

# Molecular determinants of PI3K $\gamma$ -mediated activation downstream of G-protein-coupled receptors (GPCRs)

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**Phosphoinositide 3-kinase gamma (PI3K $\gamma$ ) has profound roles downstream of G-protein-coupled receptors in inflammation, cardiac function, and tumor progression. To gain insight into how the enzyme's activity is shaped by association with its p101 adaptor subunit, lipid membranes, and G $\beta\gamma$  heterodimers, we mapped these regulatory interactions using hydrogen-deuterium exchange mass spectrometry. We identify residues in both the p110 $\gamma$  and p101 subunits that contribute critical interactions with G $\beta\gamma$  heterodimers, leading to PI3K $\gamma$  activation. Mutating G $\beta\gamma$ -interaction sites of either p110 $\gamma$  or p101 ablates G-protein-coupled receptor-mediated signaling to p110 $\gamma$ /p101 in cells and severely affects chemotaxis and cell transformation induced by PI3K $\gamma$  overexpression. Hydrogen-deuterium exchange mass spectrometry shows that association with the p101 regulatory subunit causes substantial protection of the RBD-C2 linker as well as the helical domain of p110 $\gamma$ . Lipid interaction massively exposes that same helical site, which is then stabilized by G $\beta\gamma$ . Membrane-elicited conformational change of the helical domain could help prepare the enzyme for G $\beta\gamma$  binding. Our studies and others identify the helical domain of the class I PI3Ks as a hub for diverse regulatory interactions that include the p101, p87 (also known as p84), and p85 adaptor subunits; Rab5 and G $\beta\gamma$  heterodimers; and the  $\beta$ -adrenergic receptor kinase.**

HDX-MS | oncogene | PIK3CG | PIK3R5 | PIP<sub>3</sub>

The phosphoinositide 3-kinase  $\gamma$  (PI3K $\gamma$ ) has far-reaching roles in the processes of mammalian biology, including inflammation, cell migration, cardiac function, response to pathogens, wound healing, olfaction, nociception, and tumor progression. Activation of p110 $\gamma$  produces the lipid second messenger phosphatidylinositol (3,4,5)-trisphosphate (PIP<sub>3</sub>), which in turn recruits downstream effectors, such as protein kinase B that bears PIP<sub>3</sub>-recognizing PH domains.

PI3K $\gamma$  plays a critical role in inflammation, with mice lacking the *Pik3cg* gene for p110 $\gamma$  having reduced inflammatory responses (1–3), increased protection from anaphylaxis (4), and protection from sepsis (5). Aberrant activation of the PI3K pathway is one of the most common events in cancer (6). Overexpression of p110 $\gamma$  induces cell transformation (7). Pharmacological inhibition of p110 $\gamma$  can prevent tumor growth and spreading by blocking myeloid-derived tumor inflammation and by suppressing breast cancer cell invasion (8–10). Depletion of p110 $\gamma$  or its regulatory subunit p101 inhibited primary tumor formation and metastasis by murine epithelial carcinoma cells (11). In pancreatic cancer, it has been proposed that p110 $\gamma$  is an important component of disease progression (12). PI3K $\gamma$  (together with PI3K $\delta$ ) is strictly required for development and maintenance of T-cell lymphoblastic leukemia that is driven by PTEN loss (13). Recently, it was also shown that PI3K $\gamma$  plays an essential role in formation of sarcomas induced by a viral GPCR (vGPCR) encoded by Kaposi's sarcoma herpes virus and that p110 $\gamma$ -deficient mice were completely resistant to vGPCR-induced sarcomagenesis (14). PI3K $\gamma$  also has major functions in the heart,

where it regulates cardiac contractility downstream of the  $\beta$ -adrenergic receptor ( $\beta$ AR) (15). In addition to its function as a lipid kinase, p110 $\gamma$  also plays a scaffolding/kinase-independent role in the heart (16–19). These results have established PI3K $\gamma$  as a target for the treatment of inflammation and cardiac diseases.

The PI3K $\gamma$  functions depend on direct, transient associations with various regulators, such as G $\beta\gamma$  heterodimers (G $\beta\gamma$ ), Ras,  $\beta$ AR kinase, PKA, and PP2A. Central to its many roles is the activation of PI3K $\gamma$  downstream of G-protein-coupled receptors (GPCRs) via direct binding to G $\beta\gamma$ . Although p110 $\gamma$  was among the first PI3Ks cloned, the mechanisms of p110 $\gamma$ 's regulation by G $\beta\gamma$  heterodimers and by its regulatory subunits are still not clear. In contrast to other class I PI3Ks, p110 $\gamma$  uniquely associates with a p101 or a p87 (also called p84) regulatory subunit (20–23). PI3K $\gamma$  shares with the class I<sub>A</sub> PI3K p110 $\beta$  the ability to be directly activated by G $\beta\gamma$  heterodimers (24–26). The p110 $\gamma$  catalytic subunit on its own can be activated only to a limited extent by G $\beta\gamma$  heterodimers, whereas maximal activation requires association with the p101 subunit and with Ras (21, 25, 27, 28). In cells, p101 is required for membrane translocation and activation

## Significance

Pathology of many diseases depends on signaling by phosphoinositide 3-kinase gamma (PI3K $\gamma$ ), the lipid kinase that is exquisitely adapted to activation downstream of heterotrimeric G-protein-coupled receptors (GPCRs). Using hydrogen-deuterium exchange mass spectrometry, we demonstrate the mechanism by which the p110 $\gamma$  catalytic subunit and its p101 regulatory subunit interact with G-protein G $\beta\gamma$  heterodimers liberated upon GPCR activation. We identify residues in both p110 $\gamma$  and p101 interacting with G $\beta\gamma$  heterodimers on membranes. This enabled us to generate G $\beta\gamma$ -insensitive p110 $\gamma$  and p101 variants that eliminate activation of PI3K $\gamma$  by G $\beta\gamma$ s without affecting the enzyme's basal activity or its activation by the small G-protein Ras. Ablating the interaction of PI3K $\gamma$  with G $\beta\gamma$  heterodimers attenuates signaling, chemotaxis, and transformation driven by a GPCR agonist in cell lines.

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of p110 $\gamma$  in response to ligand-induced G-protein activation (28). However, a p110 $\gamma$  that is constitutively localized to the plasma membrane is still sensitive to G-protein activation (28, 29).

In an effort to understand the molecular basis of PI3K $\gamma$  activation, we have investigated the structural determinants of the p110 $\gamma$ /p101 interaction with G $\beta\gamma$  heterodimers on membranes. Although there is a crystal structure of p110 $\gamma$  (30), no structural information or even domain organization for the p101 regulatory subunit is known, and similarly, there is no structural information about PI3K $\gamma$  interaction with membranes. Hydrogen–deuterium exchange mass spectrometry (HDX-MS) is a useful tool for analyzing interactions of proteins with membranes (31), and it has been important for understanding how PI3K complexes become activated on lipid membranes (24). The method also provides insight into protein dynamics that is difficult or impossible to obtain with other tools (32–35). Using HDX-MS, we have determined the interactions and conformational changes in p110 $\gamma$  that accompany binding to p101, to membranes, and to G $\beta\gamma$ . Our studies show that the helical domain of p110 $\gamma$  is a critical element in the regulation of p110 $\gamma$  activity. We also identified mutations in p110 $\gamma$  and p101 that specifically affect PI3K $\gamma$  activation downstream of GPCRs and impair its cellular functions.

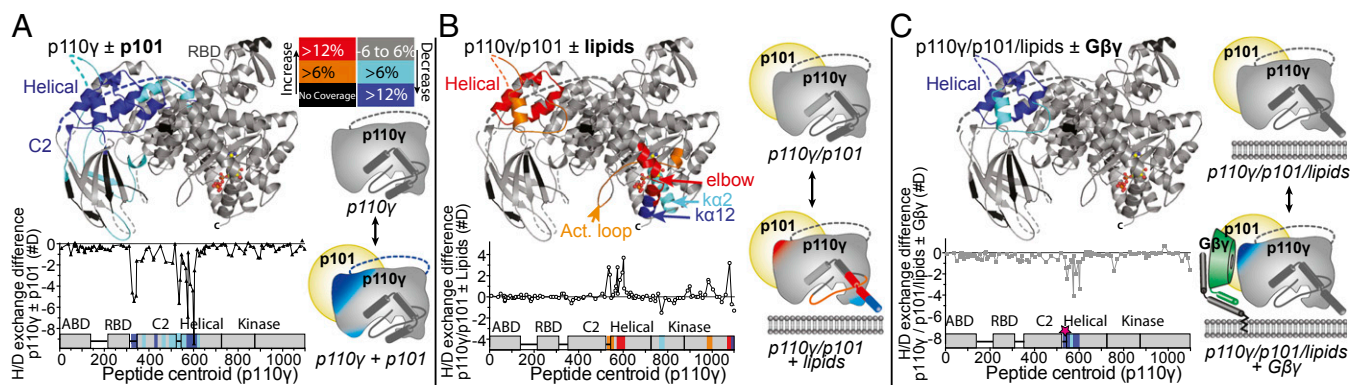
## Results

To determine the mechanism whereby G $\beta\gamma$  heterodimers activate PI3K $\gamma$  on membranes, we used a reconstituted system, consisting of purified PI3K $\gamma$  and prenylated G $\beta\gamma$  on synthetic liposomes. Using HDX-MS, we examined the following five states of the enzyme: (i) p110 $\gamma$  in solution; (ii) p110 $\gamma$  in the presence of lipids; (iii) the p110 $\gamma$ /p101 complex in solution; (iv) p110 $\gamma$ /p101 in the presence of lipids; and (v) p110 $\gamma$ /p101 with lipids and G $\beta\gamma$ . Samples of the enzyme in these various states were incubated with D<sub>2</sub>O for 3, 30, and 300 s before quenching of the reaction. The incorporation of deuterium into the protein was then determined by mass spectrometry analysis of the peptides generated by pepsin digestion. Details of the HDX-MS method are shown in Fig. S1, and total deuterium incorporation for all peptides in p110 $\gamma$  and in p101 are shown in Figs. S2–S5.

**Mapping of the p101 Binding Site on p110 $\gamma$ .** The p101 regulatory subunit is necessary for maximal activation of p110 $\gamma$  by G $\beta\gamma$  heterodimers, but how this is achieved is currently unknown. Additionally, although the interaction of class I $\alpha$  p110 $\alpha$ ,  $\beta$ , and  $\delta$  catalytic subunits with their p85 regulatory subunit is well documented, very little is known about how p101 interacts with p110 $\gamma$ . Truncation analyses have suggested that both *N*- and *C*-terminal

regions of p110 $\gamma$  are important for binding to p101 (36, 37), but studies on truncated variants are likely to have substantial destabilizing effects on the folded structure. Consequently, we wanted to understand the nature of the p110 $\gamma$ /p101 interaction for full-length proteins. To map the regions in p110 $\gamma$  required for p101 binding using full-length enzymes, we compared HDX rates of p110 $\gamma$  alone and when associated with p101. Multiple peptides are more protected in the p110 $\gamma$ /p101 complex compared with p110 $\gamma$  alone, including extensive regions in the linker between the Ras binding domain (RBD) and the C2 domain (RBD–C2 linker), in the C2 domain, in the C2-helical linker, and in the helical domain (Fig. 1A and Fig. S3). The largest changes in HDX rates occur in the RBD–C2 linker and in the helical domain, regions that were recently associated with binding to the p87 regulatory subunit (Fig. 1A and Figs. S3 and S6) (38). The involvement of the RBD–C2 linker in intermolecular interactions appears to be unique to the class I $\beta$  PI3K, whereas the helical domain of all class I PI3Ks mediate a variety of interactions. It is likely that the RBD–C2 linker makes direct contact with p101; however, several environmental influences (see below) affect the helical domain, so this domain might directly contact p101 or, alternatively, p101 binding may cause the helical domain to adopt a more rigid conformation, perhaps via an allosteric mechanism. Using HDX-MS, we have identified p110 $\gamma$  residues critical for p101 binding and also have gained insight into the dynamic changes occurring upon p110 $\gamma$ /p101 heterodimer formation. These results contrast with reports using truncated proteins (37), highlighting the benefits of working with full-length enzymes.

**Mapping Membrane-Binding Regions of p110 $\gamma$ .** Association with membranes is essential for formation of PIP<sub>3</sub> by p110 $\gamma$ , and membrane translocation is important for activation of PI3K $\gamma$ . Comparing the rate of amide exchange of p110 $\gamma$ /p101 in the absence and presence of lipids reveals regions in p110 $\gamma$  that are protected from exchange by membranes and also, more surprisingly, regions that show increased exposure upon lipid binding (Fig. 1B and Fig. S3). Two regions in the kinase domain of p110 $\gamma$  are more protected in the lipid-bound enzyme: helix  $\kappa\alpha 2$  in the *N*-lobe and the C-terminal helix ( $\kappa\alpha 12$ ). Lipid binding to p110 $\gamma$  also causes increased HDX rate in the helical and kinase domains (Fig. 1B). Exposure of the helical domain in the same region that is protected from exchange by p101 association (Fig. 1A) is not due to the p101 interaction being disrupted, because the same helical domain exposure occurs in p110 $\gamma$  alone upon lipid binding (Figs. S4 and S7). The exposure in the helical domain may represent allosteric changes that occur



**Fig. 1.** Mapping regulatory interactions of p110 $\gamma$  with p101, with membranes and with G $\beta\gamma$  using HDX-MS. (A) Mapping of the HDX changes in p110 $\gamma$  induced by binding p101. Peptides with significant changes are colored on the ribbon diagram of the p110 $\gamma$  structure (Protein Data Bank 1E8X) according to the color scheme shown (red and orange for increased HDX, cyan and blue for decreased HDX). (Lower) A linear plot highlighting changes in HDX between the two states (y axis) as a function of the central residue number for each peptic peptide (x axis). The schematic drawing on the *Right* illustrates the two states that were compared in the HDX-MS analysis. (B) Mapping of the HDX changes in p110 $\gamma$  induced by binding of p110 $\gamma$ /p101 to lipid membranes (illustrated as in A). (C) Mapping of the HDX changes in p110 $\gamma$  caused by interaction of prenylated G $\beta\gamma$  with the membrane-bound p110 $\gamma$ /p101 complex (illustrated as in A). The pink star indicates the position of residues mutated for *in vitro* and cellular characterization.

upon membrane binding, similar to what was observed for p110 $\beta$  (24). This rearrangement might represent a signature of G $\beta\gamma$ -sensitive PI3Ks because it was not seen in either p110 $\alpha$  or p110 $\delta$  upon membrane association (39, 40).

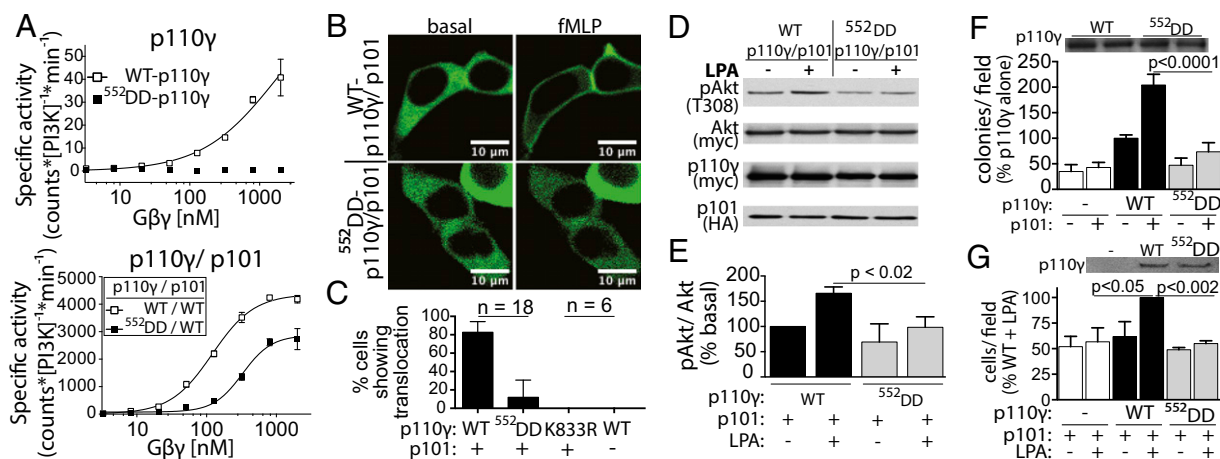
The kinase domain exposure in the “elbow” region (loop between helices  $\kappa$ 11 and  $\kappa$ 12) and of the underlying activation loop, combined with the protection of helix  $\kappa$ 12, suggests that two distinct rearrangements are linked with membrane binding. Protection of the C-terminal helix  $\kappa$ 12 is likely the consequence of direct membrane interaction, a mechanism that is supported by the interaction of that same region with membranes for the other class I PI3Ks (24, 39–41). Exposure of the elbow region and activation loop suggests that, for  $\kappa$ 12 to interact with the membrane, it has to swing out, thereby disrupting an inhibitory contact between the activation loop and the elbow. This mechanism is supported by a similar rearrangement upon activation observed for the class III PI3K Vps34 (42).

**Mapping of p110 $\gamma$  Regions Affected by G $\beta\gamma$ -Heterodimer Binding.** G $\beta\gamma$ -heterodimer binding is central to p110 $\gamma$  function (21, 25, 36, 43, 44). To map regions involved in this binding, we compared the HDX rates of the lipid-bound p110 $\gamma$ /p101 complex in the presence and absence of prenylated G $\beta_1\gamma_2$  heterodimers (G $\beta\gamma$ ). As shown in Fig. 1C, the same helical region that was protected by p101 and exposed upon lipid binding is subsequently protected by G $\beta\gamma$  (Fig. 1B and C). More precisely, the linker between the C2 domain and the helical domain (hereafter referred to as the C2-helical linker) as well as the helices lying underneath it are protected by the presence of G $\beta\gamma$ . This same region is protected by G $\beta\gamma$  in p110 $\beta$  (24), suggesting a similar mode of G $\beta\gamma$  binding for the catalytic subunits of both GPCR-sensitive PI3Ks. To confirm that the helical domain mediates G $\beta\gamma$  sensitivity, we mutated two conserved basic residues in the C2-helical linker to aspartic acid (R552D and K553D). These residues are homologous with a Lys-Lys sequence that we had shown previously as essential for the interaction of p110 $\beta$  with G $\beta\gamma$ . The p110 $\gamma$ -552-RK/DD (<sup>552</sup>DD-p110 $\gamma$ ) mutation completely abolished G $\beta\gamma$  stimulation for p110 $\gamma$  alone, and it greatly reduced sensitivity to and maximal stimulation by G $\beta\gamma$  for the p110 $\gamma$ /p101 complex (Fig. 2A), showing the importance of the

p110 $\gamma$ -552-RK motif for the interaction with G $\beta\gamma$ . The residual activation observed for the <sup>552</sup>DD-p110 $\gamma$ /p101 also confirms the contribution of p101 in G $\beta\gamma$  stimulation of p110 $\gamma$ . The inhibitory effect of the <sup>552</sup>DD-p110 $\gamma$  mutation was not due to a general destabilization of the enzyme because the <sup>552</sup>DD-p110 $\gamma$ /p101 complex had basal activity equivalent to the wild-type p110 $\gamma$ /p101 complex (Fig. S8A). Importantly, <sup>552</sup>DD-p110 $\gamma$  retains sensitivity to Ras, although, not surprisingly, it does not show the synergy of Ras and G $\beta\gamma$  activation characteristic of the wild-type enzyme (Fig. S8B and C). No stimulation of a mutant <sup>552</sup>DD-p110 $\gamma$ /p87 complex by G $\beta\gamma$  was seen (Fig. S8D). This is in sharp contrast to the <sup>552</sup>DD-p110 $\gamma$ /p101 complex (Fig. 2A), suggesting that p87 makes much less contribution to G $\beta\gamma$  interaction compared with p101, which is consistent with previous reports (22, 45, 46).

#### Importance of the G $\beta\gamma$ -Binding Site on p110 $\gamma$ for Cellular Functions.

**Effect of <sup>552</sup>DD-p110 $\gamma$  mutation on PI3K $\gamma$  signaling in cells.** To determine whether this 552-RK motif is important for signaling in cells, we used a fluorescent reporter protein, GFP-Grp1<sub>PH</sub>, which specifically binds the PIP<sub>3</sub> product of PI3Ks. This reporter molecule is a known sensor of PI3K activity in cells and was previously used to establish the roles of p101 or p87 regulatory subunits for p110 $\gamma$  sensitivity to G $\beta\gamma$  stimulation in cells (28, 36, 45, 47). We used live-cell imaging in HEK293T cells stably expressing the G<sub>i</sub>-coupled formyl-MET-LEU-PHE (fMLP) receptor to monitor PIP<sub>3</sub> formation upon stimulation by fMLP peptide. The cells were transfected with a single plasmid encoding three genes: myc-p110 $\gamma$ , FLAG-RFP-p101, and GFP-Grp1<sub>PH</sub>. This strategy enabled coexpression in each transfected cell of all proteins encoded by the plasmid, as confirmed by confocal analysis of RFP-p101 and GFP-Grp1<sub>PH</sub> fluorescence (Movie S1). Upon fMLP stimulation of cells expressing WT-p110 $\gamma$ /p101, the cytosolic GFP-Grp1<sub>PH</sub> reporter translocated to the plasma membrane within seconds (Figs. 2B; Fig. S8E; Movie S1). In contrast, almost no translocation was seen for cells expressing <sup>552</sup>DD-p110 $\gamma$ /p101, similar to what is observed with a kinase-dead p110 $\gamma$  mutant (K833R) (Fig. 2B and C and Movie S2). When comparing Akt activation upon GPCR stimulation by lysophosphatidic acid (LPA) in HEK293E cells overexpressing WT-p110 $\gamma$ /p101 or <sup>552</sup>DD-p110 $\gamma$ /p101, we observed that the mutation almost



**Fig. 2.** GPCR-mediated activities of p110 $\gamma$ /p101 in vitro and in cells. (A) Effect of the p110 $\gamma$ -<sup>552</sup>RK/DD mutation on in vitro activation by G $\beta\gamma$  of either the free p110 $\gamma$  catalytic subunit (Upper) or the p110 $\gamma$ /p101 complex (Lower). The error bars illustrate SDs of three independent replicates. (B) Representative images of HEK293T cells stably expressing the fMLP receptor and transfected with the described PI3K $\gamma$  constructs. PIP<sub>3</sub> formation in the plasma membrane was detected by translocation of the transfected GFP-Grp1<sub>PH</sub> domain. Basal and fMLP-stimulated (1–2 min) states are shown. (C) Quantification of the cellular activity assessed by GFP-Grp1<sub>PH</sub> translocation. (D) Activation of p110 $\gamma$ /p101 signaling by LPA in cells, as detected by pAkt Western blot. (E) Quantification of LPA-PI3K $\gamma$ -mediated Akt activation. The graph shows pAkt/Akt ratios normalized to unstimulated WT-p110 $\gamma$ /p101. The graph shows mean  $\pm$  SD of at least three independent experiments. *P* values calculated by two-tailed *t* test. (F) Transformation of NIH 3T3 cells measured by colony formation in soft agar resulting from transfection with the indicated constructs. (Upper) Western blot showing p110 $\gamma$  expression, using an anti-myc antibody. Graph is as in E. (G) Chemotaxis of HEK293E cells expressing WT or mutant p110 $\gamma$  toward media with or without LPA. (Upper) Western blot showing expression levels of p110 $\gamma$  as detected by anti-myc antibody. Graph is as in E with two replicates.

completely abrogates GPCR signaling downstream of PI3K $\gamma$  (Fig. 2 D and E).

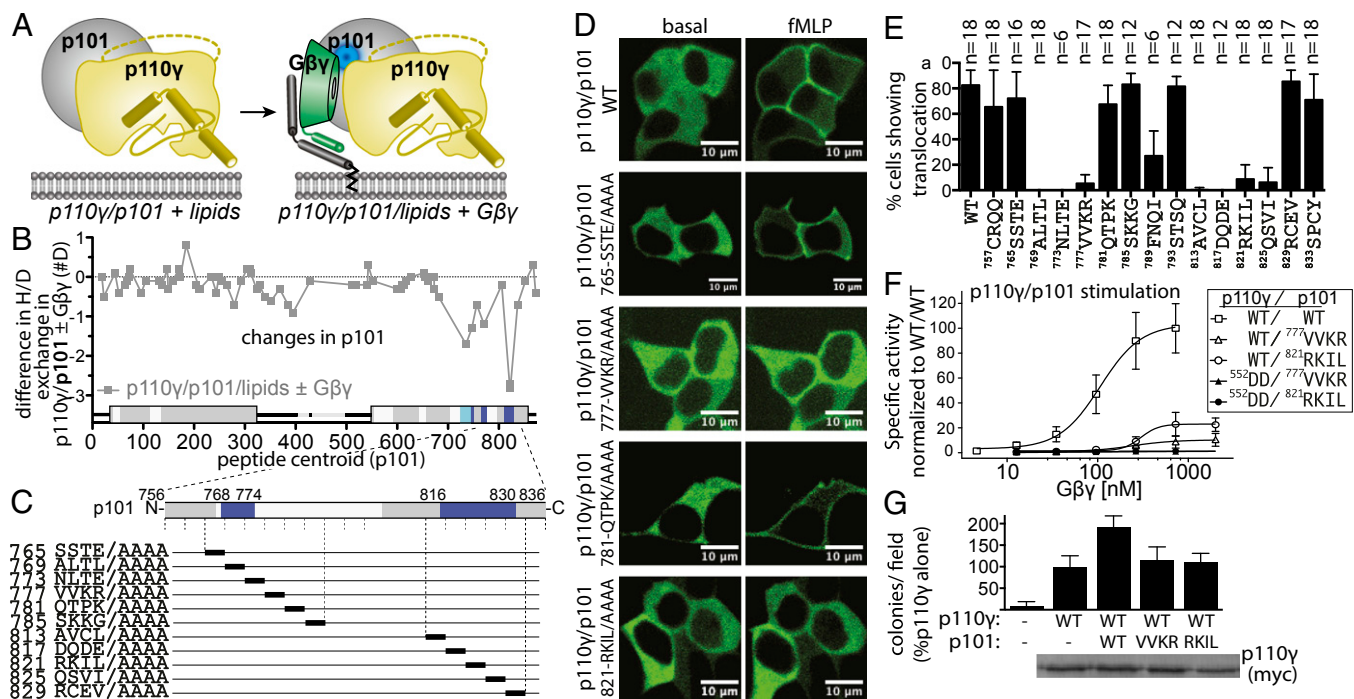
**Effect of the <sup>552</sup>DD-p110 $\gamma$  mutation on cell transformation and chemotaxis.** The wild-type p110 $\gamma$  induces cellular transformation when overexpressed, and this oncogenic potential requires binding to the small G-protein Ras (7). We wanted to determine whether activation by G $\beta\gamma$  also plays a part in transformation by PI3K $\gamma$  and what might be the role of the p101 subunit in this process. We carried out soft agar colony formation assays. As shown in Fig. 2F, the oncogenic potential was abolished for cells expressing <sup>552</sup>DD-p110 $\gamma$  and very significantly reduced for cells expressing <sup>552</sup>DD-p110 $\gamma$ /p101. In addition, to test the influence of G $\beta\gamma$  sensitivity on GPCR-driven chemotaxis, we performed Boyden chamber assays on HEK293E cells transiently transfected with WT or <sup>552</sup>DD-p110 $\gamma$  in complex with p101. Cells expressing <sup>552</sup>DD-p110 $\gamma$  showed a significant reduction in migration toward LPA compared with cells expressing WT p110 $\gamma$  (Fig. 2G). These data point to a crucial role for G $\beta\gamma$  sensitivity in PI3K $\gamma$ -mediated cellular transformation and chemotaxis.

**Mapping of p101 Regions Affected by G $\beta\gamma$  Binding on Membranes.** Despite the importance of the p101 subunit in sensitizing activation of p110 $\gamma$  downstream of GPCRs, there is no information on the structure of p101 and little information regarding how it interacts with p110 $\gamma$ . Because the p101 regulatory subunit is unstable when expressed in Sf9 cells without p110 $\gamma$ , we could not identify the regions in p101 that are affected by p110 $\gamma$  binding. However, we have been able to map regions of p101 (when associated with p110 $\gamma$ ) whose HDX rates are affected by its interaction with G $\beta\gamma$ . Previous reports, based on truncation

mutants, have proposed that the N terminus of p101 interacts with p110 $\gamma$  and that the C-terminal region is essential for G $\beta\gamma$  interaction (36, 37). However, it is not clear that these truncations did not disrupt the overall structure of the protein.

Comparison of HDX rates in p101 for the p110 $\gamma$ /p101 lipid-bound complex in the absence and presence of G $\beta\gamma$  identified several peptides in the C-terminal part of p101 that were protected from exchange in the G $\beta\gamma$  complex (Fig. 3 A and B). Two peptides in the most C-terminal region show large decreases in exchange, suggesting that these regions may be direct G $\beta\gamma$ -binding sites (Fig. 3B and Fig. S5). To determine the importance of the p101 C-terminal region for G $\beta\gamma$  stimulation of a p110 $\gamma$ /p101 heterodimer, we carried out alanine-scanning mutagenesis, mutating sets of four consecutive residues in p101 to alanine (Fig. 3C) and assayed the activity of p110 $\gamma$ /p101 mutants.

**Effect of p101 Mutations on p110 $\gamma$ /p101 Activity in Vitro and in Cells.** Some alanine mutations of p101 in regions encompassing the peptides most protected from exchange by G $\beta\gamma$  drastically diminished fMLP-induced PIP<sub>3</sub> formation in cells (Figs. 3 D and E and Fig. S9A). Other alanine mutations in these regions had no effect on GPCR sensitivity, whereas some mutations showed reduced protein expression levels, suggesting that they may affect protein stability (Fig. S9B). We selected two p101 mutants, one from each of the identified p101 regions that abrogate GPCR stimulation, for in vitro characterization. These two p101 mutants, <sup>777</sup>VVVKR/AAAA-p101 and <sup>821</sup>RKIL/AAAA-p101, expressed similarly to WT p101 but showed a much reduced G $\beta\gamma$  sensitivity in cells. However, they retained some activity in vitro upon stimulation with high G $\beta\gamma$  concentrations (Fig. 3F). Combining



**Fig. 3.** Effect of p101 mutations on cellular activity and transformation. (A) Schematic representation of the two states compared by HDX-MS to identify changes in p101 induced by prenylated G $\beta\gamma$  on membranes. In this experiment, p101 was always in complex with p110 $\gamma$ . (B) Linear plot of changes in HDX rates in p101 upon binding prenylated G $\beta\gamma$  as a function of central residue number. White indicates regions for which no peptides were detected in the HDX-MS experiment. (C) Schematic representation of p101 tetra-alanine mutants that were generated for biochemical characterization. Above the blocks illustrating positions of mutations is a bar colored according to the HDX results (blue represents a decrease in HDX, gray no change, and white regions not covered by peptides in the HDX-MS experiment). (D) Representative images of HEK293T cells transfected with the described PI3K $\gamma$  constructs. PIP<sub>3</sub> production is indicated by translocation of the GFP-Grp1<sub>PH</sub> domain construct to the plasma membrane (as for Fig. 2B). (E) A quantitation of Grp1<sub>PH</sub> translocation for all of the p101 tetra-alanine mutants. (F) In vitro lipid kinase activity as a function of G $\beta\gamma$  concentration for two selected p101 mutants in complexes with wild-type or <sup>552</sup>DD-mutated p110 $\gamma$ . Error bars show SD for at least three replicates. (G) Transformation of NIH 3T3 cells measured by colony formation in soft agar resulting from transfection with the indicated constructs.

mutations in both the p110 $\gamma$  and the p101 subunits (<sup>552</sup>DD-p110 $\gamma$ /<sup>777</sup>VVKR-p101 and <sup>552</sup>DD-p110 $\gamma$ /<sup>821</sup>RKIL-p101) resulted in a complex that could not be activated by G $\beta\gamma$  in vitro (Fig. 3F), confirming that both subunits contribute to activation of p110 $\gamma$ /p101 by G $\beta\gamma$ .

**Effect of p101 Mutants on Transformation Potential.** The transformation potential of WT p110 $\gamma$  requires binding to G $\beta\gamma$  and is enhanced by the coexpression of p101 (Fig. 2F). Because the p101 subunit also contacts the G $\beta\gamma$  subunit, we tested whether mutations in p101 that disrupt its interactions with G $\beta\gamma$  would affect p110 $\gamma$ -mediated transformation. In soft agar colony formation assays, the ability of p101 to enhance p110 $\gamma$ -mediated transformation is lost in the <sup>777</sup>VVKR/AAAA-p101 or <sup>821</sup>RKIL/AAAA-p101 mutants (Fig. 3G). This suggests that the G $\beta\gamma$  interaction with both p110 $\gamma$  and p101 is required for the full transformation potential of overexpressed p110 $\gamma$ .

## Discussion

Using HDX-MS, we have identified dynamic changes occurring during p110 $\gamma$  activation upon interaction with its p101 regulatory subunit, with lipids and with G $\beta\gamma$  heterodimers (all data are summarized in Fig. 4). The p101 regulatory subunit seems to directly contact p110 $\gamma$  in the linker between the RBD and C2 domains and possibly the helical domain itself, mediating a massive stabilization of the helical domain. Conformation of the p110 $\gamma$  helical domain seems to be central to PI3K $\gamma$  regulation. Indeed, the helical domain changes its conformation when transiting between states, being significantly protected when in a complex with p101, greatly exposed upon lipid binding, and finally protected when interacting with G $\beta\gamma$  (Fig. 4). Stabilization of the p110 $\gamma$  helical domain by p101 binding might be responsible for the higher basal activity of p110 $\gamma$ /p101 compared with p110 $\gamma$ . Interaction of p110 $\gamma$ /p101 with membrane causes a substantial exposure of the helical domain, similar to what was observed in the other GPCR-sensitive PI3K p110 $\beta$  (24). Finally, G $\beta\gamma$  heterodimers protect the helical domain, presumably by directly contacting the C2-helical linker above it. Because no exposure of the helical domain upon lipid binding was observed for the other class I PI3Ks, p110 $\alpha$  and p110 $\delta$ , this helical domain rearrangement could be required for productive interaction of p110 with membrane-resident G $\beta\gamma$  (24, 39, 40), representing a signature for G $\beta\gamma$  sensitivity of class I PI3Ks. Previously, the helical domain of p110 $\gamma$  was shown to bind the  $\beta$ -adrenergic receptor kinase, promoting PI3K $\gamma$  membrane recruitment (15). This domain also has critical functions in class I $_A$  PI3Ks, contacting the inhibitory nSH2 domain of the p85 regulatory subunit as well as G $\beta\gamma$  heterodimers and Rab5 for p110 $\beta$  (24, 41, 48–50). Taken together, these results indicate a profound role of the helical domain as a scaffold for regulatory interactions in the PI3K family.

Another region of PI3Ks important for catalysis encompasses the last two helices of the kinase domain, together with the loops that are in close proximity to these helices. The helices are part of the regulatory arch, a region previously defined as critical for

PI3K regulation (51). HDX-MS data comparing p110 $\gamma$  in solution and bound to membrane suggest a model for p110 $\gamma$  regulation by membrane binding. The last portion of helix  $\alpha$ 12 is protected by membrane, probably indicative of its partial insertion into the bilayer. At the same time, the elbow region and the activation loop underneath it are exposed upon membrane binding. In p110 $\beta$  and p110 $\delta$ , this elbow region accommodates the C-terminal SH2 domain from p85 that stabilizes these enzymes in an inactive form (40, 52). Thus, it seems that p110 $\gamma$  has evolved an inhibitory mechanism to compensate for the absence of a p85 inhibitory contact. Our HDX-MS data for p110 $\gamma$ , as well as comparison with the class III PI3K Vps34, suggest a model where this auto-inhibitory helix  $\alpha$ 12 swings from a closed, inactive conformation in solution to a more active conformation on membranes in which inhibitory contacts between the elbow and other stabilizing loops underneath it are disrupted.

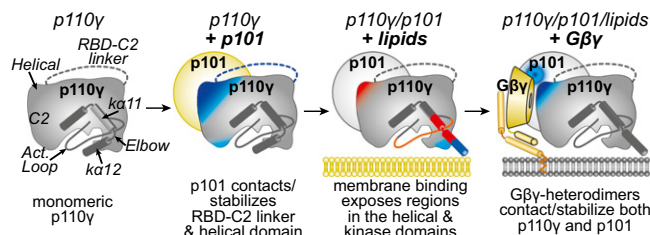
The helical domain conformation is critical for p110 $\gamma$  activity and for stimulation by G $\beta\gamma$ . We identified two conserved basic residues in the C2-helical linker of p110 $\gamma$ , Arg552 and Lys553, which, when mutated to aspartic acid, critically affect G $\beta\gamma$  sensitivity in vitro while retaining normal interaction with p101 and sensitivity to Ras. The <sup>552</sup>DD-p110 $\gamma$  mutant, when associated with p101, shows reduced downstream signaling in cells, a much lower oncogenic potential when overexpressed, and reduced chemotaxis toward LPA compared with the WT-expressing cells.

In addition to the C2-helical linker in p110 $\gamma$ , precise residues in the p101 C-terminal region that directly mediate activation by G $\beta\gamma$  were identified. Alanine-scanning mutagenesis based on results obtained by HDX-MS has identified p101 mutants that bind normally to p110 $\gamma$  but have lost G $\beta\gamma$  sensitivity. Transforming potential for these mutants was significantly reduced compared with WT p110 $\gamma$ /p101, confirming the importance of p101 for p110 $\gamma$  stimulation by G $\beta\gamma$  and for PI3K $\gamma$  oncogenicity. Combined with the data obtained for <sup>552</sup>DD-p110 $\gamma$ , these experiments establish the importance of G $\beta\gamma$  sensitivity in the oncogenic potential of PI3K $\gamma$ .

Although we have precisely mapped sites in p110 $\gamma$  and p101 that are essential for stimulation by G $\beta\gamma$ , the lack of a structure for the p110 $\gamma$ /p101 complex prevents us from forming a definitive picture of the activation mechanism. Clearly, both p110 $\gamma$  and p101 directly contact G $\beta\gamma$ . This is consistent with our previous results based on copurification studies (25, 44) and mutagenesis of G $\beta$  showing that p101 not only facilitates recruitment to membranes but also participates in the G $\beta\gamma$ -mediated activation of p110 $\gamma$  beyond recruitment (53). The previous observation that PI3K $\gamma$  retains G $\beta\gamma$  sensitivity even when it is constitutively targeted to membranes (28, 29) also suggests that membrane recruitment constitutes only part of the activation mechanism.

Given that p110 $\gamma$  makes direct contact with G $\beta\gamma$  and that p101 was previously shown to be able to bind G $\beta\gamma$  in the absence of p110 $\gamma$ , several options can be envisioned regarding the stoichiometry of the p110 $\gamma$ /p101–G $\beta\gamma$  complex (28). Each subunit of PI3K $\gamma$  could cooperate in contacting a single G $\beta\gamma$  heterodimer, or they could bind independent heterodimers. In both situations, p101 would reinforce the affinity of p110 $\gamma$  for G $\beta\gamma$  and thus for membranes. In addition to binding G $\beta\gamma$ , the p101 subunit appears to allosterically alter the conformation of the p110 $\gamma$  subunit in the same region mediating contact with G $\beta\gamma$ . This conformational change may enhance the affinity for G $\beta\gamma$ . Mutating regions in the p101 C terminus reduces the activation of p110 $\gamma$ /p101 by G $\beta\gamma$ , but mutation of both p110 $\gamma$  and p101 subunits is required to eliminate activation by G $\beta\gamma$  in vitro (Fig. 3F).

HDX-MS analysis followed by targeted mutagenesis studies has identified the molecular determinants of the G $\beta\gamma$ –PI3K $\gamma$  interaction that are critical for GPCR-dependent PI3K $\gamma$  signaling, cell transformation, and chemotaxis. Future use of the G $\beta\gamma$ -insensitive PI3K $\gamma$  construct will help decipher the biology of PI3K $\gamma$  signaling by enabling us to generate genetically modified animals with ablated activation downstream of GPCRs. Understanding the structural determinants of regulation of PI3K $\gamma$  could open avenues for generating inhibitors of GPCR-mediated



**Fig. 4.** Schematic representation of changes in HDX rate for p110 $\gamma$  and p101 observed when transiting through different activation states. Blue coloring indicates reduction in exchange, and increases are shown in red. Yellow highlights structural elements that differ between the PI3K $\gamma$  states.

PI3K $\gamma$  activation that could have useful anti-inflammatory and anti-cancer applications.

## Materials and Methods

**PIP<sub>3</sub> Reporter Translocation Assay.** After addition of fMLP, confocal microscopy was used to record a series of fields over a period of at least 5 min. For details, see *SI Materials and Methods*.

**Akt Activation.** HEK293 cells were transfected, and then lysates were analyzed by Western blotting for Akt expression and pAkt. See *SI Materials and Methods*.

**Lipid Kinase Assays.** In vitro PI3K assays used PIP<sub>2</sub> substrate in small unilamellar vesicles. Transfer of <sup>32</sup>P from labeled ATP was carried out as described in *SI Materials and Methods*.

**HDX-MS Measurements.** HDX-MS was carried out as described in *SI Materials and Methods*.

**Cellular Assays for NIH 3T3 Cells.** A soft agar colony formation as a measure of transformation was carried out as described in *SI Materials and Methods*.

**Purification of Proteins.** Purification of proteins was carried out as described in *SI Materials and Methods*.

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