Purification and Characterization of $G_{\beta\gamma}$-responsive Phosphoinositide 3-Kinases from Pig Platelet Cytosol*

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A G-protein $\beta\gamma$ subunit ($G_{\beta\gamma}$)-responsive phosphoinositide 3-kinase (PI 3-kinase) was purified approximately 5000-fold from pig platelet cytosol. The enzyme was purified by polyethylene glycol precipitation of the cytosol followed by column chromatography on Q-Sepharose fast flow, gel filtration, heparin-Sepharose, and hydroxyapatite. The major $G_{\beta\gamma}$-responsive PI 3-kinase is distinct from p85 containing PI 3-kinase as the activities can be distinguished chromatographically and immunologically and is related to p110 as it cross-reacts with anti-p110-specific antibodies. The p110-related PI 3-kinase cannot be activated by G-protein $\alpha$ subunits, and it has an apparent native molecular mass of 210 kDa. The p110-related PI 3-kinase phosphorylates phosphatidylinositol (PtdIns), phosphatidylinositol 4-phosphate (PtdIns4P), and phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2). The apparent $K_m$ values for ATP were found to be 25 $\mu$M with PtdIns, 44 $\mu$M with PtdIns4P, and 37 $\mu$M with PtdIns(4,5)P2 as the substrate. $G_{\beta\gamma}$ subunits did not alter the $K_m$ of the enzyme for ATP; however, $V_{\text{max}}$ increased 2-fold with PtdIns as substrate, 3.5-fold with PtdIns4P, and 10-fold with PtdIns(4,5)P2. Under basal conditions the apparent $K_m$ values for lipid substrates were 64, 10, and 15 $\mu$M for PtdIns, PtdIns4P, and PtdIns(4,5)P2, respectively. In the presence of $G_{\beta\gamma}$ subunits the dependence of PI 3-kinase activity on the concentrations of lipid substrates became complex with the highest level of stimulation occurring at high substrate concentration, suggesting that the binding of $G_{\beta\gamma}$ and lipid substrate (particularly PtdIns(4,5)P2) may be mutually cooperative. Wortmannin and LY294002 inhibit the $G_{\beta\gamma}$-responsive PI 3-kinase activity with $IC_{50}$ values of 10 nM and 2 $\mu$M, respectively. Unlike the p85 containing PI 3-kinase in platelets, the p110-related PI 3-kinase is not associated with a PtdIns(3,4,5)P3-specific 5-phosphatase.

The p85-associated PI 3-kinase was not activated by $G_{\beta\gamma}$ alone but could be synergistically activated by $G_{\beta\gamma}$ and phosphotyrosyl platelet-derived growth factor receptor peptides. This may represent a form of coincidence detection through which the effects of tyrosine kinase and G-protein-linked receptors might be coordinated.

Phosphoinositide 3-kinases (PI 3-kinases), EC 2.7.1.137

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1 The abbreviations used are: PI 3-kinase, phosphoinositide 3-kinase; DTT, dithiothreitol; G-protein, guanine-nucleotide binding regulatory protein; GTP, guanosine 5'-triphosphate; GTPyS, guanosine 5'-y-thio-triphosphate; PtdIns, phosphatidylinositol; PtdIns4P, phosphatidylinositol 4-phosphate; PtdIns(4,5)P2, phosphatidylinositol 4,5-bisphosphate; PS, phosphatidylserine; PEG, polyethylene glycol; PAGE, polyacrylamide gel electrophoresis; PH, pleckstrin homology; PI, phosphatidylinositol; PLC, phospholipase C; PDGF, platelet-derived growth factor.
nprecipitated with a monoclonal antibody raised against the p85 subunit of PI 3-kinase, it was thought to possess a p85-related subunit. However, Zhang et al. (25) reported a Gβγ-stimulated PI 3-kinase in platelets that was not recognized by p85-directed antibodies. The latter study established that this platelet Gβγ-stimulated enzyme was immunologically related to p110γ.

The substrate specificities of identified PI 3-kinases vary substantially (11, 14, 22, 26–28). To define the molecular characteristics and properties of Gβγ-stimulated PI 3-kinases in platelets more clearly, we have now partially purified the major form of this enzyme from pig platelets. This enzyme is a p110γ-related PI 3-kinase that is distinct from p85-associated species and that phosphorylates all three potential phosphoinositide substrates with a marked preference for PtdIns(4,5)P2 which is further enhanced by Gβγ.

As the results appeared to contradict previous detection of a Gβγ-responsive p85-containing PI 3-kinase in human platelets (24), we also studied the p85-containing PI 3-kinase in pig platelets. We found that this enzyme can be activated by Gβγ in a manner that is largely dependent upon the presence of a tyrosine-phosphorylated PDGF receptor peptide.

### EXPERIMENTAL PROCEDURES

**Materials**

Polyvinylidene difluoride membrane, Pdlns, phosphatidyl-i-serine, and protein G-Sepharose were purchased from Sigma; Pdlns(4,5)P2 and Pdlns(4,5)P3 were prepared as described (38). γ32P[ATP (3000Ci/ mmol) and enhanced chemiluminescence were purchased from Amersham. Partisphere SAX high performance liquid chromatography columns were from Whatman; Superose 12 HR 10/30, Mono Q, Q-Sepharose fast flow, heparin-Sepharose CL-6B and PD-10 column were from Pharmacia Biotech Inc. Centronic-30 was from Amicon. GTPγS was from Boehringer Mannheim; mouse monoclonal antibodies to PI 3-kinase p85 were from Upstate Biotechnology Inc. Phosphotyrosyl peptide was based on the sequence of PDGF receptor was provided by Dr. S. Car- tilej, Zeneca Pharmaceutical. p110γ anti-peptide antibodies were provided by Dr. R. Wetzk (Friedrich-Schiller-Universitat Jena).

**Methods**

**Preparation of Gβγ—**The major G-protein βγ subunits were purified from cholate extracts of bovine brain membranes as described by Sternweis and Robishaw (39). The Gβγ subunits were stored in 20 mM Tris, 1 mM EDTA, 0.1% Genaple C-100 and were more than 95% pure as determined by SDS-PAGE. The Gβγ preparation was flash-frozen in 10-μl aliquots and stored at −80 °C until use.

**Purification of the Gβγ-responsive PI 3-Kinase from Platelet Cytosol—**Platelet cytosol was derived from 12 liters of freshly drawn pig blood. The detailed procedure for preparation of platelet cytosol was essentially the same as described (24). Platelets were sonicated in 120 mM of lysis buffer (10 mM HEPE, pH 7.4, 1 mM EDTA, 0.2 mM EDTA, 3 mM MgCl2, 10 mM each of antipain and pepstatin, 1 mM each of DTT, sodium orthovanadate, phenylmethylsulfonyl fluoride, and benzamidine). The platelet lysate was centrifuged at 35,000 rpm for 1 h, and the resulting supernatant (platelet cytosol, 105 mg of protein) was kept. Gβγ-responsive PI 3-kinase was precipitated by 5–15% PEG in buffer A (20 mM HEPE, pH 7.4, 0.2 mM EDTA, 3 mM MgCl2, 10 mM each of antipain and pepstatin, 1 mM each of DTT, sodium orthovanadate, phenylmethylsulfonyl fluoride, and benzamidine). The pellet was resuspended in 36 ml of buffer A. The PEG sample (36 mg) was loaded onto a Q-Sepharose Fast Flow column (150 × 15 mm), pre-equilibrated with buffer A, and eluted with a gradient of 0–0.5 M NaCl (150 ml). Gβγ-responsive PI 3-kinase fractions (12 mg) were pooled and loaded onto a gel filtration column (Sepharose CL-4B, 100 × 2.6 cm) pre-equilibrated with buffer B (buffer A plus 100 mM NaCl and 10% suc- charose). The Gβγ-responsive PI 3-kinase activity fractions (1.35 mg protein) were pooled and loaded onto a heparin-Sepharose column (50 × 10 mm) pre-equilibrated with buffer B. The column was washed with 30 ml of buffer B and eluted with a linear gradient of 100–500 mM NaCl (60 ml). Fractions (0.36 mg) containing Gβγ-responsive PI 3-kinase were pooled and concentrated to 6 ml with Microconcentrator 30 (Amicon, Inc. Beverly, MA). Half of the sample was loaded onto a hydroxyapatite column (100 × 10) pre-equilibrated with buffer C (20 mM KH2PO4, pH 7.0, 5 mM DTT, 0.1 mM each of phenylmethylsulfonyl fluoride and benzamidine); protein was eluted with 20–750 mM K2HPO4 during which the Gβγ-responsive PI 3-kinase was separated from the tyrosine kinase-regulated PI 3-kinase. Separation of the Gβγ-responsive PI 3-kinase from the tyrosine kinase-regulated PI 3-kinase can also be achieved by incubating half of the concentrated enzyme with 2 ml of protein G-Sepharose pre-coupled with anti-p85 antibodies overnight at 4 °C with gentle agitation. The Gβγ-responsive PI 3-kinase prepared by either method did not contain any p85 protein nor any other detectable lipid kinase activity.

**PI 3-Kinase Assay—**Generally the enzyme activity was measured by adopting the following assay procedure. 10 μl of platelet cytosol or column fractions were mixed with 30 μl of lipid vesicles, which had been premixed with Gβγ or their vehicle for 10 min on ice. 10 μl of MgATP was added to start the reaction. The enzyme reaction was terminated after incubating at 37 °C for 5 min by adding 200 μl of 1 M HCl. To prepare lipid vesicles, equimolar amounts of PS and substrate lipid (PtdIns, Pdlns(4,5)P2, or Pdlns(4,5)P3) were dried onto a film under vacuum and probe-sonicated (3 × 15 s with 1 min on ice between sonication, at setting 20–30 on a Jencons Ultrasonic Processor) into kinase assay buffer (40 mM HEPE, pH 7.4, 1 mM EDTA, 1 mM DTT, 50 mM NaCl, 4 mM MgCl2). The standard assay contained 100 μM PtdIns(4,5)P2 and PS, 10–100 μM ATP (10 μM with PtdIns(4,5)P3 and crude extract), 1 μM Gβγ or its vehicles and 10 μCi of γ32P[ATP. Lipid extraction and analysis was performed as described (24). The products of the PI 3-kinase reactions were identified by deacylation and separation of their glycerol derivatives by high performance liquid chromatography and compared with deacylated 1H-labeled standards. Note that assays were done under first order conditions with respect to ATP as substrate (to optimize assay of radioactive product) ensuring that no more than 10% of ATP was consumed during a reaction.

For assays requiring activated Ga-proteins, Ga-proteins were incubated for 1 h on ice in the presence of 100 μM GTPγS and 5 μM MgCl2, mixed with Pdlns-containing lipid vesicles, and incubated again for 10 min on ice, before adding to the assay.

**p85 (p85 Subunit of PI 3-Kinase) and p110γ Immunoblot—**Samples of column eluate (for detection of p85 subunit of PI 3-kinase) or purified Gβγ-responsive PI 3-kinase (for p110γ) were mixed with 4 × SDS sample buffer, boiled for 5 min, and resolved by SDS-PAGE with 7.5% acrylamide in the separating gel. Proteins were then transferred to polyvinylidene difluoride membranes using a dry-blotting device (Bio-Rad). Western blots were performed as described (22) using either anti-p85 antibody (1:1000 dilution) or p110γ antibodies (1:200) followed by horseradish peroxidase-conjugated secondary antibodies (1:2000 dilu- tion). Blots were then developed using enhanced chemiluminescence according to the manufacturer’s instructions.

### RESULTS

**Partial Purification of Gβγ-responsive PI 3-Kinase from Platelet Cytosol—**A Gβγ-responsive PI 3-kinase was purified approximately 5000-fold, with an overall yield of 30%, from porcine platelet cytosol using PEG precipitation, Q-Sepharose, gel filtration, heparin-Sepharose, and hydroxyapatite. A typical purification is summarized in Table I. PEG precipitation resulted in a 30-fold enrichment with 100% recovery of Gβγ-responsive activity. Elation of this sample from Q-Sepharose using a continuous salt gradient revealed two distinct peaks of Gβγ-responsive PI 3-kinase (Fig. 1A). The earlier eluting, minor peak of activity was inconsistently observed in a number of purifications from different batches of platelets and was not studied further. Analysis of fractions eluting from the Q-Sepharose column by Western blotting with an anti-p85 monoclonal antibody revealed that the second peak of Gβγ-responsive PI 3-kinase co-eluted with p85 immunoreactive (Fig. 1B). p85 and Gβγ-responsive activity were separated to co-migrate through gel filtration and heparin-Sepharose (data not shown). However, separation of p85 and the Gβγ-responsive PI 3-kinase was achieved through hydroxypatite eluted with a linear gradient of KNH4PO4/K2HPO4 as shown in Fig. 2, A and B. Separation could also be achieved by immunodepletion of p85 using protein G-Sepharose that had been pre-coupled with anti-p85 antibo-
ies (Fig. 3A). The Gβγ-responsive PI 3-kinase is related to p110γ as it can be recognized by an anti-p110γ anti-peptide antibody (Fig. 3B).

Characterization of Gβγ-responsive PI 3-Kinase—The native molecular mass of the Gβγ-responsive PI 3-kinase was determined using a calibrated Superose 12 size exclusion column. As shown in Fig. 4, the activity eluted at a volume indicating a size of approximately 210 kDa. The enzyme was not pure at that stage as revealed by silver-stained SDS-PAGE gel of active fractions (not shown). This indicated that the enzyme is a very minor protein in platelet cytosol.

The substrate specificity and kinetic characteristics of the enzyme were consistent between several different preparations. As the enzyme after hydroxyapatite cannot survive freezing and thawing, activity purified through heparin-Sepharose and immunodepleted to remove p85 was used for most experiments. Such preparations were purified approximately 2000-fold with respect to platelet cytosol and contained no detectable PtdIns 4-kinase, PtdIns4P 5-kinase, or PLC (EC 3.1.4.3) activ-

### Table I

| Step                        | Total Gβγ stimulatable activity | Total protein | Specific activity | Yield | Purification |
|-----------------------------|---------------------------------|---------------|-------------------|-------|--------------|
| Crude platelet cytosol      | 4.50 nmo1 PIP₂ · min⁻¹          | 1049 mg       | 0.0042 nmo1 PIP₂ · min⁻¹ | 100   | 1            |
| PHG 8000                    | 4.60 nmo1 PIP₂ · min⁻¹          | 36 mg         | 0.13 nmo1 PIP₂ · min⁻¹ | 102   | 30           |
| Q-Sepharose                 | 3.83 nmo1 PIP₂ · min⁻¹          | 12 mg         | 0.32 nmo1 PIP₂ · min⁻¹ | 85    | 76           |
| Gel filtration              | 3.60 nmo1 PIP₂ · min⁻¹          | 1.35 mg       | 2.67 nmo1 PIP₂ · min⁻¹ | 80    | 623          |
| Heparin-Sepharose           | 3.02 nmo1 PIP₂ · min⁻¹          | 0.36 mg       | 8.40 nmo1 PIP₂ · min⁻¹ | 67    | 2000         |
| Hydroxyapatite              | 1.35 nmo1 PIP₂ · min⁻¹          | 0.06 mg       | 21.00 nmo1 PIP₂ · min⁻¹ | 30    | 5000         |

Fig. 1. Purification of Gβγ-responsive PI 3-kinase on Q-Sepharose. A, PEG sample (36 mg) was loaded onto a Q-Sepharose column. Protein was eluted with a gradient of NaCl (solid line). Aliquots of the individual fractions were immediately assayed for PI(4,5)P₂ 3-kinase activity in the presence (closed cycles) and absence (open cycles) of 1 μM Gβγ. Further details are given under “Experimental Procedures.” ●, stimulated; ○, basal. B, aliquots of fractions were analyzed by SDS-PAGE and blotted with anti-p85 antibodies.

Fig. 2. A-B: purification of Gβγ-responsive PI 3-kinase on hydroxyapatite. A, the heparin-Sepharose-eluted sample (0.18 mg) was loaded onto a hydroxyapatite column. Protein was eluted with a gradient of K₂HPO₄. Aliquots of individual fractions were immediately assayed for PI(4,5)P₂ 3-kinase activity in the presence and absence of 1 μM Gβγ. ●, stimulated; ○, basal; —, K₂HPO₄. B, aliquots of fractions were analyzed by SDS-PAGE and blotted with anti-p85 antibodies.

Fig. 3. A-B, immunoprecipitation (IP) of the p85-containing PI 3-kinase. A sample of heparin-Sepharose-purified enzyme was mixed with protein G-Sepharose that had been pre-coupled with anti-p85 antibodies. Aliquots of the heparin-Sepharose sample, supernatant, and precipitate were analyzed by SDS-PAGE and blotted with either anti-p85 antibodies (A) or blotted with anti-p110γ antibodies (B).
was completely blocked in the presence of a 3-fold calculated molar excess of a preparation of GDP-liganded Go_{i/o} and by 85% at an equimolar concentration of these proteins (Fig. 8), suggesting that the activation requires free Gβγ and hence would require activated heterotrimeric G-proteins in vivo. The effects of 1 μM Gβγ were next examined over a range of substrate concentrations. The presence of Gβγ did not significantly affect the Km for ATP whichever lipid substrate was employed. However, the effects of increasing lipid substrate concentrations on activity deviated from Michaelis-Menten kinetics in the presence of the activator. Thus, Gβγ subunits were most effective at high concentrations of lipid especially when PtdIns(4,5)P_2 was used as the substrate. The apparent cooperativity with respect to increasing substrate concentration prevented the determination of Km values and allowed only estimation of V_{max} values. Nevertheless, Gβγ enhanced V_{max} by approximately 2-, 3.5-, and 10-fold with PtdIns, PtdIns4P, and PtdIns(4,5)P_2, respectively, as substrates (Fig. 5, A and B).

The fungal metabolite, wortmannin, is a potent inhibitor of several PI 3-kinase isoforms including the Gβγ-sensitive PI 3-kinase from U937 cells (22, 26, 27), whereas the quercetin analogue, LY294002, was developed as a PI 3-kinase inhibitor that lacked the chemical instability of wortmannin (37). Both of these compounds inhibited the platelet Gβγ-sensitive enzyme with IC_{50} values of 10 nM and 2 μM for wortmannin and LY294002, respectively (data not shown). The value for wortmannin is very similar to that reported by Stephens et al. (22) for the enzyme from U937 cells and that for LY294002 is almost identical to its IC_{50} when assayed with RBL-2H3 cells (36).

Recombinant p110γ was recently shown to respond to both Gβγ and the GTPγS-ligated α subunits of G_i and transducin. We repeated these observations using a p110γ-glutathione S-transferase fusion protein expressed in Sf9 cells and immobilized on glutathione-agarose (Fig. 9B). 1 nM Go_{i/S}-GTPγS, but not GTPγS alone, induced a 50% increase in p110γ activity, whereas 1 μM Gβγ enhanced activity 4-fold. By contrast, the platelet enzyme was insensitive to Go_i, but was more sensitive to Gβγ being activated approximately 8-fold in the experiment shown in Fig. 9A.

The isolation from platelet cytosol of a Gβγ-responsive PI 3-kinase that is distinct from a p85-associated enzyme appeared to contradict our previous observation that Gβγ activated a PI 3-kinase which associated with a biotinylated phosphotyrosyl peptide related to the p85 binding region of the PDGF receptor. We therefore investigated the possibility that Gβγ could activate the p85-associated enzyme in a manner that depended on coincident association with the PDGF receptor peptides. This was indeed found to be the case. As shown in Fig. 10A, Gβγ alone did not activate immunoprecipitated p85-associated PI 3-kinase but greatly augmented the response to the phosphopeptide when assays were carried out under standard conditions. This effect was even more dramatic when substrate lipid was presented against a background of phosphatidylinositolamine rather than PS as described by Okada et al. (40). With PIP_2/phosphatidylinositolamine vesicles, basal activity was found to be very low and was slightly activated by either Gβγ or phosphopeptides. The combination of Gβγ and phosphopeptides, however, resulted in a greater than 50-fold enhancement of PI 3-kinase activity (Fig. 10B).

**DISCUSSION**

A Gβγ-sensitive PI 3-kinase has been purified 5000-fold from pig platelet cytosol. A second form of Gβγ-responsive enzyme eluted as an early peak on Q-Sepharose, but the appearance of this peak and its magnitude relative to the major peak of activity were variable between platelet preparations. For these
reasons the early eluting peak was not analyzed further in this study; whether it represents a distinct species of PI 3-kinase, a processing variant, or a proteolytic fragment are not clear from these studies. The major Gbg-sensitive enzyme co-eluted with p85 immunoreactivity through Q-Sepharose, gel filtration, and heparin-Sepharose but could be separated from the latter protein on hydroxyapatite. Moreover, separation of the major Gbg-responsive enzyme from p85-associated PI 3-kinase can also be achieved by p85 immunoprecipitation. One further line of evidence that excludes the association of the major Gbg-sensitive PI 3-kinase with p85 was the lack of PtdIns(3,4,5)P3 5-phosphatase activity which was previously shown to be complexed to p85 in human platelets (30). Nevertheless, since a polypeptide of approximately 120 kDa was observed on Western blots probed with a p110g-specific anti-peptide antibody, and its native molecular weight was found to be in excess of 200 kDa, Gbg-sensitive PI 3-kinase presumably exists either as a dimer or in complex with one or more additional polypeptides. This situation is similar to that reported for the partially purified enzyme from U937 cells (22).

In agreement with the previous report (24) on the Gbg-responsive PI 3-kinase in human platelet cytosol, the p85-associated PI 3-kinase in pig platelet cytosol can also be activated by Gbg under certain assay conditions. Moreover, this Gbg-responsive activity can be further synergistically augmented by phosphorytrosine PDGF receptor peptide. This may represent a form of coincidence detection through which the effects on cellular functions of tyrosine kinase and G-protein-linked receptors might be coordinated. Similar findings have also been reported in human monocytic THB-1 cells (40).

Although the platelet enzyme that was sensitive to Gbg alone co-purified with a p110g-immunoreactive component, it differed from the latter in its native molecular weight as noted above. It also differed in terms of its regulation, being insensitive to GTPγS-ligated Gai/o and being stimulated to a greater degree by Gbg compared with recombinant p110g. Whether these distinguishing features reflect the differences between the native and recombinant proteins, the catalytic subunit itself or the presence of an additional complexed polypeptide(s) cannot be discerned at present.

The partially purified platelet enzyme phosphorylated PtdIns, PtdIns4P, and PtdIns(4,5)P2 to give the corresponding 3-phosphorylated lipids as determined by co-chromatography of their deacylation products with authentic standards. The phosphorylation of PtdIns and PtdIns4P could be inhibited by an excess of PtdIns(4,5)P2 suggesting that a single enzyme species was responsible for the observed phosphorylation of all three substrates (29). However, the efficiency with which these substrates were utilized varied substantially, with polyphosphoinositides exhibiting lower K m values than PtdIns and with V max values being greatest with PtdIns(4,5)P2 as substrate. These features were exaggerated in the presence of Gbg subunits that enhanced PtdIns(4,5)P2 phosphorylation more markedly than either PtdIns or PtdIns4P. Assuming these features are relevant to the situation in cell membranes, then this enzyme would be expected to synthesize mainly PtdIns(3,4,5)P3 in vivo, consistent with the observed effects of thrombin on 3-phosphorylated inositol phospholipids in intact platelets (5).

In the absence of Gbg the PI 3-kinase activity obeyed Michaelis-Menten kinetics with respect to both ATP and lipid substrates. This was not the case in the presence of Gbg which

**Fig. 5. Effects of substrate concentrations on the activity of Gbg-responsive PI 3-kinase.** A, purified Gbg-responsive PI 3-kinase was assayed in the presence and absence of 1 μM Gbg and in the presence of 100 μM of either PIP2, PIP-4-P, or PIP(4,5)P2 with various concentrations of [γ-32P]ATP. B, 10 μM [γ-32P]ATP throughout in the presence of various concentrations of PIP/PS, PIP-4-P/PS, or PIP(4,5)P2/PS. Data shown are means of duplicate ± range of one experiment. Two other experiments gave similar results. ○, basal; ●, stimulated.
induced sigmoidal kinetics for lipid substrates, especially in the presence of PtdIns(4,5)P$_2$. This suggests that this form of PI 3-kinase might possess more than one PtdIns(4,5)P$_2$ binding site; by analogy with $\beta$ (31) and $\delta$ (32) isoforms of phospholipase C (PLC) the additional site(s) might be non-catalytic and function to associate PI 3-kinase at a substrate-bearing membrane and thus allow processive catalysis to occur. Because we only observed sigmoidal kinetics in the activated state, it is proposed that G$\beta$g regulates the binding of substrate lipid at such a non-catalytic site. The results with different lipid substrates predict that the putative regulatory lipid site prefers PtdIns(4,5)P$_2$ over PtdIns$_4$P which in turn is preferred over PtdIns. Interestingly this matches the expected rank order of binding of these lipids to some PH domains (33), a structural feature that has been proposed to occur in p110$\gamma$ but not other published forms of PI 3-kinase. A further interesting regulatory feature was the observation that the EC$_{50}$ for activation by G$\beta$g also was affected by the nature of the lipid substrate suggesting that the binding of lipid (especially PtdIns(4,5)P$_2$) and G$\beta$g are mutually cooperative. Such an observation is again reminiscent of the ligand binding properties of some PH domains and closely associated sequences C-terminal to the PH domain proper that appear to possess distinct binding sites for anionic lipids and G$\beta$g subunits, respectively. However, it should be pointed out that without structural studies, the proposal that p110$\gamma$ possesses a PH domain remains speculative.

An alternative explanation for the observed cooperative kinetics for lipid substrates in the presence of G$\beta$g might be that G$\beta$g is complexed with vesicles at the higher lipid concentrations. There are two lines of evidence suggesting that the membrane localization of G$\beta$g is important for its function. Katz et al. (34) reported that transfection of COS-7 cells with cDNA for PLC$\beta$2 and G-protein $\beta$1$\gamma$1 subunits caused an increase in PLC activity as evidenced by the accumulation of...

FIG. 6. Double-reciprocal plots of kinetic data of G$\beta$g-responsive PI 3-kinase in the absence of G$\beta$g. A, various concentrations of PI/PS, PI-4P/PS, or PI(4,5)P$_2$/PS. B, various concentrations of $\gamma$-32P[ATP. □, PI; ◊, PI4P; ○, PI(4,5)P$_2$.

FIG. 7. G$\beta$g dose dependence of PI 3-kinase activity. Purified G$\beta$g-responsive PI 3-kinase activity was determined in the presence of various concentrations of G$\beta$g. The data shown are the means (± S.E., n = 3) of one experiment. Similars results were obtained with one other experiment.

FIG. 8. Inhibition of G$\beta$g-responsive PI 3-kinase by Ga$_i$-GDP. Purified G$\beta$g-responsive PI 3-kinase activity (purified 2000-fold and devoid of any other lipid kinase activity) was determined in the presence of 1 μM G$\beta$g that had been preincubated with various concentrations of Ga$_i$ liganded with GDP. The data shown are means (± S.E., n = 3) of one experiment. Similar results were obtained in one other experiment.

FIG. 9. Regulation of G$\beta$g-responsive PI 3-kinase (A) and recombinant p110$\gamma$ by Ga$_i$ and G$\beta$g. Partially purified G$\beta$g-responsive PI 3-kinase (after hydroxypatite) (A) and recombinant p110$\gamma$ (B) were incubated with Ga$_i$-GTP$\gamma$S, GTP$\gamma$S, and G$\beta$g, respectively, and enzyme activity was assayed with PtdIns(4,5)P$_2$ as substrate.
Another experiment. Two further experiments gave similar results. and prevented the increase in cellular inositol phosphates. Such approaches identified p110 families, such as the PI 3-kinases, using cloning strategies that cooperativity observed and the extent of activation of PI 3-kinase. mark differences between lipid substrates in terms of the membrane location and functionality of the complex. However, the transfected insect cells has shown that only C-terminally mod-
ifications of PI 3-kinase present in both platelets and myeloid cells is distinct from p85 and which confers unknown properties on the enzymes, further studies are required to define the molecular components of these native proteins. Such studies are also required to understand the structural basis for the regulatory mechanisms that we have described.

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FIG. 10. Activation of the p85-containing PI 3-kinase by Gβγ and phosphorylony PE DGFR receptor peptide. The PI 3-kinase activity in the immunoprecipitates was assayed in the presence or absence of 0.5 μM Gβγ and/or 1 μM dual-phosphorylated PDGF receptor peptide. A, assays were performed in the presence of both 100 μM PtdIns(4,5)P2 (PIP2) and 100 μM PS. B, assays were performed in the presence of 1 mM phosphatidylyethanolamine (PE) and 100 μM PtdIns(4,5)P2 (PIP2). Data shown are means of duplicate (± range) of one experiment. Two further experiments gave similar results.

inositool phosphates. The use of a mutant cDNA encoding a γ subunit lacking the essential cysteine residue required for iso-
prénylation resulted in a shift of the γ subunit to the cytosol and prevented the increase in cellular inositol phosphates. Furthermore, the purification of γ dimers from baculovirus transfected insect cells has shown that only C-terminally modified γ subunits confer PLCβ2-activating function on γ complex. (35), suggesting that the isoprenylation and carboxyl methylation of γ subunits may be important for both mem-
brane location and functionality of the complex. However, the extent of membrane insertion of Gβ is unlikely to explain the marked differences between lipid substrates in terms of the cooperation observed and the extent of activation of PI 3-kinase.

Much can be learned about the molecular diversity of protein families, such as the PI 3-kinases, using cloning strategies that exploit sequence relationships among the family members. Such approaches identified p110α and have also revealed a wider family that encompasses both inositol phospholipid and protein serine/threonine kinases. The initial observations that revealed the presence of G-protein-regulated forms of PI 3-ki-
nase, however, were made using partially purified protein preparations from cell extracts. Since the major Gβγ-sensitive forms of PI 3-kinase present in both platelets and myeloid cells appear to be complexed to at least one other polypeptide, which is distinct from p85 and which confers unknown properties on the enzymes, further studies are required to define the molecular components of these native proteins. Such studies are also required to understand the structural basis for the regulatory mechanisms that we have described.