Characterizing the Interactions between the Two Subunits of the p101/p110γ Phosphoinositide 3-Kinase and Their Role in the Activation of This Enzyme by G\textsubscript{βγ} Subunits*

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Recently, we have reported the purification and cloning of a novel G protein βγ subunit-activated phosphoinositide 3-kinase from pig neutrophils. The enzyme comprises a p110γ catalytic subunit and a p101 regulatory subunit. Now we have cloned the human ortholog of p101 and generated panels of p101 and p110γ truncations and deletions and used these in in vitro and in vivo assays to determine the protein domains responsible for subunit interaction and activation by βγ subunits. Our results suggest large areas of p101 including both N- and C-terminal portions interact with the N-terminal half of p110γ. While modifications of the N terminus of p110γ could modulate its intrinsic catalytic activity, binding to the N-terminal region of p101 was found to be indispensable for activation of heterodimers with G\textsubscript{βγ}.

Phosphoinositide 3-kinases (PI 3-kinases)\textsuperscript{1} are responsible for the phosphorylation of inositol phospholipids in the D-3 position of the inositol ring. Their lipid products (PtdIns(3,4)P\textsubscript{2}, PtdIns(3,4,5)P\textsubscript{3}) function as second messengers in eukaryotic cells. Indeed, PI 3-kinases appear to be involved in the control of a host of cellular responses ranging from intracellular transport to cell motility and the suppression of apoptosis (see Refs. 1–3, for reviews).

Three classes of PI 3-kinases are distinguished (4). Type I PI 3-kinases can be rapidly activated by cell-surface receptors and in vivo make predominantly PtdIns(3,4,5)P\textsubscript{3} (5). They are heterodimeric enzymes comprising a 110-kDa catalytic subunit and a regulatory subunit. Type IA PI 3-kinases contain an α, β, or δ p110 catalytic subunit (6, 7) and a p50, p55, or p85 (α or β) regulatory subunit (8–10). The regulatory subunits contain two SH2 domains which allow the enzyme to bind to, and be activated by key phosphotyrosine residues found in the cytoplasmic tails of growth factor receptors and various adapter proteins (11).

Type IB PI 3-kinase is made up of a p110γ catalytic subunit and a p101 regulatory subunit (12). This PI 3-kinase seems to be specifically stimulated by receptors capable of activating heterotrimic G proteins (12, 13). It appears, that this effect is mediated by G protein βγ subunits which can directly activate p101/p110γ PI 3-kinase (12). Although several reports show that both the biological effects of p110γ and its intrinsic sensitivity to G\textsubscript{βγ} are substantially amplified by the presence of p101, some data suggest that p110γ alone can be substantially catalytically activated by G\textsubscript{βγ} and have clear biological effects (14, 15). We have begun to address the issue of the role of p101, if any, in these events by analyzing the regions of p101 involved in interactions with p110γ and furthermore, for those constructs that bind, how they affect the activation of the complex by G\textsubscript{βγ}. We analyzed also the part played by p110γ in the process of G\textsubscript{βγ} activation and p101 binding.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis of p101 and p110γ—All point mutations, novel N or C termini, and internal deletions within porcine p101 cDNA were done using polymerase chain reaction-based strategies with mutagenic primers and Taq polymerase (Promega). Polymerase chain reaction-generated fragments were ligated back into pCMV3 with an N-terminal (EE)- or Myc-tag for expression in mammalian cells and into pAcOG3 with an N-terminal (EE)-tag for constructing baculovirus transfer vectors. All polymerase chain reaction-generated inserts were sequenced.

N- and C-terminal deletions of the human p110γ were either done by digesting with the appropriate restriction enzymes at designated sites. The isolated DNA fragments were religated into pcDNA3 containing N-terminal Myc-tag linkers. The Ras-binding domain deletions were done by fusing the internal fragments in-frame to the N-terminal 169 amino acids, creating a three amino acid linker. Full-length human p101 was cloned into an N-terminal EE-tag containing pcDNA3 vector.

Cell Culture—SF9 cells were grown in TNM-FH medium with 11% fetal bovine serum in spinner flasks at 27 °C at 0.5–2 × 10\textsuperscript{6} cells/ml. COS-7 cells were grown in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum.

SF9 Transfections and Production of Recombinant Protein—SF9 cells were lipofected using Insectin (Invitrogen) liposomes with baculogold (Pharmingen) linearized baculoviral DNA and a baculovirus transfer vector. Viral particles were plaque purified prior to amplification. Infection times were 2.3 days for p101 and 1.8 days for p110γ. Harvested cells were pelleted, washed in 7 mM NaH\textsubscript{2}PO\textsubscript{4} (pH 6.2), 20 mM MgCl\textsubscript{2}, 0.7% KCl, 2.66% sucrose, and stored at −80 °C.

** Purification of Proteins from Sf9 Cells—** 6H-tagged porcine p110γ and EE-tagged porcine p110γ were purified as described previously (12) with metal ion chelate columns or immunoprecipitation, respectively. The p110γ purification was modified as follows. Buffer C was 50 mM sodium phosphate (pH 7.1, 4 °C), 1% betaine, 0.05% Tween 20, 0.1 M NaCl; buffer D was 1% betaine, 30 mM Tris-Cl (pH 7.5, 4 °C), 0.15 M NaCl, 0.02% Tween 20, 10% ethylene glycol; buffer E was as C supplemented with 7 mM imidazole (pH 7.5, 4 °C); elution was in buffer F which was as C supplemented with 100 mM imidazole (pH 7.5, 4 °C). Eluted p110γ in buffer F was supplemented with 1 mM dithiothreitol and 1 mM EGTA (F\textsuperscript{−}) or, if it was to be bound to p101, passed through a PD10 column (Pharmacia) equilibrated in buffer H (10 mM Tris-Cl (pH 7.5, 4 °C), 0.15% NaCl, 1% betaine, 0.02% Tween 20, 10% ethylene glycol, 1 mM MgCl\textsubscript{2}, 1 mM dithiothreitol, 1 mM EGTA, 1% Triton X-100).

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1 The abbreviations used are: PI 3-kinase, phosphoinositide 3-kinase; G protein, guanosine nucleotide-binding protein; PtdIns(3,4)P\textsubscript{2}, phosphatidylinositol (3,4)-bisphosphate; (EE)-tag, glutamine-tag; (6H)-tag, hexa-histidine-tag; TNM-FH, insect cell medium; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; PEI, polyethylenimine cellulose; SF9 cells, Spodoptera frugiperda cells.

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In some cases, the p110γ (6H)-tag was cleaved with thrombin (Sigma) at 12 units/ml at 4 °C for 12 h.

The p101 purification was modified as follows, cells were sonicated into 0.15 M NaCl, 25 mM Hepes (pH 7.2, 4 °C), 2 mM EGTA, 1 mM MgCl₂ plus antiproteases; cytoplasmic fractions were not pre-cleared with antibodies. N-terminal truncations were made after the anti-EE beads (Onyx Pharmaceuticals) were five times in 0.4 mM NaCl, 20 mM Hepes (pH 7.4, 4 °C), 1 mM EGTA, 1% Triton X-100, 0.4% cholate, and three further times in buffer H. To bind the subunits, p101 bound to packed anti-EE beads was mixed on end for 2.5 h with a 25-fold molar excess of free p110γ in a small volume of buffer H. Heterodimer on anti-EE beads was washed in buffer J (1% Triton X-100, 0.15 M NaCl, 20 mM Hepes (pH 7.4, 4 °C), 1 mM EGTA) and in buffer F. p101-p110γ heterodimer was eluted from buffer F supplemented with 125 μM/ml EY peptide (EYMPTD). Typical yields were 2 mg of p110γ and 25 μg of p101/500 ml of SF9 culture.

GST-p110γ (the relevant recombinant baculovirus was a gift from R. Wetzker and encoded the human form of the protein) was purified from cytosolic fractions or Triton X-100 lysates of SF9 cells as described by Leopold et al. (Ref. 15 and references therein). This GST construct could not be cleaved by thrombin to yield full-length untagged p110γ.

Transient Transfections in COS-7—Exponentially growing COS-7 cells were trypsinized, washed twice in phosphate-buffered saline, and resuspended in 30 mM NaCl, 0.12 mM KCl, 8.1 mM Na₂HPO₄, 1.46 mM KH₂PO₄, 5 mM MgCl₂ at 1 × 10⁶ cells/ml. They were mixed with 30 μg of pCMV3-EE/101 constructs and 15 μg of pCMV3(Myct)1D0 or 15–50 μg of irrelevant plasmid DNA per 10⁷ cells aliquot. Aliquots were placed into 0.4-cm gap electroporation cuvettes (Bio-Rad) and electroporated in a single pulse (250 V, 980 microfarads). Cells from each electroporation were seeded into 175-ml tissue culture flasks in full growth medium. After 28 h, cells were harvested by trypsinization, washed once in phosphate-buffered saline, and cell pellets lysed in 1 × phosphate-buffered saline, 1 mM EGTA, 1% Triton X-100. Cytoplasmic fractions were immunoprecipitated with anti-EE beads followed by washes in 2 × phosphate-buffered saline, 1 mM EGTA, 1% Triton X-100. Samples for PI 3-kinase assays were washed further in 25 mM Hepes (pH 7.4, 4 °C), 1 mM EGTA. Remaining samples were resolved by SDS-PAGE. Gels were either Coomassie stained or wet-blotted onto polyvinylidene fluoride membranes (Millipore) and immunoblotted according to their tags with anti-Myc antibody (obtained from Onyx Pharmaceuticals) and anti-EE ascites fluid (Babco) or with an anti-p101 antisero (Microchemical Facilities, Babraham Institute).

PI 3-Kinase Assays—Free, purified protein from SF9 cells or COS-7 derived protein on anti-EE beads was diluted in sample dilution buffer (2 mM Tris/NaCl, 10% FBS, 0.1 mM EDTA, 0.1 mM dithiothreitol) (pH 7.4, 4 °C), 1 mM dithiothreitol). Lipid mixtures containing phosphatidylinositol and PtdIns(4,5)P₂ were dried down and sonicated into 0.1 M NaCl, 25 mM Hepes (pH 7.4, 4 °C), 1 mM EGTA, 0.1% cholate for final concentrations of 50 μM phosphatidylinositol and 5 μM PtdIns(4,5)P₂ (13). They were supplemented with G protein βγ subunits (prepared as in Ref. 16) or its vehicle (1% cholate, 1% bovine serum albumin, 0.1 M KCl, 20 mM Hepes (pH 7.4, 4 °C)) and resolved on PEI TLC plates as described before (13). Alternatively, assays were conducted precisely as described by Leopold et al. (15) with PtdIns acting as the substrate.

RESULTS

First, we simply reproduced the results of previous work showing purified p110γ could bind to p101 both in vitro and in vivo, but found that this association substantially increased the scale of activation of the PI 3-kinase with Gbg, from 1–2-fold to 50–150-fold, but now using modified procedures and porcine and human versions of the proteins. Human p101 (GenBank accession number AF128881) was 88% identical to porcine p101 (c.f. human p110γ is 94% identical to porcine p110γ). We found the species orthologs behaved interchangeably in these assays and gave results identical to those of the earlier work (Ref. 12, data not shown).

Analysis of p101 Structure/Function in Vitro—In order to understand the interactions between p101 and p110γ and the ability of the heterodimer to respond to GαS subunits, we made panels of p101 and p110γ constructs. Analysis of p101 structures involved in binding to p110γ was first approached with the following assay. NH₂-terminal (EE)-tagged p110γ constructs were purified from SF9 cells using anti-(EE)-beads. After washing, an excess of purified, NH₂-terminal (6H)-tagged full-length p110γ from SF9 cells was mixed with the immobilized p101 constructs. The beads were washed again and eluted with an epitope (EY) peptide. The released proteins were analyzed by SDS-PAGE and PI 3-kinase assays with and without GαS subunits. This analysis revealed that all of the p101 constructs tested (Fig. 1A) had reduced, but still significant capacity to specifically bind p110γ relative to full-length. Thus both the large N-and C-terminal truncations of p101 (Δ1–163 and 1–733) resulted in a 50% reduction in the recovery of p110γ, while 1283–581 reduced recovery by about 25% (Fig. 1B). Some p110γ specifically bound to N-terminal (p101 1–163) and C-terminal fragments of p101 (p101 1–574) (about 10 and 30% of wild-type, respectively) (Fig. 1C).

All p101-p110γ heterodimers were also assayed for PI 3-kinase activity in the presence or absence of GαS (Fig. 1D). All of the heterodimers created containing p101 truncations and deletions had reduced activation by G protein βγ subunits. It is striking, however, that the N-terminal truncation (Δ1–163) completely abrogated activation by Gbg, while the remaining truncations resulted merely in decreased ability of the respective heterodimers to be activated by Gbg. Neither N- nor C-terminal p101 peptides bound to p110γ yielded considerable activation when assayed in the presence of GαS subunits. Overall this suggests a number of regions in p101 are involved in its ability to interact with p110γ, particularly the N and C termini. Similarly, multiple areas in p101 are required for heterodimers to give maximal activation by GαS subunits, however, the N terminus is absolutely required for this process.

Analysis of p101 Structure/Function in Vivo—To address issues such as (a) the possibility that purification and our handling of the p101 constructs had resulted in varying degrees of denaturation and that this effect generated the differential binding we observed, and (b) the possibility that Gbg only occurs in vitro in the absence of competing proteins found in the cell, we examined the ability of p101 derivatives to bind to p110γ when the proteins are transiently co-expressed in COS-7 cells. In addition, to allow for more detailed mapping of the impact of different areas of p101 on both binding to p110γ and activation of the heterodimer with Gbg, a number of further constructs were introduced (Fig. 2A).

All p101 constructs were (EE)-tagged and co-expressed with (Myc)-tagged p110γ in COS-7 cells in transient transfection assays. Cytosolic fractions of harvested cells were subjected to anti-(EE) immunoprecipitations, half of which were used to estimate binding stoichiometries on gels or blots (see Fig. 2B, for an example) while the other half was assayed for PI 3-kinase activity in the presence or absence of G protein βγ subunits.

The data describing binding of p101 derivatives to p110γ in this assay is summarized in Fig. 2C. Binding to p110γ was affected in all p101 constructs except for the point mutations and DE328–341STP constructs, supporting the conclusion from the in vitro studies indicating that more than one area of p101 contributes to binding p110γ. It appears, however, that in the COS-7 cell assays compared with the in vitro assays, the N-terminal deletions lead to a larger decrease in p110γ binding.
ability than C-terminal deletions (25 versus 70% binding) and N-terminal peptides can rescue more binding than C-terminal peptides (50 versus 12% binding).

We also assayed all constructs immunoprecipitated from COS-7 cells for PI 3-kinase activity. The resulting data (Fig. 2D) underlines the data already obtained from the in vitro assays from Sf9-produced protein. Again, we found that both C and N termini of p101 contribute to full activation of the complex by G protein βγ subunits. Any deletion within p101 interfered with the ability of the heterodimer to be completely activated. Interestingly, deletion of the C-terminal 150 amino acids (1–733 and p84) and deletion of much larger C-terminal portions (1–314, 1–265, and 1–163) lead to a similar decrease in activation by G<sub>βγ</sub> to less than 25% of that of full-length p101, stressing the role for the very C-terminal part. The most dramatic result, however, is induced by deletion of the very N-terminal portion of p101 (constructs Δ1–153 and Δ1–263) which lead to complete loss of activation, confirming the conclusions from the in vitro assays that this region of p101 is essential for activation of the complex by G protein βγ subunits.

**Analysis of p110γ Structure/Function**—To define the sites in p110γ involved in both interaction with p101 and activation by G<sub>βγ</sub>, we prepared a set of constructs shown in Fig. 3 for expression in COS-7 and Sf9 cells. These constructs were used in assays aimed at defining the regions that interacted with p101 that were relevant to the G<sub>βγ</sub> activation of p101-p110<sub>γ</sub> heterodimers and were required for basal PI 3-kinase activity.

**PI 3-Kinase Activity of p110γ Constructs**—Increasing N-terminal truncation of p110γ systematically reduced its basal catalytic activity (Fig. 4A). Deletions beyond residue 369 had no detectable PI 3-kinase activity. N-terminal tags seemed to increase the basal catalytic activity of p110γ. The activity of the N-terminal (6H)-tagged p110γ (6H-p110γ) was reduced 2-fold by removal of the tag with thrombin and the activity of defined p110γ constructs was reduced by half by switching from an...
N-terminal to a C-terminal (6H)-tag. Furthermore, N-terminal (GST)-tagged p110 \( g \) (GST-p110 \( g \)) possessed an approximately 10–15-fold higher specific activity than wild-type p110 \( g \). In contrast, and as previously reported (12), binding of p101 reduced the basal catalytic activity of p110 \( g \) by 5-fold.

Reports that GST-p110 \( g \) can be substantially activated by \( G_b \) in vitro (15) lead us to test the effect of manipulating p110 \( g \) structure on its responsiveness to \( G_b \), despite the fact that we have never detected significant effects of \( G_b \) on porcine p110 \( g \) activity in the absence of p101. Testing a range of catalytically active porcine and human p110 \( g \) constructs with or without N-terminal or C-terminal (6H) tags in the presence and absence of \( G_b \) showed up to 2-fold activations of human (but not porcine) proteins with either our standard assay procedure or that of Leopoldt et al. (15; see below). However, with human GST-p110 \( g \) we found that although our assay procedure (using PtdIns(4,5)\( P_2 \) and 3.5 mM MgCl\(_2\)) failed to reveal any activation by \( G_b \), using PtdIns and 10 mM MgCl\(_2\) in the assay (15), we could reproducibly detect 6–7-fold activations by \( G_b \) (Fig. 4B); this was unaffected if the constructs were purified from membrane or cytosolic fractions (data not shown).

**Discussion**

It is clear from the above results that multiple regions of p101 are involved in either direct (i.e. physical contact) or indirect interactions with p110\( g \). Interestingly, the \( G_b \) sensitivity of the p110\( g \) heterodimers formed by using various p101

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**Fig. 2.** In vivo association of p101 constructs with p110\( g \) and activation of resulting heterodimers by \( G_b \). A, further p101 constructs for use in COS-7 transient transfection assays included further N- and C-terminal peptides and larger deletions. Furthermore, an unusual acidic region was replaced by a stretch of serines, threonines, and prolines (DE328–341STP); a region bearing a vague similarity to PH domains of known signaling proteins as well as potential key residues therein were mutated (Δ161–263, Y193C, W252A); a possible spliced variant of p101 is also included (p84, this sequence diverges from p101 at residue 733 resulting in a truncated version of the protein; P. Hawkins and A. Eguinoa, unpublished data). Again, all p101 constructs included N-terminal (EE)-tags (C). B, COS-7 cells were transiently transfected with mammalian expression vectors encoding a (EE)-p101 construct and a (Myc)-p110\( g \) (for controls, the total amount of DNA was made up to with irrelevant DNA). Cytoplasmic fractions of transfected cells were subjected to immunoprecipitations with (EE)-beads. The amount of co-immunoprecipitated (Myc)-p110\( g \) was visualized on Coomassie-stained protein gels. Binding ratios were estimated by eye with a minimum of three independent transfections being taken into account for each p101 construct. C, graphical illustration of all binding data obtained from the COS-7 transient transfection assays. D, aliquots of the COS-7-derived p101-p110\( g \) heterodimers (on the beads) were assayed for PI 3-kinase activity in the presence or absence of \( G_b \). Fold activation obtained with \( G_b \) for the different constructs is detailed comparatively in this graph. The legend on the y axis indicates with which construct p110\( g \) had been expressed.

**Interaction of p110\( g \) Constructs with p101 and Activation of Heterodimers by \( G_b \).** Experiments performed with proteins expressed in either COS-7 or Sf9 cells indicated that the N-terminus of p110\( g \) was critical for binding to p101. p110\( g \) deletions through the series Δ1–122, Δ1–133, and Δ1–144 (Fig. 5A, for examples) showed decreasing ability to bind p101, a Δ1–169 construct showed no detectable binding (Fig. 5B). However, other regions were clearly involved in binding p101 (see Fig. 5C for an overview). Hence, N-terminal peptides (e.g. 1–169) had low p101 binding potential and further deletion of either the Ras-binding domain (178–330) or the central regions (330–775) of p110\( g \) also significantly reduced p101 binding. It is significant that those p110\( g \) constructs capable of binding p101, once bound, were all very similarly activated by \( G_b \) (Fig. 5D). Catalytically active p110\( g \) constructs incapable of binding p101 remained insensitive to \( G_b \) when assayed in the presence of p101 (Fig. 5C).
constructs clearly correlated with how tightly they formed heterodimers, but absolutely depended on the presence of the N terminus of p101. We have previously published some binding data showing $G_{bg}$ can apparently bind about five times more effectively to p101 than p110y (12), but we cannot discount important binding of $G_{bg}$ to p110y, as others have identified clear effects of $G_{bg}$ on GST-p110y (13, 15; see also Fig. 4B). There are, therefore, two types of explanation for this data. First, that a lot of the contacts between p101 and p110y are required for $G_{bg}$ to have their full effects on p110y catalytic activity (whether the $G_{bg}$ bind to p101, p110y, or both). Second, that the primary sites of $G_{bg}$ binding that influence the activity of the heterodimer are located on p101 and are “co-incidentally” disrupted by changes which impinge on p101-p110y interaction. This apparently highly interwoven structure-function relationship between p101, $G_{bg}$, and p110y is not surprising if it is accepted that binding of p101 and $G_{bg}$ can have such a profound effect on the catalytic site in p110y. The binding site for p101 in p110y, although primarily involving the N terminus of the protein, is a far larger region extending deeper into the protein. Considering the fact that several parts of p101 are involved in binding to p110y, it would seem reasonable that a substantial piece of p110y is involved in binding p101 (note that in p110a a relatively short region of the N terminus is thought to bind to a short inter-SH2 domain segment of p85 (17, 18)). In contrast to the situation with p101, where deletions affecting the total contact area and strength of binding to p110y also affected the sensitivity of the heterodimer to $G_{bg}$, deletions through the N terminus of p110y that reduced the efficiency of binding to p101, had no effect on the activation of heterodimeric enzyme that could be recovered. Clearly, this analysis cannot be complete because deletions removing more than 200 N-terminal residues begin to strongly reduce the intrinsic catalytic activity of p110y, such that deletions beyond residue 369 activity is completely lost (see below), yet these deeper regions are clearly involved in binding to p101. However, the implication of this data is that some of the contacts between p101 and p110y, involved in their binding, can be eliminated by disruptions on the p110y side without effect on $G_{bg}$-activation of the heterodimer, but that contacts broken by p101 disruptions reduce the ability of $G_{bg}$ to activate the enzyme. The simplest explanation for these observations is that p101 disruptions are also interfering with $G_{bg}$-binding sites that are most important for activation of the heterodimer; i.e., that the latter of our proposed models (see above) is most likely to be correct.

In the process of engineering and assaying a range of p110y constructs it became clear that the N terminus of the protein had significant potential to influence catalytic activity. Hence the addition of N-terminal GST and (6H)-tags apparently increased the activity of the enzyme. In contrast, binding of p101,
which appears to interact most strongly with the N terminus of p110γ lead to a 5-fold inhibition of basal catalytic activity. This effect of p101 appears to have some parallels with observations that p85 binding to p110α suppresses its PI 3-kinase catalytic activity (19). Clearly the scale of activation of the p101/p110γ heterodimer by Gbg in vitro indicates this "simple model" cannot apply here, however, this phenomenon lead us to test whether N-terminal tagging also influenced the sensitivity of p110γ alone to Gbg subunits.

We found that of a large range of constructs GST-p110γ alone was clearly activated by Gbg, but only under the conditions previously reported to show this effect (15). The very fact that Gbg can have some significant effect on GST-p110γ in the absence of p101 may be taken to suggest one primary point of interactions of Gbg with p110γ is through direct binding to the p110γ subunit. This gains some support from work indicating Gbg can bind to p110γ alone. We have previously found that although five times more Gbg could be rescued from in vitro binding assays associated with p101 than with p110γ we could detect above background binding to p110γ (12). Furthermore, Leopoldt et al. (15) have shown Gbg can be recovered with p110γ immunoprecipitated from SF9 cells, and they went on to map this binding to two distinct regions within p110γ.

As a consequence of these considerations and in the light of (i) lack of effect of Gbg on other, non-GST-tagged p110γ constructs, (ii) the unphysiological nature of the substrate and ionic conditions required to see Gbg activation of GST-p110γ, (iii) the inevitable problem that in using p110γ alone rather than in a com-
plex with p101 further hydrophobic regions of protein interaction may be exposed, and (iv) that the presence of the GST fusion will drive homodimerization of p110γ, we consider the evidence that the effects of Gβγ on p101/p110γ PI 3-kinase are only via direct interaction with p110γ to be very weak.

Overall, our data suggests that p101 and p110γ interact primarily through large areas covering the N and C termini of p101 and the N-terminal half of p110γ and that the areas which bind Gβγ giving the major effect on PI 3-kinase activity are probably located on p101. Given the substantial difficulties encountered in studying specific, low affinity interactions of Gβγ subunits with various effectors in vitro (e.g. studying Gβγ activation of PLCβs can be seen as an analogous problem), it is likely that a combination of further technologies, including detailed structural information, will be required to yield further insight into the mechanism of action of Gβγ on p101/p110γ PI 3-kinase.

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