Mechanism of the regulation of type IB phosphoinositide 3OH-kinase by G-protein $\beta\gamma$ subunits

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Type IB phosphoinositide 3OH-kinase (PI3K) is activated by G-protein $\beta\gamma$ subunits (G$\beta\gamma$s). The enzyme is soluble and largely cytosolic *in vivo*. Its substrate, PtdIns(4,5)P_2, and the G$\beta\gamma$s are localized at the plasma membrane. We have addressed the mechanism by which G$\beta\gamma$s regulate the PI3K using an *in vitro* approach. We used sedimentation assays and surface plasmon resonance to determine association of type IB PI3K with lipid monolayers and vesicles of varying compositions, some of which had G$\beta\gamma$s incorporated. Association and dissociation rate constants were determined. Our results indicated that in an assay situation *in vitro* the majority of PI3K will be associated with lipid vesicles, irrespective of the presence or absence of G$\beta\gamma$s. In line with this, a constitutively active membrane-targeted PI3K construct could still be activated substantially by G$\beta\gamma$s *in vitro*. We conclude that G$\beta\gamma$s activate type IB PI3K by a mechanism other than translocation to the plasma membrane.

Key words: heterotrimeric G-protein, neutrophil, PI3K, PtdIns(3,4,5)P_3, signal transduction.

INTRODUCTION

Phosphoinositide 3OH-kinases (PI3Ks) are a large family of enzymes capable of phosphorylating the 3-hydroxyl group of phosphoinositides, leading to the production of 3-phosphorylated lipid molecules [PtdIns3P, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3] [1]. PI3Ks are divided into classes according to their substrate specificity and structure. Type I PI3Ks respond rapidly to activated cell-surface receptors and, *in vivo*, produce predominantly PtdIns(3,4,5)P_3 [2] on the inner leaflet of the plasma membrane. This in turn causes the stimulation of multiple downstream events, initiated by the membrane recruitment and activation of PtdIns(3,4,5)P_3-binding proteins (see [3] for a recent review). Type I PI3Ks are heterodimeric enzymes comprising a p110 catalytic subunit [4–6] and a smaller regulatory subunit. Two subclasses are distinguished.

Type IA PI3Ks consist of p110α, p110β or p110δ catalytic subunits which associate with an SH2 domain containing regulatory subunit (p85α or its spliced variants, or p55 [7–9]). These regulatory subunits are responsible for its activation by phosphotyrosine-containing proteins, such as the cytoplasmic tails of activated growth factor receptors [10,11]. Binding of phosphotyrosine motifs by the SH2 domains modestly activates the PI3K [12,13] and also leads to the translocation of the cytosolic enzyme complex to the plasma membrane, the location of the substrate PtdIns(4,5)P_2 ([14] and references therein). Further, type IA PI3Ks are stimulated directly by GTP-bound Ras. This activation is synergistic with p85-mediated stimulation by phosphotyrosyl peptides [15]. Interactions between the PI3K and GTP–Ras occur through a clearly defined Ras-binding domain in the N-terminus of the catalytic subunit.

Type IB PI3K consists of a p110γ catalytic subunit, the crystal structure of which has been reported recently [16], and a p101 regulatory subunit [6,17]. It is activated directly by G-protein $\beta\gamma$ subunits (G$\beta\gamma$s) *in vivo* and *in vitro* [6,17]. Sensitivity of type IB PI3K to G$\beta\gamma$-dependent stimulation is enhanced significantly by the presence of the regulatory p101 [18]. Whereas p101 is devoid of any known protein–protein interaction motifs, p110γ contains an N-terminal Ras-binding domain. p110γ alone or p101/p110γ heterodimers are activated directly by GTP–Ras [19]. Ras stimulation of the PI3K is synergistic with its activation by G$\beta\gamma$s [19]. However, the precise mode of activation by G$\beta\gamma$s or Ras is as yet unresolved. It has been assumed to be linked to a translocation event by analogy to the situation seen with class IA PI3Ks. However, many other G$\beta\gamma$-regulated effectors are membrane-associated proteins and appear to be modulated via allosteric mechanisms [20,21].

We present experiments here addressing the mode of activation of type IB PI3K by G$\beta\gamma$s *in vitro*. By studying the effects of G$\beta\gamma$s on the distribution of the PI3K in a variety of assays, we show that G$\beta\gamma$s do not cause the PI3K to translocate into phospholipid membranes.

MATERIALS AND METHODS

Cell culture

Sf9 cells (derived from *Spodoptera frugiperda* cells) were grown in TNM-FH medium (Sigma) with 11% fetal bovine serum in spinner flasks at 27 °C at (0.5–2) × 10^6 cells/ml. COS-7 cells were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum.

Purification of recombinant proteins from Sf9 cells

Hexahistidine-tagged (6H-tagged) porcine p110γ and glutamine-tagged (EE-tagged) porcine p101 were produced individually and purified from Sf9 cells as described previously [17], with metal ion chelate columns and immunoprecipitation, respectively. G$\beta\gamma$s were $\beta_1\gamma_2$ heterodimers from Sf9 cells; the β subunit was EE-tagged.

Abbreviations used: PI3K, phosphoinositide 3OH-kinase; 6H tag, hexahistidine tag; EE tag, glutamine tag; SPR, surface plasmon resonance; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdSer, phosphatidylserine; G$\beta\gamma$, G-protein $\beta\gamma$ subunit.

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Purification of recombinant proteins from COS-7 cells

Heterodimers of Myc-tagged p110γ or of a p110γ fused to a combined myristoylation/palmitoylation signal from the tyrosine kinase Yes (Myr-p110γ) together with EE-tagged p101 were produced in transiently transfected COS-7 cells as described previously [17]. Pellets of frozen, transiently transfected COS-7 cells were resuspended by vortexing into ice-cold 150 mM NaCl, 40 mM Hepes, pH 7.4 (at 4 °C), 5 mM EGTA, 1 mM $MgCl_2$ and a cocktail of protease inhibitors (0.1 mM PMSF and leupeptin, antipain, aprotinin and pepstatin A, all at 10 μg/ml; Sigma). This was followed by probe sonication at a very low setting (four bursts of 10 s with 30 s intervals on ice). Undisrupted cells and nuclei were pelleted (500 ***g***, for 15 min at 4 °C) and discarded, whereas the remaining sample was subjected to ultracentrifugation (40 min at 100000 ***g*** and 4 °C). Supernatants thereof were used as cytoplasmic fractions for immunoprecipitations after addition of Triton X-100 to a 1 % final concentration. Membrane-containing pellets were washed twice in sonication buffer before being resuspended slowly in the same buffer supplemented with 1 % Triton X-100; insoluble material was pelleted by ultracentrifugation. Fractions were immunoprecipitated with anti-EE beads followed by extensive washes in 150 mM NaCl, 20 mM Hepes, pH 7.4 (at 4 °C), 1 mM EGTA, 1 % Triton X-100 and 0.1 % azide. Samples were washed further in 150 mM NaCl, 30 mM Tris/HCl (pH 7.5 at 4 °C), 10 % ethylene glycol, 0.02 % Tween-20, 100 mM imidazole (pH 7.5 at 4 °C), 1 mM dithiothreitol, 1 mM EGTA and 0.1 % azide prior to elution in the same buffer supplemented with 125 μg/ml EY peptide (Glu-Glu-Tyr-Met-Pro-Met-Glu, with the N-terminus acetylated) on ice.

Surface plasmon resonance (SPR)

The SPR instrument was a Biacore 2000 (Biacore AB, Stevenage, Herts., U.K.) used with a hydrophobic association sensor chip, which consisted of an octadecane-thiol monolayer on a gold surface. Each sensor chip contained four flow cells (2.4 mm × 0.5 mm × 0.05 mm) with a probing spot for the SPR signal of approx. 0.26 mm^2 for each flow cell. The buffer used for all experiments was 1 × PBS, pH 7.4/1 mM EGTA/1 mM $MgCl_2$. For the formation of lipid monolayers [22], small unilamellar vesicles from phosphatidylcholine (PtdCho), phosphatidylserine (PtdSer), phosphatidylethanolamine (PtdEtn; Sigma) and PtdIns(4,5)P_2 [23] were prepared in PBS as 2 mM final solutions by bath sonication.

For G$\beta\gamma$-containing monolayers (625 nM), lipid vesicles together with concentrated G$\beta\gamma$ protein were dialysed against PBS overnight at 4 °C. Gα subunit-containing vesicles were formed from PtdCho/PtdSer/PtdEtn lipid vesicles together with 10 μM Gα subunit dialysed against PBS overnight at 4 °C. The surface of the hydrophobic association chip was cleaned by a 10 min injection of 40 mM octyl-D-glucoside at a flow rate of 10 μl/min. The injection needle was cleaned by pre-dipping in water, and 20 μl of 500 μM lipid vesicles injected at a flow rate of 2 μl/min. The lipid layer was then washed at 100 μl/min with 20 μl of 10 mM NaOH. The degree of lipid bound was determined from the amount of lipid bound at a stable level after the NaOH wash and from the extent of non-specific binding of 0.1 mg/ml BSA (in PBS) over a 5 min injection. For SPR binding assays, proteins were serially diluted in PBS/1 mM EGTA/1 mM $MgCl_2$ and passed sequentially over flow cells containing different lipid monolayers. For Gα subunit-binding assays, Gα subunit-containing vesicles were diluted in PBS/1 mM EGTA/1 mM $MgCl_2$ and passed over different lipid monolayers. Following injection of the sample, the sample solution was then replaced by running buffer and the protein–receptor complex allowed to dissociate. Lipid monolayers were stripped by a 10 min injection of 100 mM octyl-D-glucoside and reloaded prior to each individual binding experiment.

Data analysis

Data were prepared for analysis by adjusting the average response recorded 20 s prior to injection and the time of each injection to zero. The corrected data were analysed by non-linear fitting using BIAeval 3.0 global software based on algorithms for numerical integration [24,25]. This gave the observed rate k_{obs} (s^{-1}) for the association phase of the binding curve. As proteins were binding to lipid, to PtdIns(4,5)P_2 and to G$\beta\gamma$s, no attempt was made to determine the family of individual association and dissociation rate constants that underlie binding to this heterogeneous surface. Instead, affinities were calculated from analysis of equilibrium binding levels at various protein concentrations. By measuring the response units attained at equilibrium as a function of protein concentration (C), affinities can be determined from a Scatchard analysis using the equation:

$$R_{eq}/C = (R_{max} - R_{eq})/K_d \quad (1)$$

where R_{eq} is the response at equilibrium, R_{max} is the maximum possible response (proportional to the amount of receptor immobilized on the sensor surface) and K_d is the affinity constant. A plot of R_{eq}/C versus R_{eq} has a slope of $-1/K_d$. Where binding had not reached a true equilibrium, a pseudo-first-order fit of the data was used to extrapolate binding curves to equilibrium.

Association of PI3K with sucrose-loaded vesicles

A dry lipid film of PtdEtn and PtdIns(4,5)P_2 was bath-sonicated into 0.2 M sucrose, 20 mM KCl, 20 mM Hepes, pH 7.4 (at room temperature), and 0.01 % azide to give a lipid stock [12.5 μM PtdIns(4,5)P_2/123 μM PtdEtn in the final assay mixture], which was mixed with EE-p101/6H-p110γ protein (0.4–0.6 μg of protein, which was prespun for 40 min at 200000 ***g***) and 4 vol. of reaction buffer [0.3 mg/ml BSA, 0.12 M NaCl, 1 mM EGTA, 1 mM $MgCl_2$, 1 mM dithiothreitol, 5 mM KCl, 20 mM Hepes pH 7.4 (at room temperature), and 0.01 % azide]. Protein–lipid vesicle complexes were allowed to form on ice for 15 min, and then spun to tight pellets (200000 ***g*** for 10 min). Control reactions were set up with mixtures lacking lipids. Portions of supernatants and of washed aspirated pellets were used for immunoblotting. Bands corresponding to EE-p101 were detected using an anti-EE ascites fluid (Babco), a secondary anti-mouse horseradish peroxidase conjugate and enhanced chemiluminescence. They were quantified by densitometric scanning (Gel-Doc 2000; BioRad).

Immunofluorescence staining

Transfected cells were seeded on to glass coverslips and fixed 28 h later in 4 % paraformaldehyde in PBS. Cells were permeabilized in 0.2 % Triton X-100 in PBS and blocked by 1 % goat serum in PBS. p110γ protein was visualized using a mouse monoclonal anti-p110γ antibody (clone LS1/38) followed by a goat anti-mouse FITC-conjugated secondary antibody (Jackson Immunoresearch Laboratories). Coverslips were mounted on to a small drop of antifade mountant (Aqua Polymount; Polysciences, Warrington, PA, U.S.A.). Cells were examined on a Zeiss axiophot microscope using × 40 and × 63 (1.4 numerical aperture) oil immersion lenses. Fluorescent images were recorded on 400 ASA Fujichrome film.

PI3K assays

PI3K assays were done as described previously [17]. Prior to starting the assays, PI3K aliquots were incubated with the lipid vesicle mixtures on ice for 1 h to allow the association of the enzyme and vesicles to reach an equilibrium.

RESULTS

We have previously defined *in vitro* assay conditions under which heterodimeric p101/p110γ PI3K, but not the p110γ catalytic subunit alone, can be activated substantially (up to 80-fold) by G$\beta\gamma$s [6,17]. In an attempt to test whether inclusion of G$\beta\gamma$s in these assays activates p101/p110γ by driving its translocation into the vesicles containing phospholipid substrate, PtdIns-(4,5)P_2-containing vesicles were prepared as described previously [17], except that they contained 0.2 M sucrose. Dilution of these vesicles into osmotically balanced sucrose-free buffer allowed them to be sedimented by centrifugation. Addition of G$\beta\gamma$s to these preparations could still substantially stimulate p101/p110γ PI3K activity (53% of the observed fold activation in control assays using the same G$\beta\gamma$s; results not shown). The distribution of p101/p110γ heterodimers or p110γ alone in the presence and absence of G$\beta\gamma$s between the soluble and vesicle compartments was assessed by analysis of pellet and supernatant fractions using quantitative immunoblotting or silver-staining of SDS/PAGE gels. These experiments were carried out in the absence of ATP but under conditions otherwise identical to PI3K activity assays [12.5 μM PtdIns(4,5)P_2 at 9.2 mol%]. Although in control reactions lacking lipids all PI3K remained soluble, where lipid vesicles were present, 70% of p101/p110γ PI3K was found in the vesicle fraction even in the absence of G$\beta\gamma$s (Figure 1A). G$\beta\gamma$s clearly inserted into the lipid vesicles (Figure 1B, panel i), but their presence did not significantly alter the fraction of p110γ catalytic subunits alone or of heterodimeric p101/p110γ PI3K, which associated with the vesicles (Figure 1B, panels ii and iii).

To obtain an experimentally independent and more quantitative estimate of these protein–phospholipid interactions, we used a SPR instrument to assess the binding of soluble-phase p110γ and p101/p110γ proteins to phospholipid monolayers, either with or without EE-tagged G$\beta\gamma$s, coated on to hydrophobic association chips. G$\beta\gamma$s inserted into lipid monolayers in this way were clearly exposed to the mobile phase, as specific association of an anti-EE antibody with the G$\beta\gamma$-loaded monolayer surfaces was readily detected (Figure 2A). Furthermore, phospholipid vesicles containing Gα_i subunits purified from bovine brain, but not vesicles without Gα_i subunits, bound specifically only to surfaces with G$\beta\gamma$s, compared with surfaces without G$\beta\gamma$s (Figure 2A, and results not shown). These results suggest that the G$\beta\gamma$s immobilized in the lipid monolayer are functional and exposed to the mobile phase and hence potentially able to interact with effectors.

Initial experiments addressing interactions between PI3K preparations (Figure 2B) and lipid monolayers showed significant interactions between p110γ and p101/p110γ preparations with the lipid surfaces, while control proteins such as BSA did not bind (results not shown). The p110γ catalytic subunit displayed higher affinities than the heterodimeric p101/p110 γ PI3K (Table 1). The extent of these interactions was reduced by including 1 mM Mg^{2+} in the mobile phase in subsequent experiments (results not shown). We derived data sets describing p110γ and p101/p110γ binding to mixed lipid surfaces (equimolar PtdCho, PtdSer and PtdEtn), which did or did not contain PtdIns(4,5)P_2 (10 mol% for p110γ; 1 or 10 mol% for p101/p110γ) with and without G$\beta\gamma$s (Figure 2C). All results indicated that binding of both p110γ and p101/p110γ to the lipid surfaces was increased substantially by inclusion of PtdIns(4,5)P_2, but that inclusion of G$\beta\gamma$s promoted only very small increases in the association of these proteins with the lipids. G$\beta\gamma$s were also unable to promote significant association of p101/p110γ to PtdCho-only lipid surfaces, conditions in which background binding of the proteins to the lipids was minimal (results not shown).

Affinities and observed rate constants were determined from long injections, in which equilibrium positions had been reached, using the algorithms described in the Materials and methods section. The affinity constants (K_d) describing the binding of p101/p110γ to surfaces containing 1 or 10 mol% PtdIns(4,5)P_2 were near identical (Table 1, and results not shown). The surfaces containing 10 mol% displayed approx. 3-fold higher effective

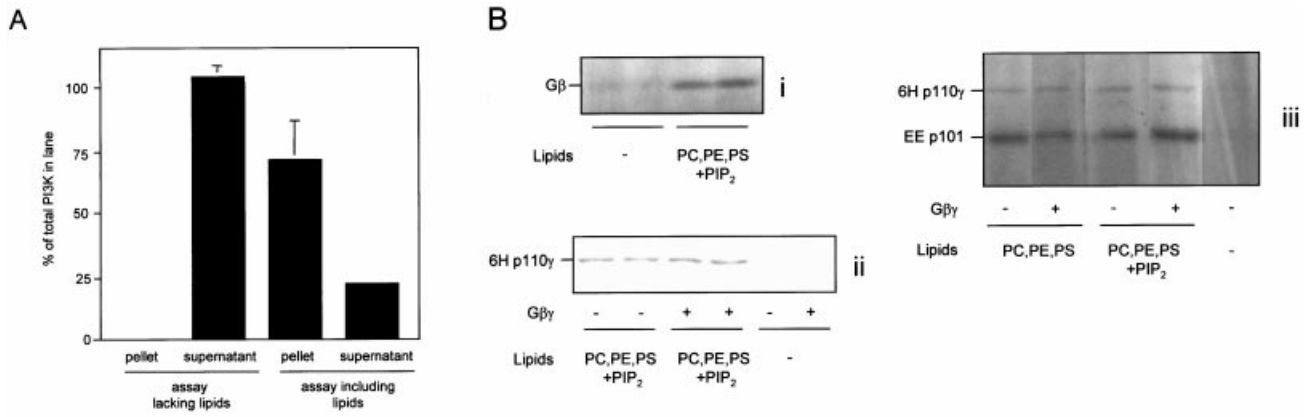

Figure 1 Association of p101/p110γ PI3K with sucrose-loaded lipid vesicles

Recombinant 6H-tagged p110γ monomers or EE-tagged p101/6H-tagged p110γ PI3K heterodimers were allowed to interact with sucrose-loaded vesicles [90% PtdEtn/10% PtdIns(4,5)P_2] prepared with or without 0.3 μM G$\beta\gamma$s or their vehicle. After 15 min on ice, vesicles were sedimented by ultracentrifugation. (**A**) Distribution of EE-p101/6H-p110γ PI3K between the pellet and supernatant fractions in assays with or without lipid vesicles (in the absence of G$\beta\gamma$s). The amounts of EE-p101 in the fractions were quantified by immunoblotting with an anti-EE antibody and densitometric scanning. (**B**) Association of proteins with the vesicle fraction of protein components in the sucrose-loaded vesicle pellets were visualized by SDS/PAGE followed by silver staining. Panel i: the incorporation of G$\beta\gamma$s into the vesicle fraction. Panel ii: the association of 6H-p110γ with vesicles prepared with or without G$\beta\gamma$s. Panel iii: the association of EE-p101/6Hp110γ with vesicles prepared with or without G$\beta\gamma$s. PC, PtdCho; PE, PtdEtn; PS, PtdSer; PIP_2, PtdIns(4,5)P_2.

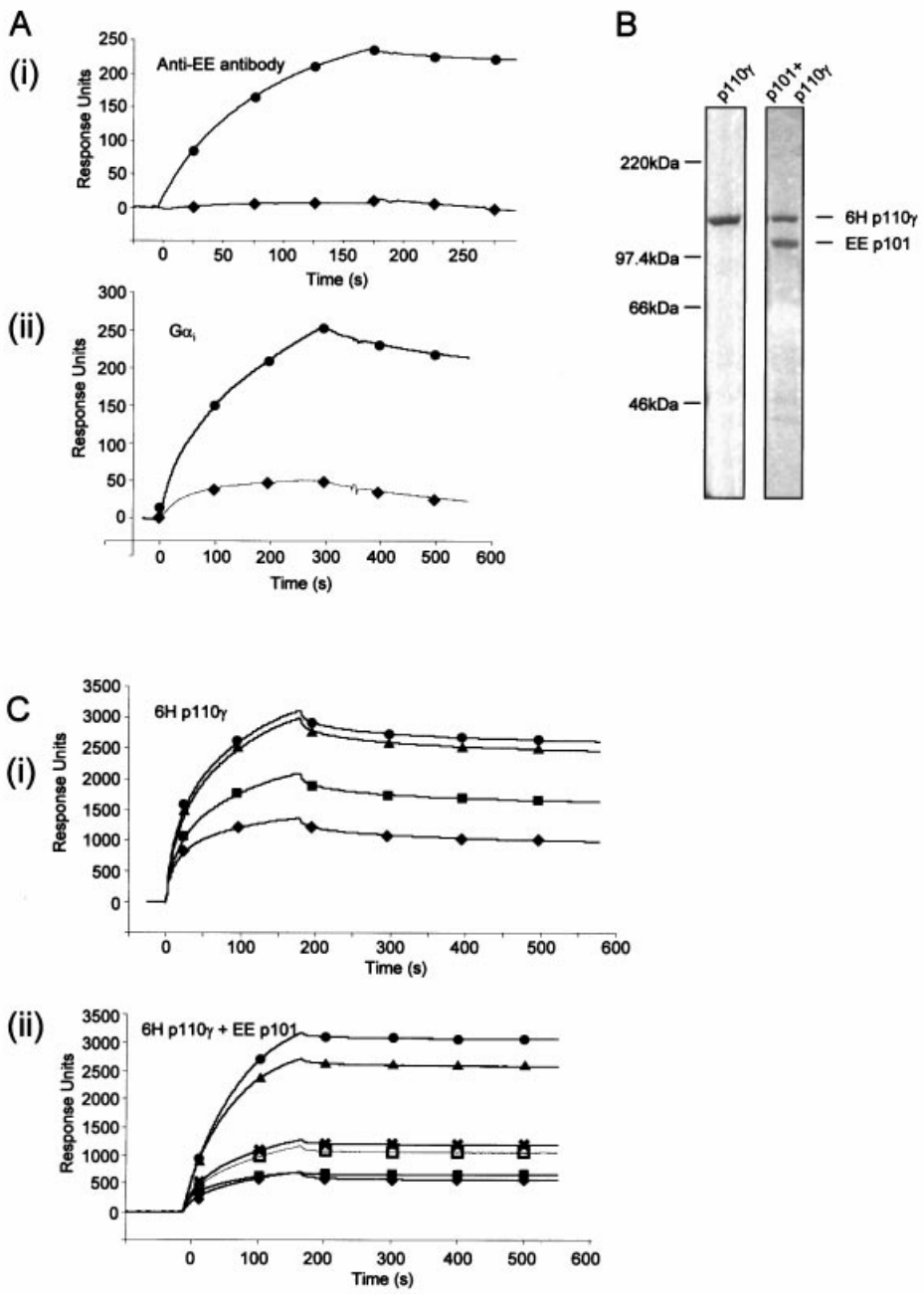

Figure 2 Association of recombinant p110γ and p101/p110γ proteins with lipid surfaces of various compositions measured using SPR

(**A**) Presentation of G$\beta\gamma$s in lipid surfaces on hydrophobic association sensor chips. Monitoring of real-time binding of the association of an anti-EE antibody (panel i) and lipid vesicles incorporating 10 μM Gα_i subunits (panel ii) to control lipid surfaces (equimolar PtdCho, PtdSer and PtdEtn, ◆) or lipid surfaces incorporating 10 μM G$\beta\gamma$s (●). Sample injections were 0–180 s and 0–300 s, respectively, followed by washing. (**B**) 6H-tagged p110γ was prepared from Sf9 cells in a one-step purification procedure as described previously [17]. The example shown is at an estimated purity of greater than 90%. Sf9-cell-derived EE-tagged p101 and independently prepared 6H-tagged p110γ were mixed for *in vitro* binding. Complexes were purified further on a size-exclusion column to ensure 1:1 stoichiometry of the two subunits. The preparation shown was estimated to be 80% pure. (**C**) Monitoring of real-time binding of 125 nM monomeric p110γ (panel i) and of 250 nM p101/p110γ heterodimer (panel ii) to lipid surfaces of different compositions coated on to a hydrophobic association sensor chip. Sample injections were 0–180 s followed by washing. Lipid surfaces for monomeric p110γ: ◆, 33% PtdEtn/PtdSer/PtdCho; ■, as ◆ plus 625 nM G$\beta\gamma$s; ▲, 30% PtdEtn/PtdSer/PtdCho and 10% PtdIns(4,5)P_2; ●, as ▲ plus 625 nM G$\beta\gamma$s. Lipid surfaces for p101/p110γ heterodimer: ◆, ■, ▲ and ●, as for monomeric p110γ; □, 33% PtdEtn/PtdSer/PtdCho and 1% PtdIns(4,5)P_2; ×, as □ plus 625 nM G$\beta\gamma$s. All SPR experiments were repeated at least twice using fresh, independently prepared PI3K proteins with the same results.

maximum binding capacity (manifest as an approx. 3-fold increase in R_{eq} and k_{obs}). This suggests that PtdIns(4,5)P_2 concentration in the membrane is an important determinant of PI3K binding in the absence of ATP. The effects of G$\beta\gamma$s on binding of PI3K to the lipid monolayer with either 1 or 10 mol% PtdIns(4,5)P_2 were relatively small compared with the scale of the activations of the PI3K that occur under similar conditions. Indeed, this mismatch is at its greatest in the presence of PtdIns(4,5)P_2, the conditions under which we can assay substantial G$\beta\gamma$ stimulation of PI3K activity. In some respects the

Table 1 Kinetic and binding constants for association of p110γ and p101/p110γ to lipid monolayers in the presence of 1 mM $MgCl_2$

All results presented were reproducible and were demonstrated at least twice from independent protein preparations. Values for PtdIns(4,5)P_2-containing surfaces refer to 10 mol% PtdIns(4,5)P_2. Surfaces containing 1 mol% PtdIns(4,5)P_2 exhibited the same trends with rate constants within the same errors as 10 mol% PtdIns(4,5)P_2-containing surfaces. k_{obs} refers to the observed rate of association during injection of protein at a concentration of 125 nM. K_d represents an affinity constant to the surface as a whole, which takes into account the contributions of each individual binding event [binding to PtdEtn/PtdCho/PtdSer, binding to PtdIns(4,5)P_2 and binding to G$\beta\gamma$s] (means ± S.D. are shown).

Background lipids	PtdIns(4,5)P_2	G$\beta\gamma$s	k_{obs} (s^{-1})	K_d (nM)
p110γ				
PtdCho/PtdEtn/PtdSer (33% each)	−	−	0.0061	45 ± 8
PtdCho/PtdEtn/PtdSer (33% each)	−	+	0.0094	17 ± 4
PtdCho/PtdEtn/PtdSer (30% each)	+	−	0.0196	3.8 ± 1.1
PtdCho/PtdEtn/PtdSer (30% each)	+	+	0.0225	2.4 ± 0.6
p101/p110γ				
PtdCho/PtdEtn/PtdSer (33% each)	−	−	0.0029	85 ± 12
PtdCho/PtdEtn/PtdSer (33% each)	−	+	0.0038	28 ± 9
PtdCho/PtdEtn/PtdSer (30% each)	+	−	0.013	2.1 ± 0.7
PtdCho/PtdEtn/PtdSer (30% each)	+	+	0.0188	0.8 ± 0.2

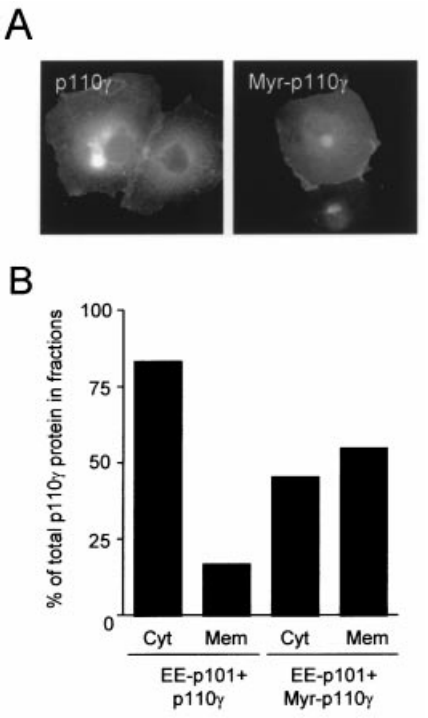

Figure 3 Distribution of p101/p110γ and p101/Myr-p110γ in transfected COS-7 cells

(**A**) Immunofluorescent staining of COS-7 cells transiently transfected with a wild-type p110γ (p110γ) or a membrane-targeted p110γ construct (Myr-p110γ). Cells were stained using a monoclonal anti-p110γ antibody. (**B**) COS-7 cells were transiently transfected with expression vectors encoding EE-p101 and Myc-tagged p110γ (p110γ) or with EE-p101 and untagged, myristoylated p110γ (Myr-p110γ). Cytosolic (Cyt) and membrane (Mem) fractions of the cells were subjected to immunoprecipitations with EE beads. Aliquots of immunoprecipitated proteins were visualized on a Coomassie Brilliant Blue-stained protein gel and quantified by densitometric scanning. The experiment was repeated three times with similar results.

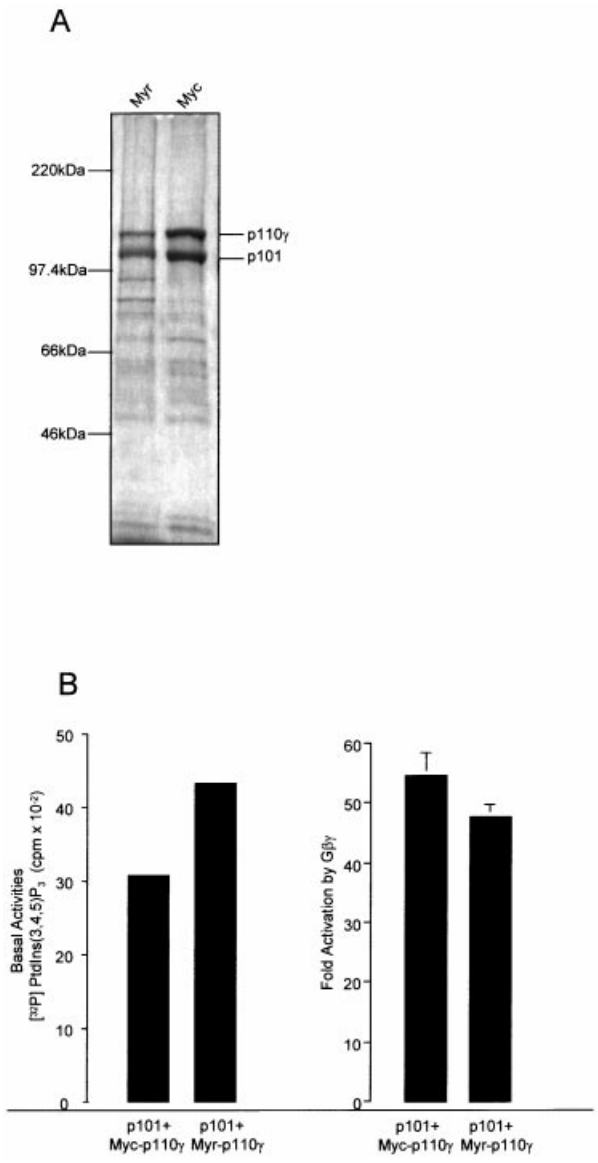

Figure 4 Intrinsic activity and activation by G$\beta\gamma$s of a membrane-targeted p101/p110γ PI3K

(**A**) Cytoplasmic fractions of COS-7 cells transiently transfected to produce p101/p110γ (Myc, lane 2) or pools (48×175 cm^2 flasks of transfected cells) of membrane preparations of those producing p101/Myr-p110γ (Myr, lane 1) were subjected to immunoprecipitations with EE beads. Bound heterodimer was eluted and aliquots subjected to SDS/PAGE and Coomassie Brilliant Blue staining. (**B**) Equal amounts of the proteins shown in (**A**) were assayed for PI3K activity in the presence and absence of G$\beta\gamma$s. Basal catalytic activities (left-hand panel) as well as fold activations obtained in the presence of G$\beta\gamma$s (right-hand panel) are shown. The ranges from duplicate assays are shown throughout; they were within the lines of the graph for the basal activities. Similar results were obtained in two further experiments.

effects of PtdIns(4,5)P_2 on PI3K binding to these monolayers represent a positive control for the type of effect that G$\beta\gamma$s may have exerted on p101/p110γ, which could be called 'translocation-based'. In the context of our evidence that G$\beta\gamma$s in these monolayers are functional, i.e. can bind Gα_i subunits specifically, these results suggest that G$\beta\gamma$s do not cause a significant shift of p101/p110γ into a PtdIns(4,5)P_2-containing mixed phospholipid monolayer.

Both of our experimental approaches produced results that were internally consistent in suggesting that p101/p110γ can be activated substantially by G$\beta\gamma$s in assays where there is no evidence for a large change in the macroscopic distribution of the

PI3K. However, these results seemed at odds with evidence that membrane-targeted p110 constructs (including myristoylated/palmitoylated p110γ) are apparently catalytically activated when expressed in cells [26,27]. To address whether such constitutively activated constructs could be activated further by G$\beta\gamma$s *in vitro*, we expressed EE-tagged p101 together with Myr-p110γ (a p110γ with an N-terminal myristoylation/palmitoylation consensus derived from Yes) transiently in COS-7 cells. Immunofluorescent staining of transfected cells with an antibody against p110γ showed a predominantly cytoplasmic distribution of the control enzyme, whereas the membrane-targeted p110γ was predominantly located at the cellular membranes (plasma and nuclear membranes; Figure 3A). Similarly, cell-fractionation experiments (Figure 3B) revealed a predominantly cytoplasmic localization of control p110γ whereas 55% of the lipid-modified p110γ was found to be associated with cellular membranes. Aliquots of heterodimer preparations of EE-p101/Myc-p110γ and of EE-p101/Myr-p110γ (Figure 4A) were subjected to *in vitro* PI3K assays in the presence and absence of G$\beta\gamma$s. Membrane-targeted PI3K displayed a moderately increased (1.4-fold; Figure 4B) basal catalytic activity compared with the control enzyme. However, in line with our lipid-association data, both PI3K preparations were activated by G$\beta\gamma$s to a comparable extent (54-fold for p101/Myc-p110γ compared with 47-fold for p101/Myr-p110γ; Figure 4B).

In a final set of experiments we attempted to establish entirely soluble assays in which there was no phospholipid phase into which the PI3K could be translocated. These experiments were based on use of either non-lipid modified G$\beta\gamma$s or short-term, large dilutions of fully modified G$\beta\gamma$s along with short-chain (C_4), water-soluble PtdIns(4,5)P_2 and PtdIns substrates. We could not detect significant appropriate phosphorylation of any short-chain lipid up to concentrations of 5 mM and enzyme concentrations of 0.1 μM (results not shown).

DISCUSSION

Any soluble enzyme whose substrate is localized at the plasma membrane could potentially be regulated by control of its association with the membrane. The work presented here addressed whether this is the mechanism by which G$\beta\gamma$s regulate type IB PI3K in an *in vitro* assay situation.

In a centrifugation-based assay under conditions where the enzyme was catalytically active and clearly substantially activated by G$\beta\gamma$s, the majority of PI3K associated with lipid vesicles irrespective of the presence or absence of G$\beta\gamma$s. Using SPR, closer analysis of the interactions involved showed that binding of type IB PI3K to mixed phospholipid monolayers with or without G$\beta\gamma$s was influenced by the background lipids, the mol% of PtdIns(4,5)P_2 and the presence of G$\beta\gamma$s. Importantly, the effects of G$\beta\gamma$s on the overall strength of binding of type IB PI3K to the monolayers were relatively small and, under the conditions most resembling our assays of G$\beta\gamma$ activation of the PI3K in the presence of PtdIns(4,5)P_2, were over an order of magnitude less than their effects on catalytic activity. Clearly these results concur with those derived by a second, completely independent, strategy using sucrose-loaded phospholipid vesicles. Further, a third approach to this question lead us to the same conclusion. We used purified, lipid-modified, membrane-associated type IB PI3K and assayed it in comparison with the wild-type PI3K *in vitro*. The palmitoylated/myristoylated enzyme displayed a modest increase in basal activity compared with its non-modified counterpart, possibly associated with its more efficient membrane association, but was still very substantially activated by G$\beta\gamma$s. We conclude that membrane targeting and G$\beta\gamma$s activate the PI3K by distinct mechanisms.

Taken together, these results suggest that G$\beta\gamma$-driven translocation of type IB PI3K plays at most a minor role in activation of the enzyme in *in vitro* assays. The implication of this is that G$\beta\gamma$s activate type IB PI3K in these assays very largely either by allosteric modulation of the enzyme or by re-orientating it within the membrane to enable more effective interaction with ATP and/or PtdIns(4,5)P_2. Interestingly, activation of type IB PI3K by GTP–Ras does not appear to rely on translocation of the PI3K either (S. Suire and L. R. Stephens, unpublished work). Crystallographic studies of the p110γ–GTP-bound Ras complex have shown that Ras causes the catalytic subunit to undergo a conformational change which might allosterically activate the PI3K [19].

It would seem that *in vivo* only a minor fraction of wild-type type IB PI3Ks is membrane-associated; the reason for the difference in distribution between our *in vitro* assays and the situation *in vivo* is not clear. It may be related to issues such as competition with other proteins *in vivo*, or the relatively more open, and hence more easily penetrated, structure of the phospholipid surfaces we prepare, or, finally, the differences in the mol% PtdIns(4,5)P_2 between these environments. This may enable membrane-targeting devices to cause more substantial changes in the membrane versus cytosol distribution of the enzyme *in vivo*. We would argue this then leads to substantial increases in PtdIns(3,4,5)P_3 formation because PtdIns(4,5)P_2 availability within the membrane is not limiting. Hence membrane targeting or translocation can be apparently activating [26,27]. Yet our data indicate that this is probably not the primary and certainly not the sole means by which G$\beta\gamma$s activate type IB PI3K-catalysed accumulation of PtdIns(3,4,5)P_3.

This conclusion is consistent with the rapid kinetics of G-protein coupled signalling events. Having a pool of PI3K ready in the membrane should enable very rapid increases and decreases in PtdIns(3,4,5)P_3 formation [e.g. PtdIns(3,4,5)P_3 levels peak approx. 10 s after stimulation of human neutrophils with *N*-formyl-methionyl-leucyl-phenylalanine (FMLP)] [2]. In this regard, our findings are reminiscent of the mechanism of activation of phospholipase C-β_2 isotypes by G$\beta\gamma$s. It has been shown independently by several groups [20,21,28] that distribution of phospholipase C-β_2 in an *in vitro* system does not change upon activation by G$\beta\gamma$s and that the enzyme fraction which is being activated is already membrane-localized at the time of activation, whereas the majority of the phospholipase C enzyme is cytoplasmic.

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