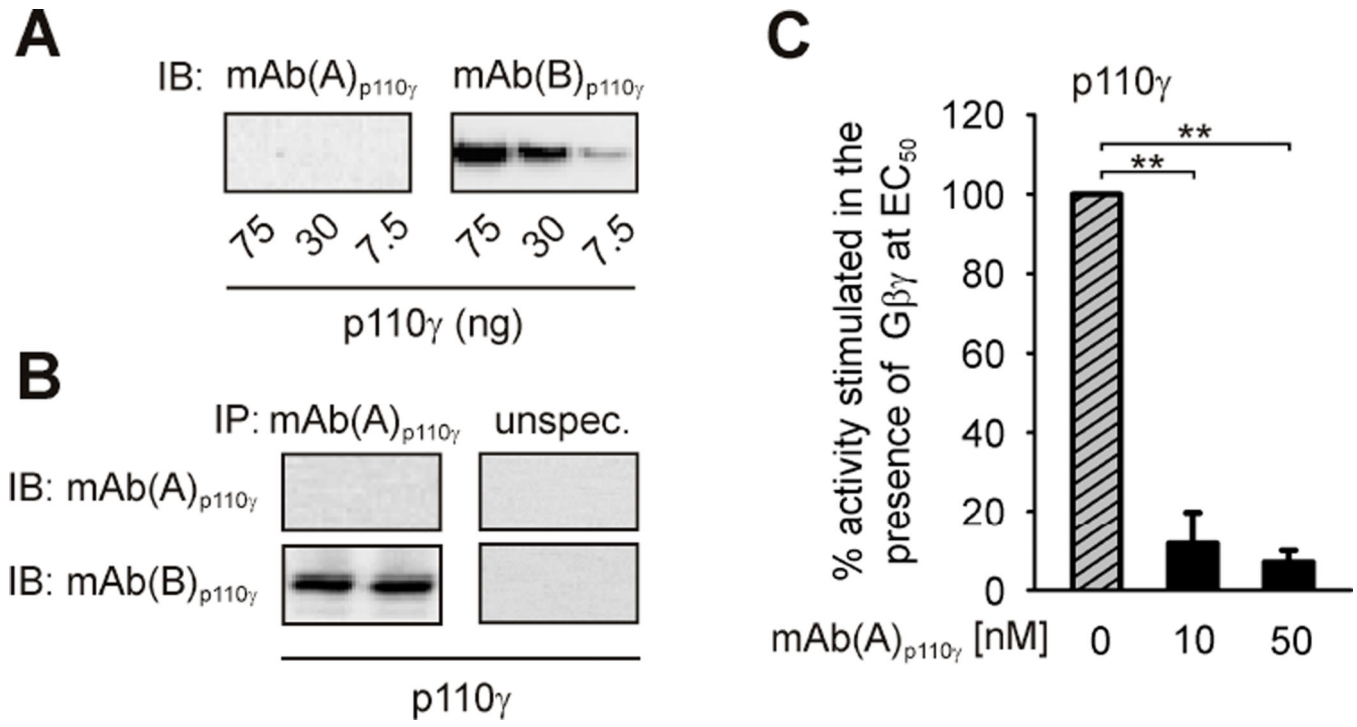


Figure 1. mAb(A)$_{p110\gamma}$ inhibits enzymatic activity of monomeric p110γ

(**A**) mAb(A)$_{p110\gamma}$ does not interact with denatured catalytic p110γ subunit in immunoblots. Monomeric p110γ was expressed in and purified from Sf9 cells. Different amounts of the protein were subjected to SDS/PAGE (10% acrylamide) followed by immunoblotting (IB) using monoclonal anti-p110γ antibodies, mAb(A)$_{p110\gamma}$, mAb(B)$_{p110\gamma}$. (**B**) mAb(A)$_{p110\gamma}$ binds intact p110γ. Purified recombinant p110γ was subjected to immunoprecipitation (IP) using mAb(A)$_{p110\gamma}$ p110γ-unspecific antibody as detailed in the Experimental section. Duplicates of immunoprecipitates were separated by SDS/PAGE (10% acrylamide) followed by immunoblotting (IB) with mAb(A)$_{p110\gamma}$ or mAb(B)$_{p110\gamma}$. (**C**) mAb(A)$_{p110\gamma}$ was tested for its ability to affect $G\beta_1\gamma_2$-induced lipid kinase activity of purified recombinant monomeric p110γ. The lipid kinase activity of enzyme (1.5 nM) was measured in the presence of 300 nM $G\beta_1\gamma_2$ (EC_{50} value) and in the absence or presence of increased concentrations of mAb(A)$_{p110\gamma}$. The data shown here are mean values ± S.E. (n = 3).

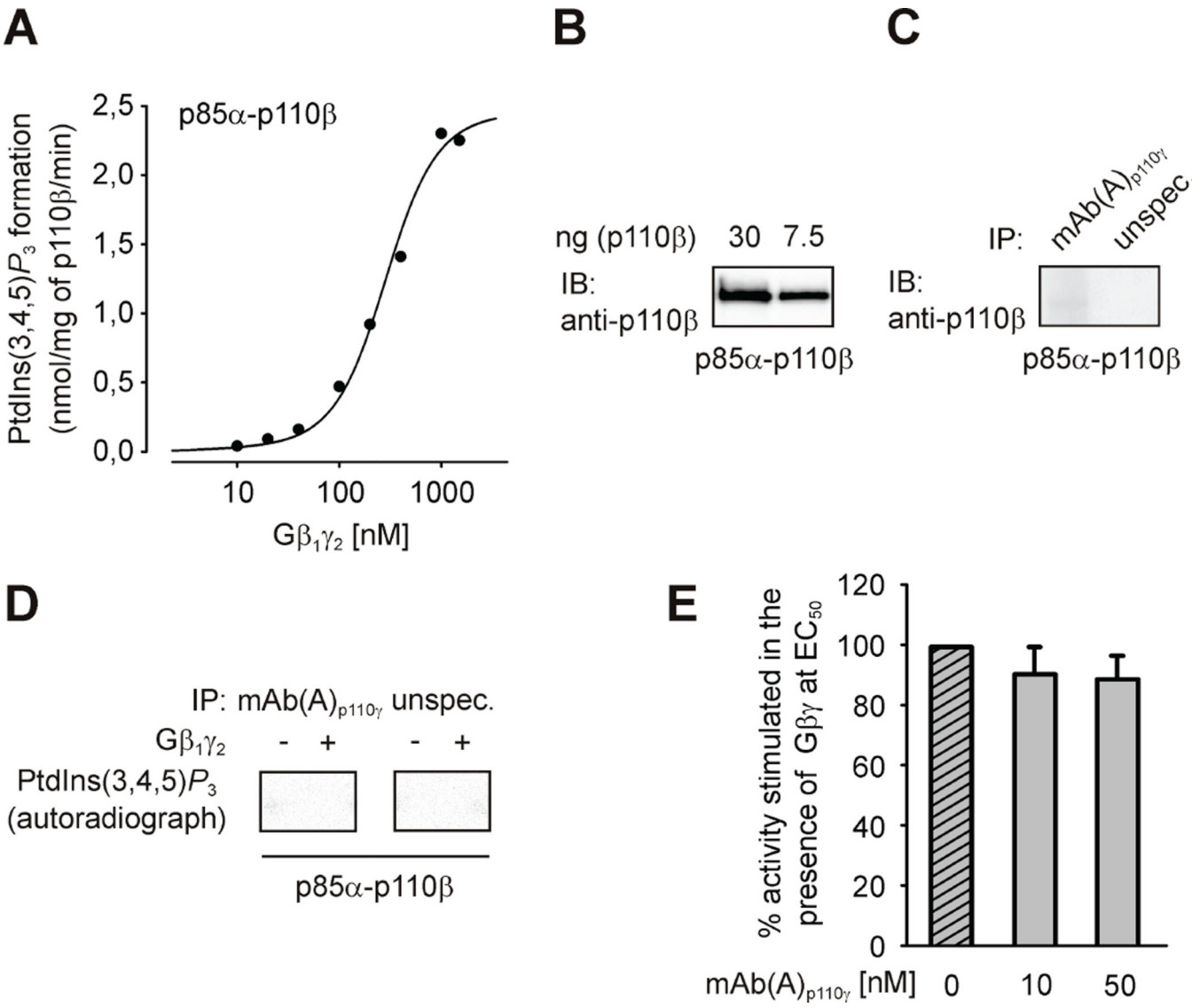


Figure 2. mAb(A)$_{p110\gamma}$ does not interact with Gβγ-sensitive PI3Kβ

(**A**) Stimulation of recombinant class I_A PI3Kβ (p85α-p110β) lipid kinase activity in response to increasing concentrations of $G\beta_1\gamma_2$. The data shown here represent the average of three independent experiments. (**B**) Different amounts of purified recombinant p85α-p110β were subjected to SDS/PAGE (10% acrylamide) followed by immunoblotting (IB) using specific anti-p110β antibody. (**C**) and (**D**) Purified p85α-p110β was immunoprecipitated (IP) using mAb(A)$_{p110\gamma}$ or p110β-unspecific antibody as described in the Experimental section. Obtained immunoprecipitates were analyzed by immunoblotting using specific anti-p110β antibody (shown in **C**) and tested in the lipid kinase assay in the absence or presence of 120 nM $G\beta_1\gamma_2$ (shown in **D**). Shown here are one typical immunoblot and autoradiograph out of three independent experiments. (**E**) mAb(A)$_{p110\gamma}$ was tested for its ability to affect $G\beta_1\gamma_2$-induced lipid kinase activity of purified recombinant p85α-p110β. The lipid kinase activity of enzyme (1.5 nM) was measured in the presence of

300 nM $G\beta_1\gamma_2$ (EC_{50} value) and in the absence or presence of increased concentrations of $mAb(A)_{p110_\gamma}$. The data shown here are mean values ± S.E. (n = 3).

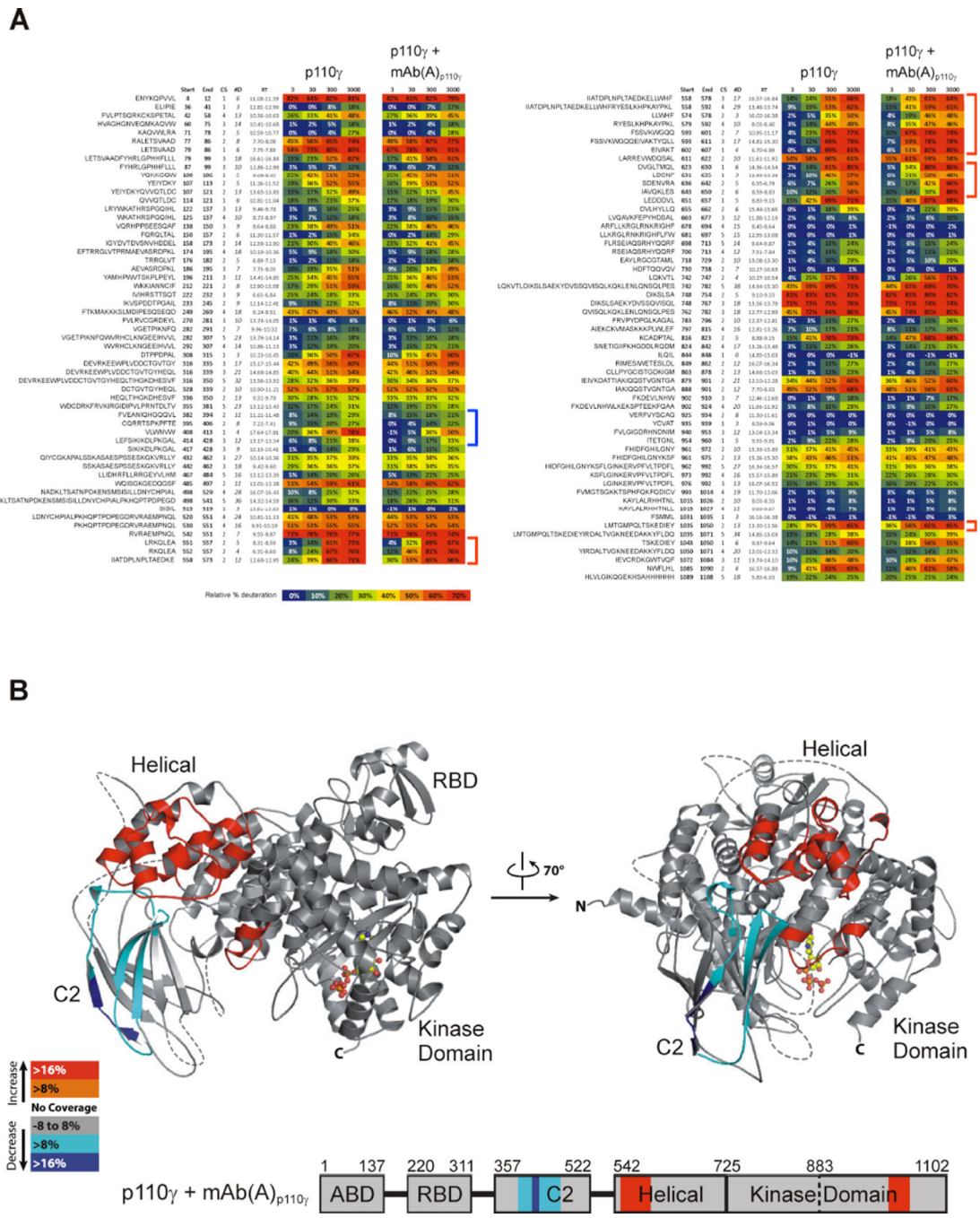


Figure 3. Binding of the of mAb(A)$_{p110\gamma}$ to the C2 domain of p110γ promotes allosteric changes in distinct domains

(**A**) Global hydrogen-deuterium exchange (HDX) in p110γ were analyzed for the following states: p110γ alone and p110γ associated to mAb(A)$_{p110\gamma}$. 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)$_{p110\gamma}$ are indicated with brackets. (**B**) Mapping of

the changes in deuteration levels between free p110γ and p110γ bound to $mAb(A)_{p110_\gamma}$ are visualized on p110γ crystal structure (top, PDB ID 1e8x) and on a schematic representation of p110γ sequence (bottom). Peptides with significant changes are coloured on p110γ model according to the color scheme shown (red and orange indicate increased exposure on binding, and cyan and blue represent decreased exposure).

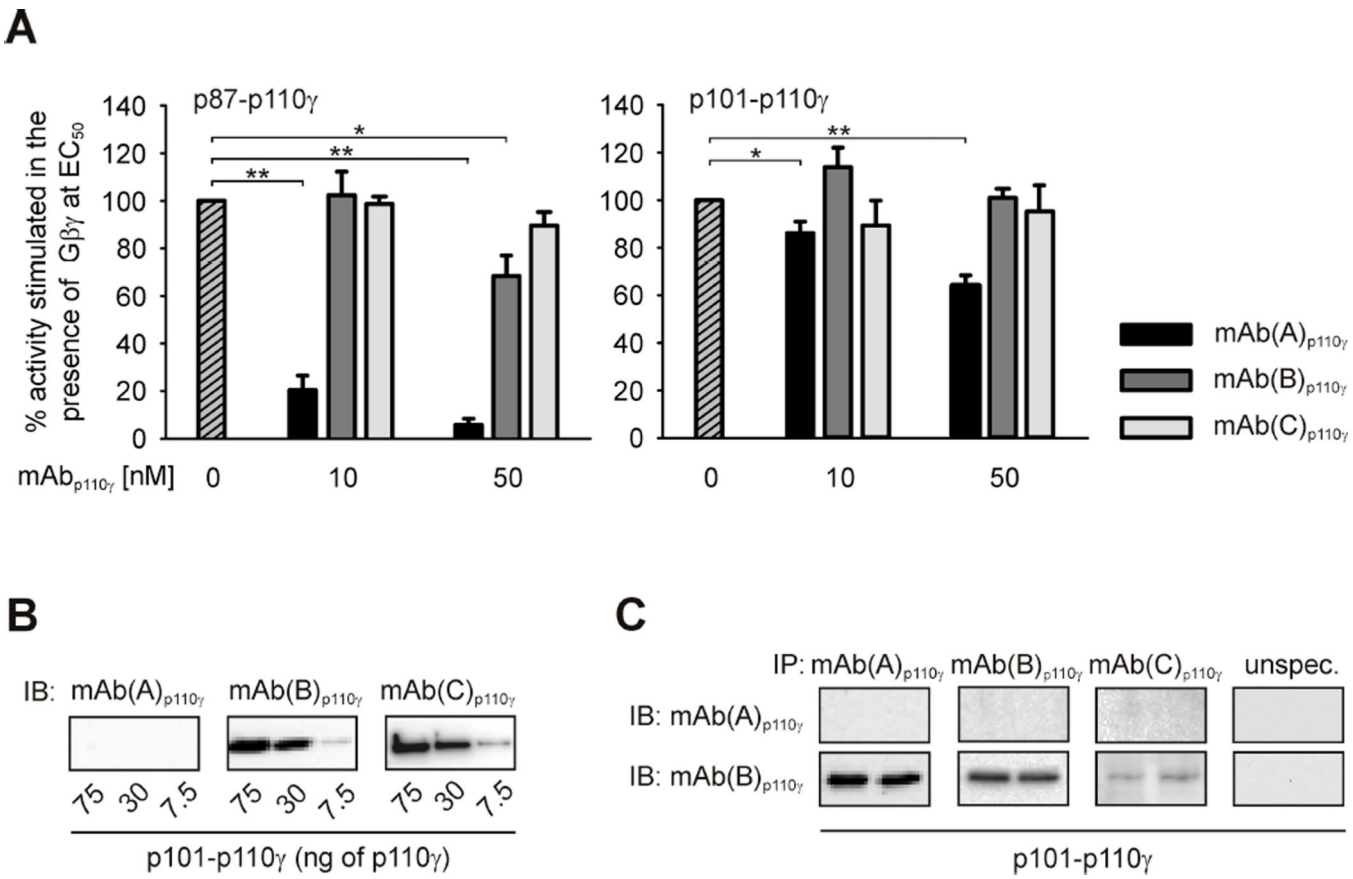


Figure 4. Different impact of monoclonal anti-p110γ antibodies on enzymatic activity of heterodimeric PI3Kγ variants

(**A**) Monoclonal anti-p110γ antibodies, $mAb(A)_{p110\gamma}$, $mAb(B)_{p110\gamma}$ and $mAb(C)_{p110\gamma}$, were tested for their ability to affect $G\beta_1\gamma_2$-induced lipid kinase activity of purified recombinant p87-p110γ and p101-p110γ. PI3Kγ variants (1.5 nM) were stimulated by $G\beta_1\gamma_2$ at EC_{50} values, *i.e.* 300 nM for p87-p110γ and 30 nM for p101-p110γ. The data shown here are mean values ± S.E. (n = 3). (**B**) $mAb(A)_{p110\gamma}$ does not interact with 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 immunoblotting (IB) using monoclonal anti-p110γ antibodies, $mAb(A)_{p110\gamma}$, $mAb(B)_{p110\gamma}$ or $mAb(C)_{p110\gamma}$. (**C**) $mAb(A)_{p110\gamma}$ binds catalytic subunit of intact p101-p110γ. Purified recombinant p101-p110γ was subjected to immunoprecipitation (IP) using $mAb(A)_{p110\gamma}$, $mAb(B)_{p110\gamma}$, $mAb(C)_{p110\gamma}$ or p110γ-unspecific antibody as described in the Experimental section. Duplicates of immunoprecipitates were separated by SDS/PAGE (10% acrylamide) followed by immunoblotting (IB) with $mAb(A)_{p110\gamma}$ or $mAb(B)_{p110\gamma}$.

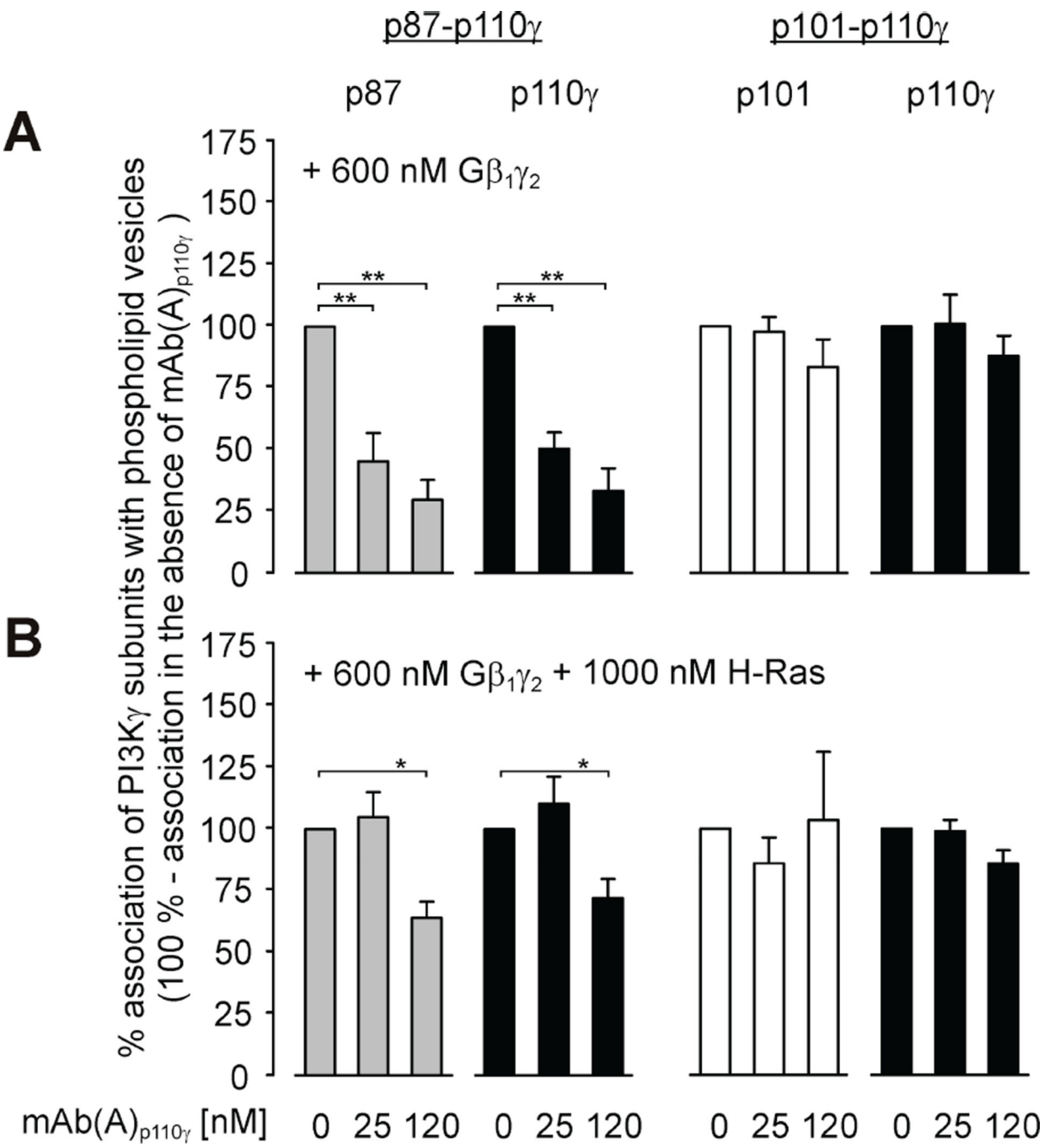


Figure 5. Effect of mAb(A)$_{p110\gamma}$ on association of PI3Kγ variants with phospholipid vesicles

mAb(A)$_{p110\gamma}$ was tested for its ability to affect Gβ$_1$γ$_2$-mediated association (600 nM Gβ$_1$γ$_2$) 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 subunits with phospholipid vesicles was analyzed by immunoblotting using mAb(A)$_{p110\gamma}$ and antibodies specific against p87 or p101. Chemiluminescence signals were estimated with a VersaDoc™ 4000 MP imaging system (Bio-Rad). For calculation of

phospholipid vesicle-associated subunits of PI3Kγ variants, signal intensities in the sedimented phospholipid vesicles and their supernatants were added and considered as 100 %. Shown here are the mean values ± S.E. of at least three separate experiments.

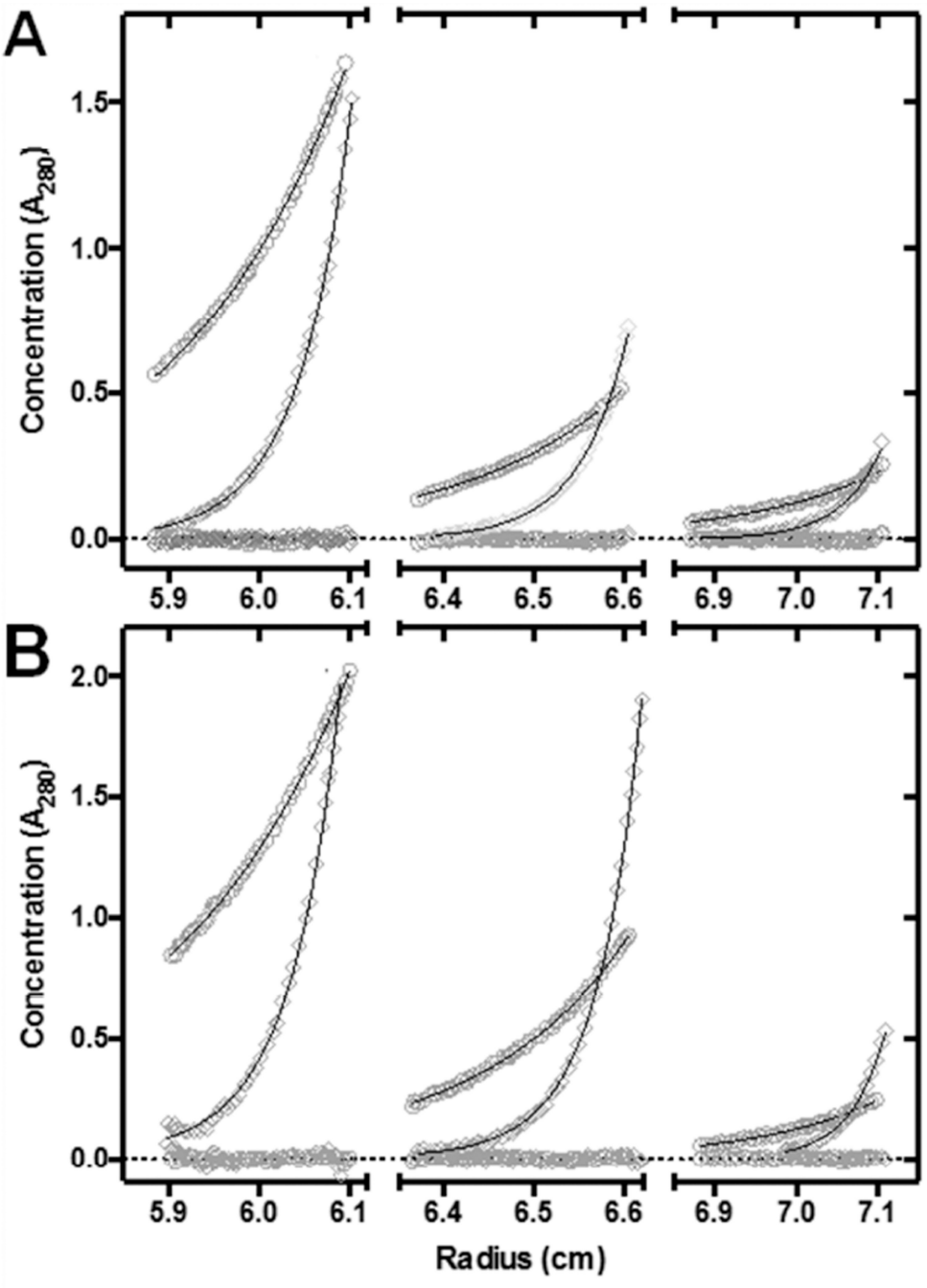


Figure 6. Comparable complex stability of p87-p110γ and p101-p110γ measured by analytical sedimentation equilibrium

Three concentrations (0.5, 2.0, and 4.0 μM) of p87-p110γ (**A**) and p101-p110γ (**B**) were centrifuged and analyzed 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 center of the rotor at 6,000 rpm (○) and 11,000 rpm (◊) for each of the three sample channels. The solid lines depict the best nonlinear least squares fit of the heteroassociation 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 weights (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 K_d values determined from these data are presented in the “Results” section.

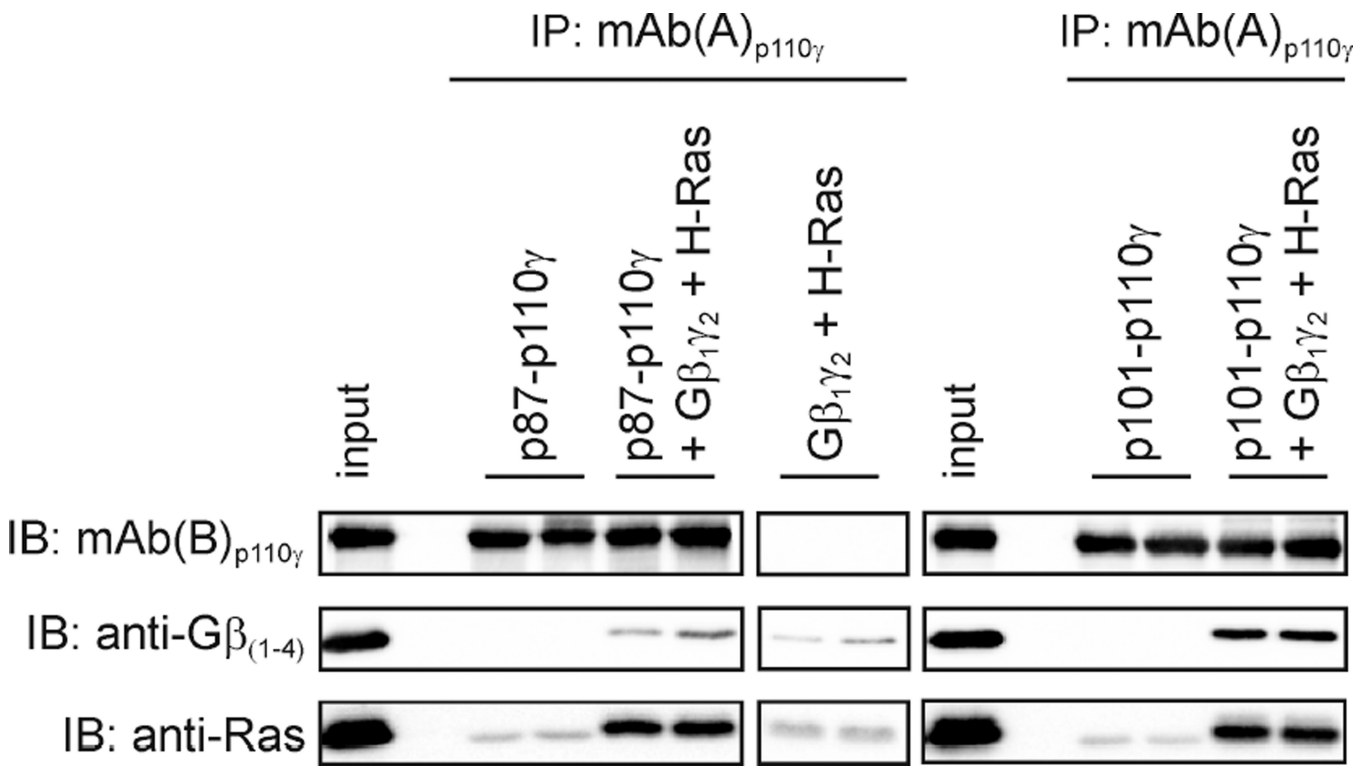
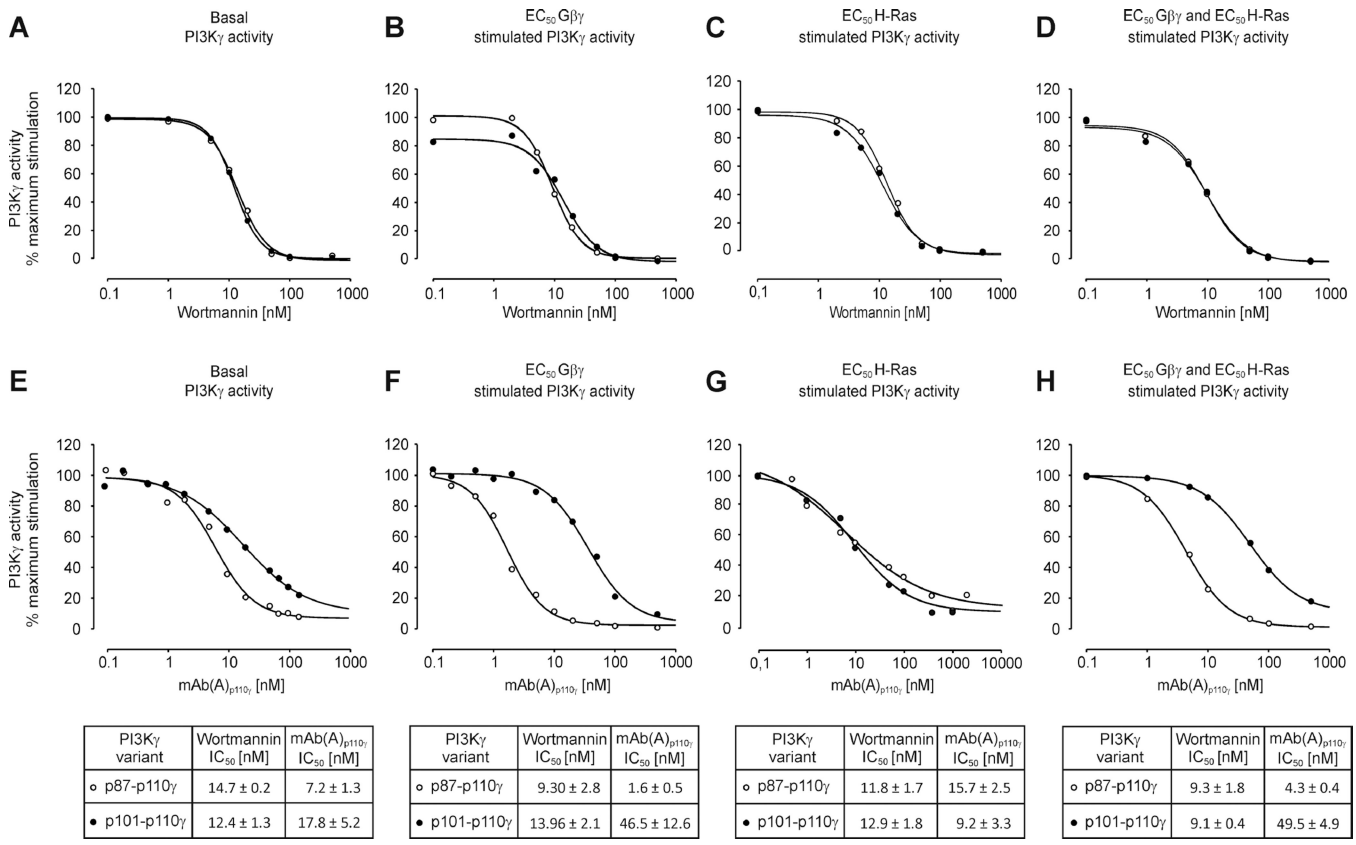


Figure 7. mAb(A)$_{p110\gamma}$ affects binding of G$\beta_1\gamma_2$ to p87-p110γ

Purified recombinant p87-p110γ or p101-p110γ (0.375 μg of catalytic p110γ subunit) in the absence or presence of G$\beta_1\gamma_2$ (1.25 μg) and H-Ras (1.25 μg) were subjected to immunoprecipitation (IP) using mAb(A)$_{p110\gamma}$ as described in the Experimental section. Duplicates of immunoprecipitates were separated by SDS/PAGE (10% acrylamide) followed by immunoblotting (IB) with mAb(B)$_{p110\gamma}$. Co-immunoprecipitated G$\beta_1\gamma_2$ and H-Ras were visualized using specific anti-G$\beta_{(1–4)}$ and anti-Ras antibodies. Weak unspecific chemiluminescence signals detected by anti-Ras antibody in PI3Kγ immunoprecipitates in the absence of G$\beta_1\gamma_2$ and H-Ras are caused by light chains of mAb(A)$_{p110\gamma}$.



PI3Kγ variant	Wortmannin IC_{50} [nM]	mAb(A)$_{p110\gamma}$ IC_{50} [nM]
○ p87-p110γ	14.7 ± 0.2	7.2 ± 1.3
● p101-p110γ	12.4 ± 1.3	17.8 ± 5.2

PI3Kγ variant	Wortmannin IC_{50} [nM]	mAb(A)$_{p110\gamma}$ IC_{50} [nM]
○ p87-p110γ	9.30 ± 2.8	1.6 ± 0.5
● p101-p110γ	13.96 ± 2.1	46.5 ± 12.6

PI3Kγ variant	Wortmannin IC_{50} [nM]	mAb(A)$_{p110\gamma}$ IC_{50} [nM]
○ p87-p110γ	11.8 ± 1.7	15.7 ± 2.5
● p101-p110γ	12.9 ± 1.8	9.2 ± 3.3

PI3Kγ variant	Wortmannin IC_{50} [nM]	mAb(A)$_{p110\gamma}$ IC_{50} [nM]
○ p87-p110γ	9.3 ± 1.8	4.3 ± 0.4
● p101-p110γ	9.1 ± 0.4	49.5 ± 4.9

Figure 8. Discriminative inhibition of heterodimeric PI3Kγ variants by mAb(A)$_{p110\gamma}$

The activities of p87-p110γ or p101-p110γ either in the basal condition or in the presence of $G\beta_1\gamma_2$, Ras and $G\beta_1\gamma_2$ together with Ras were measured in the presence of increasing concentrations of pan-PI3K inhibitor wortmannin (**A–D**) or mAb(A)$_{p110\gamma}$ (**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) of kinase in the assay. (**B**) and (**F**) The activities of PI3Kγ variants (1.5 nM) were measured in the presence of EC_{50} concentrations of $G\beta_1\gamma_2$ (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 EC_{50} concentrations of 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 EC_{50} concentrations of Ras (450 nM for p87-p110γ and 850 nM for p101-p110γ) and EC_{50} concentrations of $G\beta_1\gamma_2$ (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. of at least three separate experiments.

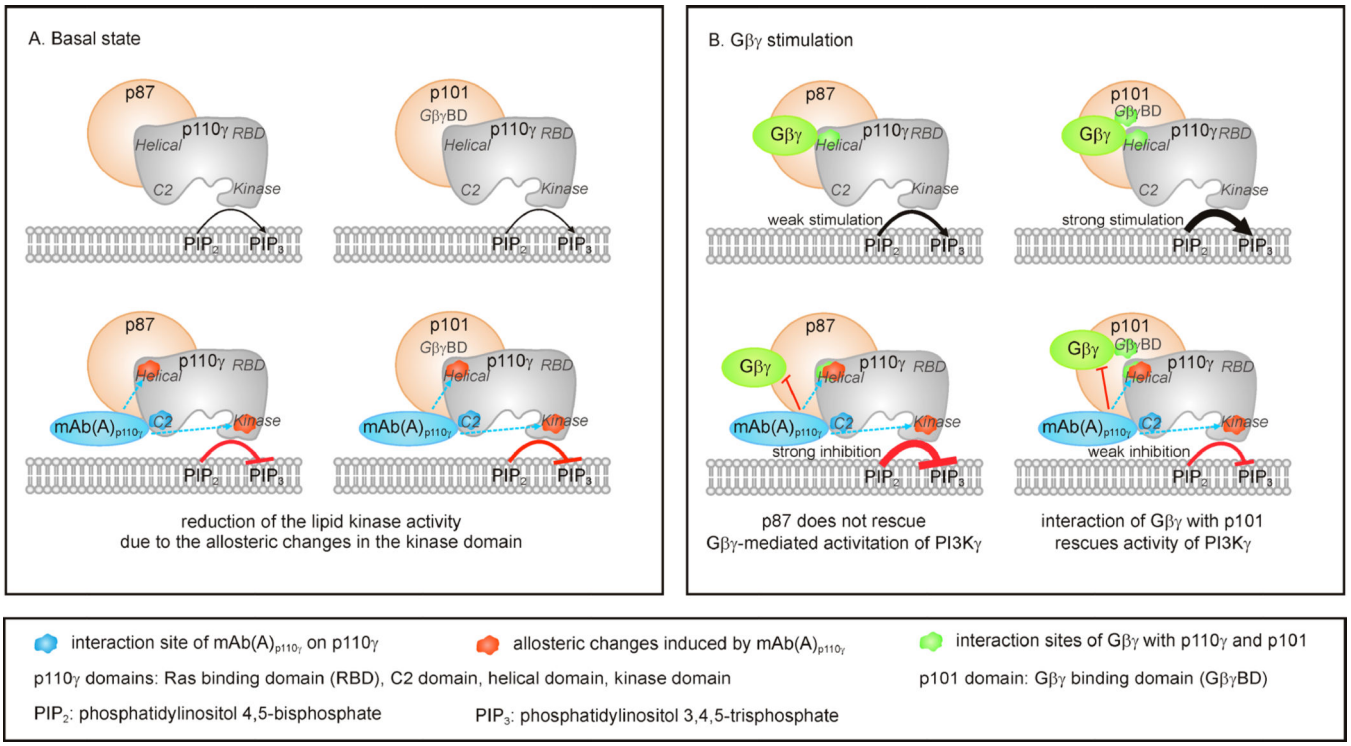


Figure 9. Schematic representation of putative molecular mechanisms induced by mAb(A)$_{p110\gamma}$ resulting in discriminative inhibition of the PI3Kγ variants

(**A**) Effect of mAb(A)$_{p110\gamma}$ on the basal states of the PI3Kγ variants. Binding of mAb(A)$_{p110\gamma}$ to the C2 domain mediates allosteric modulation of residues 551–650 in the helical domain and residues 1035–1050 located in the helices kα9 and kα10 of the C-terminal lobe of the kinase domain. These helices play important role in allosteric activation of p110γ, as it was shown in the case of Ras stimulation [35]. mAb(A)$_{p110\gamma}$ -induced structural change of the kinase domain may interfere and reduce the basal lipid kinase activities of p87-p110γ and p101-p110γ. Slight protection of p101-p110γ basal lipid kinase activity from the inhibitory effect of mAb(A)$_{p110\gamma}$ is in line with the previous data showing stimulatory modulation of p110γ by p101 independently of its Gβγ adaptor function [41]. (**B**) Effect of mAb(A)$_{p110\gamma}$ on the PI3Kγ variants stimulated by $G\beta_1\gamma_2$. Binding of mAb(A)$_{p110\gamma}$ 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, Arg_{552} and Lys_{553} [48]. This results in allosteric interference of mAb(A)$_{p110\gamma}$ with Gβγ binding to p110γ. p101 was shown to be also involved in interaction with Gβγ *via* putative Gβγ binding domain (GβγBD) located in the C-terminal region of p101 [48]. In contrast to p101, p87 contributed much lesser (if at all) to Gβγ interaction [28,38,41,48]. In the scenario of discriminative inhibition, mAb(A)$_{p110\gamma}$ disrupts p110γ-Gβγ interaction in a similar way for each PI3Kγ variant, whereas unaltered Gβγ binding capacity of p101 still allows effective translocation of p101-p110γ and regulatory activity. In contrast, p87-p110γ showed reduced capability to interact with Gβγ in the presence of mAb(A)$_{p110\gamma}$ resulting in drastic reduction of enzymatic activity.

Indicated are phosphatidylinositol 4,5-bisphosphate (PIP_2), phosphatidylinositol 3,4,5-trisphosphate (PIP_3), 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 [58], and putative Gβγ binding domain (GβγBD) of p101 [48].

Table 1

$mAb(A)_{p110\gamma}$ does not change association of $G\beta_1\gamma_2$ and H-Ras with phospholipid vesicles

Recombinant purified $G\beta_1\gamma_2$ 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 $mAb(A)_{p110\gamma}$. Aliquots of sedimented phospholipid vesicles and their supernatants were subjected to SDS/PAGE (10 % acrylamide) followed by immunoblotting using antibodies specific for $G\beta_{1–4}$ and H-Ras proteins. Chemiluminescence signals were estimated with a VersaDoc™ 4000 MP imaging system (Bio-Rad). 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. of at least three separate experiments.

Incubation with PI3Kγ variants	$mAb(A)_{p110\gamma}$, [nM]	Association with lipid vesicles	
		$G\beta_1\gamma_2$	H-Ras
		%	
p87-p110γ	0	29.2 ± 5.3	35.2 ± 6.9
	25	24.7 ± 5.2	34.1 ± 7.3
	120	23.7 ± 4.5	34.8 ± 8.1
p101-p110γ	0	31.4 ± 4.8	32.2 ± 7.2
	25	30.2 ± 7.9	28.2 ± 8.1
	120	32.1 ± 7.9	29.3 ± 5.9