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The Gβγ Sensitivity of a PI3K Is Dependent upon a Tightly Associated Adaptor, p101

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Summary

Two highly similar, PtdIns(4,5)P_2-selective, Gβγ-activated PI3Ks were purified from pig neutrophil cytosol. Both were heterodimers, were composed of a 101 kDa protein and either a 120 kDa or a 117 kDa catalytic subunit, and were activated greater than 100-fold by Gβγs. Peptide sequence–based oligonucleotide probes were used to clone cDNAs for the p120 and p101 species. The cDNA of p120 is highly related to p110γ, while the cDNA of p101 is not substantially related to anything in current databases. The proteins were expressed in and purified from insect and mammalian cells. They bound tightly to one another, both in vivo and in vitro, and in so doing, p101 amplified the effect of Gβγs on the PI3K activity of p120 from less than 2-fold to greater than 100-fold.

Introduction

A variety of extracellular transmitters, which utilize receptors that activate heterotrimeric GTPases or protein tyrosine kinases, can stimulate accumulation of phosphatidylinositol (3,4,5)-trisphosphate [PtdIns(3,4,5)P_3] in their target cells (Traynor-Kaplan et al., 1988; Auger et al., 1989). This event is now considered an important intracellular signal upstream of a variety of responses such as insulin-stimulated glucose uptake, ruffling, superoxide production, activation of $p70^{S6K}$, and activation of AKT/PKB, among others (Chung et al., 1994; Hara et al., 1994; Thelen et al., 1994; Wennström et al., 1994; Franke et al., 1995). PtdIns(3,4,5)P_3 is known to be a potential product of phosphoinositide 3-kinases (PI3Ks).

PI3Ks form a large family of enzymes capable of 3-phosphorylating (Whitman et al., 1988) at least one, but often all, of the cellular phosphoinositides: PtdIns, PtdIns4P, and PtdIns(4,5)P_2 (Auger et al., 1989). The first and best characterized subfamily is comprised of heterodimers containing a regulatory subunit (see below) and a 110 kDa catalytic subunit (including 110α and 110β) (Hiles et al., 1992; Hu et al., 1993). Both p110α and p110β are covalently modified and potently inhibited by the fungal metabolite wortmannin (Acaro and Wymann 1993; Yano et al., 1993) and can phosphorylate all three lipid substrates in vitro. However, they appear to be PtdIns(4,5)P_2-selective, and hence PtdIns(3,4,5)P_3-producing, in vivo (Stephens et al., 1991). The known regulatory adaptors (p55α, p55 PIK, p85α, and p85β; Otsu et al., 1991; Pons et al., 1995; Inukai et al., 1996) contain a large collection of protein–protein interaction domains, the best studied of which are protein tyrosine phosphate–binding SH2 domains (Songyang et al., 1993). The binding of tyrosine phosphates to the SH2 domains of PI3K directly activates the PI3K catalytic subunit (although this effect is relatively small in vitro; Carpenter et al., 1993) and translocates the cytosolic PI3K to a source of its phospholipid substrate, leading to an increase in PtdIns(3,4,5)P_3 production. Clearly, this family of PI3Ks is structurally adapted to function as dedicated signal transducers downstream of receptor-regulated tyrosine kinases, much like the way the γ family of PI-PLCs is regulated by receptor-sensitive tyrosine kinases (Lee and Rhee, 1995). These p85/p110-type PI3Ks have been classified as type I PI3Ks. Type II enzymes contain a C-terminal C2 domain and prefer PtdIns and/or PtdIns4P as substrates. Type IIIs, which are relatives of the VPS34p PI3K in yeast, associate with a protein kinase and only phosphorylate PtdIns (MacDougall et al., 1995; Zvelebil et al., 1996).

The accumulation of PtdIns(3,4,5)P_3 that occurs in response to activation of heterotrimeric G protein–linked receptors, particularly ATP receptors on U937 cells (Stephens et al., 1994), is unlikely to involve a p85/p110 PI3K (although some work suggests that FMLP receptors on fully primed neutrophils may be able to activate a p85/p110 PI3K after long 0.5 min periods of stimulation; Stephens et al., 1993; Ptasznik et al., 1996). Recent evidence has identified a chromatographically distinct form of wortmannin-sensitive, PtdIns(4,5)P_2-selective PI3K present in U937 cells and neutrophils. This form of PI3K can be specifically and substantially activated by heterotrimeric GTPase βγ subunits (Gβγs, but not Gα-GTP subunits) and possesses a native, relative molecular mass of about 220 kDa (Stephens et al., 1994). A similar PI3K activity has also been described in an osteosarcoma cell line (Morris et al., 1995). Stoyanov et al. (1995) have recently published the cloning and expression of a wortmannin-sensitive, PtdIns(4,5)P_2-selective PI3K from a human bone marrow cDNA library (p110γ). It is clearly distinct from p110α and p110β, particularly in its lack of an NH_2-terminus-binding domain for a member of the p85 adaptor family, and has been classified as a type IB PI3K (p85/p110-type PI3Ks becoming IA PI3Ks).

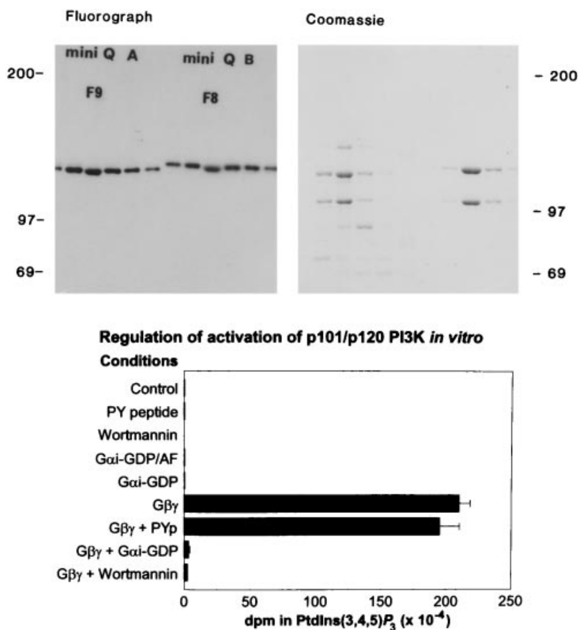

Figure 1. Characterization of PI3K-A and PI3K-B

(Upper) Aliquots (4%, v/v) of each of the fractions across the peaks of PI3K activity eluting from the final purification steps for both PI3K-A and PI3K-B were incubated with [^{3}H]-17-hydroxy-wortmannin, resolved by SDS–PAGE (6% acrylamide), and stained with colloidal Coomassie G250 (see right gel; note that photographs of the same, identically aligned gel are shown). PI3K activity peaked in fraction 9 for PI3K-A and in fraction 8 for PI3K-B. Protein-bound [^{3}H]-17-hydroxy-wortmannin was localized by fluorography (see left gel; note that in the image shown, the peak fractions were allowed to overexpose the film to demonstrate the specificity of binding). The position to which a set of molecular size standards migrated is marked.

(Lower) The effects of various reagents on the lipid kinase activity of PI3K-B. Assays were conducted with PtdIns(4,5)P_2 as a substrate as described below, with or without 30 μM doubly tyrosine-phosphorylated peptide (PY peptide), 100 nM wortmannin, 1 μM Gα_i (with or without 10 mM sodium fluoride and 30 μM aluminum chloride), and 0.5 μM Gβγs.

It was speculated that p110γ constituted the PI3K activity downstream of heterotrimeric GTPase-linked receptors, although this hypothesis clearly left several unresolved questions regarding earlier work (e.g., the apparent native, relative molecular mass).

This paper describes experiments that were aimed at characterizing the Gβγ-sensitive PI3K activity we previously detected in myeloid-derived cells.

Results

Purification and Characterization of Gβγ-Activated PI3K Activities

Analysis of pig neutrophil cytosol by ion-exchange chromatography showed that it contained a Gβγ-activated PI3K activity of similar properties to one already described in U937 cells. The Gβγ-activated PI3K activity was purified further and split into two peaks that both displayed native, relative molecular masses of 220 kDa (we intend to publish the details of this purification separately). Once essentially pure, as assessed by Coomassie-stained SDS–PAGE gels (see Figure 1), it was clear that both activities comigrated with two proteins: PI3K-A with proteins of 117 kDa (which specifically bound [^{3}H]-17-hydroxy-wortmannin and were therefore assumed to be catalytic) and 101 kDa, and PI3K-B with proteins of 120 kDa (which also bound [^{3}H]-17-hydroxy-wortmannin) and 101 kDa, hence indicating that the PI3K activities were p117/p101 and p120/p101 heterodimers in their native state. In their final forms, PI3K-A and PI3K-B had been purified approximately 180,000-fold and 370,000-fold from neutrophil lysates with 5.5% overall recovery of activity. Purified preparations of PI3K-A and PI3K-B were indistinguishable on the basis of their lipid kinase activities. They were both found to be: activated over 100-fold by Gβγ subunits in a Gα-GDP-sensitive fashion; completely inhibited by 100 nM wortmannin (with 5 μM ATP in the assays); insensitive, either in the presence or absence of Gβγs, to tyrosine-phosphorylated peptides (which activate p85/p110 family PI3Ks 5-fold); insensitive to aluminum fluoride–activated, GDP-bound Gα_i subunits; and capable of phosphorylating PtdIns, PtdIns4P, and PtdIns(4,5)P_2. However, they displayed the lowest apparent K_M for PtdIns(4,5)P_2 (8 μM and 10 μM for PI3K-A and PI3K-B, respectively), utilizing the γ-phosphate of ATP as the phosphate donor (see Figure 1).

Peptide Sequencing of Gβγ-Activated PI3K-A and PI3K-B and Cloning of p120 and p101

PI3K-A and PI3K-B, purified from the equivalent of 45 g of cytosolic protein, were Western blotted onto nitrocellulose and stained with Ponceau S. The bands corresponding to all four subunits were excised, treated with trypsin, and processed for internal amino acid sequence analysis. Peptide sequence data resolved several issues regarding the relationships between these proteins. The p101s derived from both PI3K-A and PI3K-B were identical, and furthermore, a relatively common allelic variant was identified at 483 in the ORF (marked in Figure 2). p117 and p120 displayed virtually identical tryptic HPLC profiles, and all apparently common peptides that were sequenced from both species were identical, with the exception of an NH_2-T (NH_2-terminal)-blocked peptide from p117 (see below). A degenerate oligonucleotide probe, based on the sequence of a peptide from p120, was used to screen an oligo(dT)-primed pig neutrophil cDNA library. Two clones defined a full-length ORF encoding all of the p117/p120 tryptic peptides (see Figure 2). Of two potential start methionines, the 5′ one was identified as active on the basis of the precise match between the measured mass of an NH_2-T-blocked, p120-derived peptide and the theoretical masses of NH_2-T tryptic peptides that would be derived as a result of initiating translation at the alternative methionines. This was confirmed by postsource decay (PSD) mass spectrometric sequencing using a MALDI-reflectron TOF mass analyzer (data not shown). As such, p120 has a theoretical size of 126 KDa. The mass of a blocked, and therefore presumably N-terminally located, tryptic peptide from p117 could not be satisfactorily matched with any sequence (assuming acetylation of the first residue) in the N-terminal region of the *p120* ORF. Putative sequence differences between p117 and p120, likely

p101

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   1  MQPGATTCTEDRIQHALERCLHGLSLSRRSTSWSAGLCLNCWSLQELVSR
  51  DPGHFLILLEQILQKTREVQEKCTYDLLAPLALLFYSTVLCTPHFPPDSD
 101  LLLKAGRTYHRFLTWPVPYCSICQELLTFIDAELKAPGISYQRLVRAEQG
 151  LSTRSHRSSTVTVLLVNPVEVQAEFLDVADKLSTPGPSPHSAYITLLLHA
 201  FQATFGAHCDLSGLHRRLQSKTLAELEAIFTETAEAQELASGIGDAAEAR
 251  QWLRTKLQAVGEKAGFPGVLDTPKPGKLRTIPIPVARCYTYSWNQDSFDI
 301  LQEILLKEQELLQPEILDDEEDEDEEDEEEDLDADGHCAERDSVLSTGSA
 351  ASHASTLSLASSQASGPTLSRQLLTSFVSGLSDGVDSGYMEDIEESAYER
 401  PRRPGGHERRGHRRPGQKFNRIYKLFKSTSQMVLRRDSRSLEGSPDSGPP
                                       *
 451  LRRAGRLCSPLDSPTLPPSRAQGSRSWPQPKLSPQLPGWFLAPASRHQGR
 501  RPFLSGDEDPKASTLRVVVFGSDRISGKVVRAYSNLRRLENNRPLLTRFF
 551  KLQFFYVPVKRSRGTGTPTSPAPRSQTPPLPTDAPRHPGPAELGAAPWEE
 601  STNDISHYLGMLDPWYERNVLGLMHLPPEVLCQSLKAEPRPLEGSPAQLP
 651  ILADMLLYYCRFAARPVLLQVYQTELTFITGEKTTEIFIHSLELGHSAAT
 701  RAIKASGPGSKRLGIDGDREAVPLTLQIIYSKGAISGRSRWSNMEKLCTS
 751  VNLSKACRQQEELDSSTEALTLNLTEVVKRQTPKSKKGFNQISTSQIKVD
 801  KVQIIGSNSCPFAVCLDQDERKILQSVIRCEVSPCYKPEKSSLCPPPQRP
 851  SYPPAPATPDLCSLLCLPIMTFSGALP
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p120

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   1  MELENYEQPVVLREDNRRRRRRMKPRSTAASLSSMELIPIEFVLATSQRN
  51  TKTPETALLHVAGHGNVEKMKAQVLLRALETSVSWDFYHRFGPDHFLLVF
 101  QKKGEWYEIYDKYQVVQTLDCLRYWEVLHRSPGQIHVVQRHAPSEETLAF
 151  QRQLNALIGYDVTDVSNVHDDELEFTRRRLVTPRMAEVAGRDPKLYAMHP
 201  WVTSKPLPEYLLKKITNNCVFIVIHRSTTSQTIKVSADDTPGTILQSFFT
 251  KMAKKKSLMDIPESQNERDFVLRVCGRDEYLVGETPIKNFQWVRQCLKNG
 301  EEIHLVLDTPPDPALDEVRKEEWPLVDDCTGVTGYHEQLTIHGKDHESVF
 351  TVSLWDCDRKFRVKIRGIDIPVLPRTADLTVFVEANIQYGQQVLCQRRTS
 401  PKPFTEEVLWNVWLEFSIKIKDLPKGALLNLQIYCGKAPALSGKTSAEMP
 451  SPESKGKAQLLYYVNLLLIDHRFLLRHGEYVLHMWQLSGKGEDQGSFNAD
 501  KLTSGTNPDKEDSMSISILLDNYCHPIALPKHRPTPDPEGDRVRAEMPNQ
 551  LRKQLEAIIATDPLNPLTAEDKELLWHFRYESLKDPKAYPKLFSSVKWGQ
 601  QEIVAKTYQLLAKREVWDQSALDVGLTMQLLDCNFSDENVRAIAVQKLES
 651  LEDDDVLHYLLQLVQAVKFEPYHDSALARFLLKRGLRNKRIGHFLFWFLR
 701  SEIAQSRHYQQRFAVILEAYLRGCGTAMLHDFTQQVQVIDMLQKVTIDIK
 751  SLSAEKYDVSSQVISQLKQKLENLQNLNLPQSFRVPYDPGLKAGALVIEK
 801  CKVMASKKKPLWLEFKCADPTALSNETIGIIFKHGDDLRQDMLILQILRI
 851  MESIWETESLDLCLLPYGCISTGDKIGMIEIVKDATTIAKIQQSTVGNTG
 901  AFKDEVLSHWLKEKCPIEEKFQAAVERFVYSCAGYCVATFVLGIGDRHND
 951  NIMISETGNLFHIDFGHILGNYKSFLGINKERVPFVLTPDFLFVMGTSGK
1001  KTSLHFQKFQDVCVKAYLALRHHTNLLIILFSMMLMTGMPQLTSKEDIEY
1051  IRDALTVGKSEEDAKKYFLDQIEVCRDKGWTVQFNWFLHLVLGIKQGEKH
1101  PA
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Figure 2. The Amino Acid Sequences of p120 and p101

The nucleotide sequences of cDNAs encoding p120 and p101 are shown. The deduced amino acid sequences of p101 and p120 are shown in single letter code. The sequences of peptides obtained from tryptic digests of purified p120 and p101 are underlined in bold. Two tryptic peptides were obtained from p101 that differed only by Ser/Gly in their second residues (indicated by an asterisk), suggesting allelic variation in sequence.

to reside at the N terminus, must therefore be recognized. However, a cDNA with an ORF encoding p117 has not been isolated. The protein and DNA sequences defining p120 were used to search databases for similar structures. Similarities with all previously cloned PI3Ks were identified. The sequence was nearly identical to p110γ, allowing for species differences (Stoyanov et al., 1995). The only significant discrepancy between our sequence and that of p110γ is found in the extreme COOH-T (dashed underlining in Figure 2). During a recent, independent cross-check of this sequence, Stoyanov et al. identified a reading error that had resulted in a frameshift and predicted a premature stop codon. Their corrected sequence now agrees with ours (R. Wetzker, personal communication).

A full-length clone defining p101 has not been isolated, but by utilizing several overlapping fragments derived from both oligo(dT)- and random-primed, pig neutrophil cDNA libraries, a full-length ORF encoding all of the peptide sequence derived from p101 has been defined. A p101-derived, NH_2-T-blocked peptide was identified. Its mass was precisely equivalent to that predicted for an NH_2-T-acetylated version of the first 12 residues defined by the predicted start in the ORF described and a tryptic cleavage site (Arg) at position 12. The predicted relative molecular mass of p101 is 97 kDa (see Figure 2). The protein and DNA sequence databases were searched for similar structures or substructures; none were found.

Analysis of Northern blots of poly(A)-selected pig neutrophil RNA with probes specific for p120 and p101 (both NH_2-T-derived) revealed the presence of very low abundance messages of the expected sizes (3.8 and 4.5 kb, respectively; data not shown).

Expression of p120 and p101 in Insect Cells

Recombinant, clonal baculoviruses (RBV) harboring either an NH_2-T, 6× HIS–tagged *p120* cDNA (pAcHLT p120) or an NH_2-T, (EE)-tagged *p101* cDNA (pAcO-G1 p101) were prepared and used to drive production of the above proteins in insect cells (Sf9). Single-step purifications utilizing the tags yielded both proteins at greater than 90% purity. Their apparent sizes matched expectation, and both were recognized in Western blots by specific, COOH-T-directed and internal sequence–directed antipeptide rabbit polyclonal sera, indicating their authenticity (data not shown).

p120 bound tightly, in 1:1 molar stoichiometry, to p101 both in vitro, when both proteins had been independently purified and mixed (see Figure 3), and in vivo, when Sf9 cells were coinfected with both forms of RBV and proteins were purified via the p101 tag (data not shown).

Free, purified p120 was a PtdIns(4,5)P_2-selective, wortmannin-sensitive PI3K. Gβγs had a small, bimodal effect on free p120 PI3K activity. An equimolar mixture of Gαs (final total concentration of 2 μM of Gαs o, i_1, i_2, and i_3 bound to GDP) in the presence of aluminum fluoride had no significant effect on free p120 PI3K activity. This preparation of Gαs, when added in a 1.5-fold molar excess, could completely inhibit the effects of Gβγs on PI3K. Tyrosine-phosphorylated peptides able to activate p85/p110 PI3K family members also had no detectable effect on p120 PI3K activity (see Figure 5).

When bound to p101 (either in vivo or in vitro), the

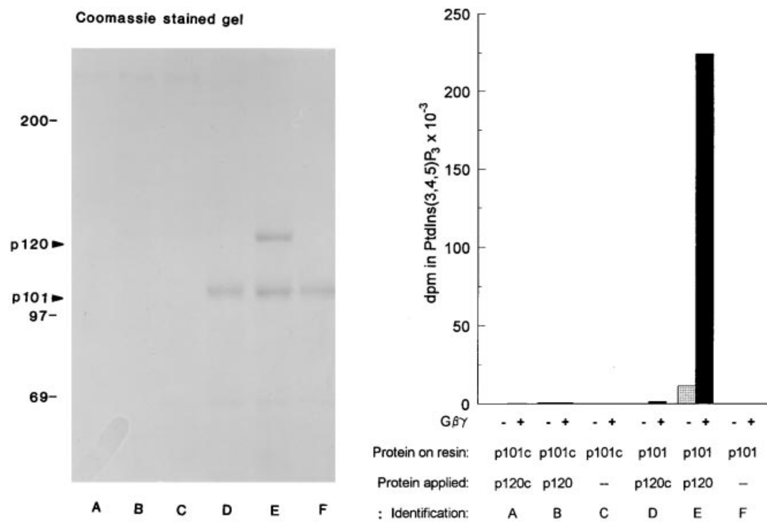

Figure 3. Immobilized p101 Binds Purified p120

Assays (A–F) were constructed. They contained 0.27 nmols (EE)-p101 (immobilized on α-[EE]-beads) or an equivalent fraction purified from Sf9 cells infected with wild-type BV (p101C), and 2.7 nmol of p120 or an equivalent fraction purified from Sf9 cells infected with wild-type BV (p120C). After 2 hr of mixing, the beads were washed and specifically bound proteins were eluted with (EE)-peptide. Each fraction (2.45, v/v) was resolved by SDS–PAGE (8% acrylamide) and stained with Coomassie R250 (see gel at left). Each fraction (0.2%, v/v) was assayed for PI3K activity with and without Gβγ subunits (1 μM final concentrations). The components added to each assay are defined beneath the graph at right.

PI3K activity of p120 could be activated greater than 100-fold by Gβγs. Tyrosine-phosphorylated peptides and Gα-GDP/aluminum fluoride had no effect on Gβγ-activated, or basal, p101/p120 PI3K activity. In the absence of Gβγs, the specific activity of p120 in a p101/p120 complex is lower than the specific activity of free p120 (24% ± 5%, n = 6) but is increased greater than 50-fold in their presence (see Figures 4 and 5). The specific activities of recombinant p101/p120 heterodimer toward PtdIns, PtdIns4P, or PtdIns(4,5)P_2 was 0.8, 0.6, or 2 fmol of product/mi/pmol of PI3K (with 500 μM, 50 μM, and 50 μM lipid concentrations, respectively, and 100 μM ATP) and 20, 60, and 300 fmol product/min/pmol of PI3K, respectively, in the presence of 0.5 μM Gβγs (other conditions as defined in the Experimental Procedures). Sf9-derived p101/p120 had a 2-fold higher specific activity than pig neutrophil–derived PI3K-B. This suggests that the bulk of the recombinant enzyme is correctly folded and processed.

To assess if Gβγs could bind to (EE)-p101 and/or (EE)-p120, the latter proteins were purified from Sf9 cells, incubated with bovine brain–derived Gβγs, and rapidly washed. Bound proteins were then analyzed by SDS–PAGE. Under a variety of conditions, 5-fold more Gβγs were recovered, per mole input protein, specifically (i.e., above control) associated with (EE)-p101 than (EE)-p120. Furthermore, binding was completely inhibited by preincubating the Gβγs with a 2-fold molar excess of GDP-bound $G\alpha_i$ (Figure 6).

Expression of p101 and p120 in Mammalian Cells

A family of mammalian expression vectors with NH_2-T epitope-tagged forms (either [myc] or [EE]) of p101 and p120 were constructed. When transiently expressed in U937 cells, (EE)-p101 and (EE)-p120 could be specifically immunoprecipitated from ^{35}S-methionine-labeled cells in approximately equal amounts (allowing for their relative content of methionines; 8:25, respectively; data not shown). Stringently washed α-(EE)-p101 immunoprecipitates contained a wortmannin-sensitive, PtdIns(4,5)P_2-selective, Gβγ-sensitive PI3K activity that was absent in controls using either an irrelevant monoclonal antibody for the immunoprecipitation or a cDNA encoding an (EE)-tagged irrelevant protein (see Figure

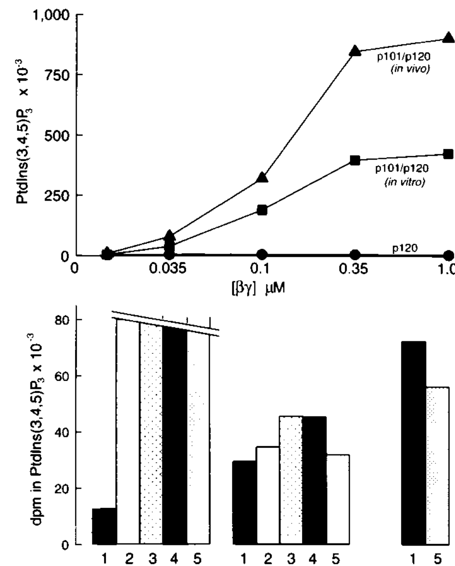

Figure 4. p101 Confers p120 Sensitivity to Gβγ Subunits

(Upper) Assays containing 12 nM purified p120 (final concentration) alone, or as p101/p120 heterodimers that had been formed by either reconstitution from purified components in vitro or by coinfection of Sf9 cells with RBV-pAcHLT p120 and RBV-pAcO-GI p101 and purification of the heterodimer via the (EE)-tag on p101. The different preparations were assayed in the presence of a range of concentrations of Gβγs. Data are means of duplicates; the ranges fell within the symbols.

(Lower) The bar graph shows data from experiments similar to those above, except that the axis scales are set to indicate the dose-dependent, bimodal effects of Gβγ on free p120 in these preparations (conditions: (1), no Gβγs; (2), 0.035 μm; (3), 0.1 μM; (4), 0.35 μm; (5), 1.0 μM Gβγs; all final concentrations), the linearity of the assays containing free p120, under these conditions, and the small but significant inhibition of p120 PI3K activity in the p101/p120 heterodimers, in the absence of Gβγ. The assays in the upper graph contained 5-fold more [γ^{32}P]-ATP. The data shown are means of duplicates (the average range of these means was 4.4%).

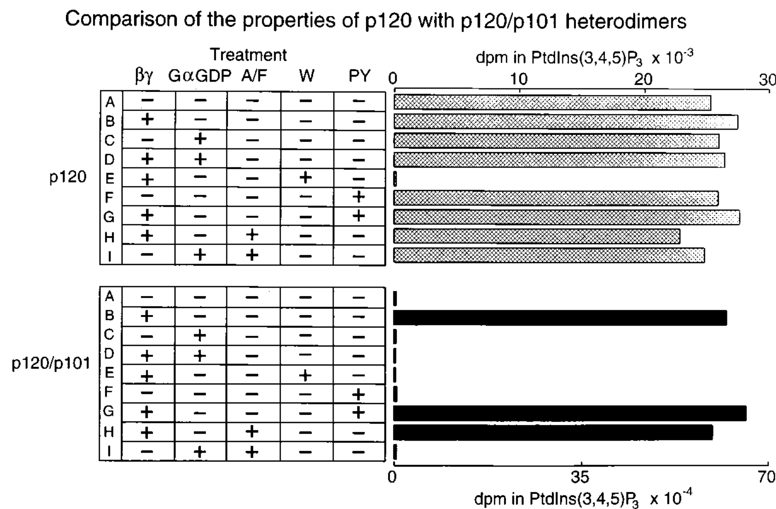

Figure 5. Pharmacological and Regulatory Properties of Free p120 and p101/p120 PI3Ks

Assays contained either 20 nM p101/p120 or 100 nM p120 alone (final concentrations) that were incubated with various reagents (10 mM NaF and 30 μM AlCl3 [A/F], 1 μM Gβγs, 2 μM Gα-GDP, 100 nM wortmannin [W], or 50 μM tyrosine-phosphorylated peptide [PY] for a total of 15 min (at 0°C) prior to starting the assays by adding [γ^{32}P]-ATP. [^{32}P] incorporated into [^{32}P]-PtdIns(3,4,5)P_3 was quantified. The data shown are means (n = 2); the average range of these means was 6.4%.

7). The activation by Gβγs was blocked by preincubation with a 2-fold molar excess of Gα-GDP. The PI3K activity in these p101 immunoprecipitates was insensitive to Gα-GDP/aluminum fluoride (data not shown). Cotransfection of (myc)-p120 with (EE)-p101 did not increase the PI3K activity specifically recovered in α-(EE) immunoprecipitates. Indeed, it decreased, probably because the expression of ^{35}S-methionine-labeled (EE)-p101 was lower in the presence of (myc)-p120 expression vectors (data not shown). In contrast, in cells transfected with (EE)-p120, α-(EE) immunoprecipitates contained barely detectable PI3K activity either in the presence or absence of Gβγs. Cotransfection with (myc)-p101 resulted in a substantial increase in the Gβγ-stimulated PI3K activity that could be recovered, despite a fall in the expression of (EE)-p120 (as judged by ^{35}S-methionine labeling) (see Figure 7). We interpret these data to indicate that U937 cells (human) contain an endogenous PI3K catalytic subunit that can bind to a transiently expressed p101 (pig), and because all of the p120 present in these immunoprecipitates is bound to p101, this catalytic subunit displays substantial regulation by Gβγs. In α-(EE) immunoprecipitates from (EE)-p120-transfected cells, much of the p120 is unassociated with p101 and hence relatively inactive even in the presence of Gβγs; however, cotransfection with (myc)-p101 amplifies the Gβγ-activated PI3K activity in (EE)-p120 immunoprecipitates. An alternative explanation for these data could be that the p120 is "denatured" unless expressed in the presence of p101 (although this p120 is soluble and capable of being immunoprecipitated). However, we consider this unlikely in view of the data obtained in experiments reconstituting independently-purified, Sf9-derived proteins.

To test whether the p101/p120 PI3K could be activated by Gβγs and produce PtdIns(3,4,5)P_3 in vivo, we transiently expressed various combinations of (myc)-γ_2, (EE)-β_1, (myc)-p101, and (myc)-p120 in cos-7 cells and measured their effects on the levels of [^{32}P]-phosphoinositides in cells 48 hr after transfection. p120 only produced significant increases in PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 in a $\beta_1\gamma_2$-dependent fashion in the presence of p101. This pattern of results could not be explained by changes in the relative expression of the different cDNAs when introduced in combinations (see Figure 8).

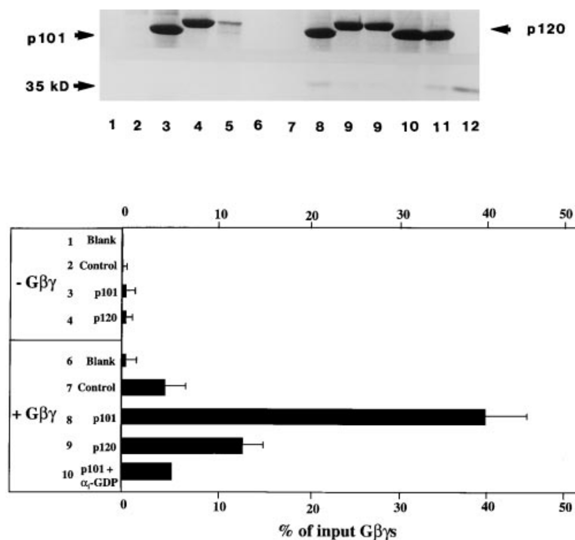

Figure 6. Gβγs Bind to p101

Gβγs were incubated with α-(EE)-beads (lanes 1 and 6) or with similar beads already bound to nonspecific Sf9-derived proteins (lanes 2 and 7), (EE)-p101 (lanes 3, 8, 10, and 11), or (EE)-p120 (lanes 4 and 9). Gβγs in some assays were preincubated with a 2-fold molar excess of either boiled (lane 11) or unboiled (lane 10) $G\alpha_i$-GDP. The beads were further washed in the binding buffer, then eluted with SDS sample buffer. The bound proteins were resolved by SDS–PAGE (12% acrylamide), detected by staining with Coomassie R250, and quantitated by densiometric scanning. The figure shows a photograph of a stained gel from a typical experiment and a bar graph with data accumulated from 5 independent experiments. Lane numbering refers to conditions defined in the bar graph, except that in lane 5, the (EE)-120-loaded beads had been eluted with (EE)-peptide prior to assay; in lane 11, the (EE)-p101 preparation was incubated with boiled α_i-GDP and Gβγs; and in lane 12, the (EE)-p101 preparation was loaded with an aliquot of Gβγs equivalent to 80% of the Gβγs that would be recovered from the assays if all bound. The data shown are means ± SE (n = 3–10) (except those including $G\alpha_i$-GDP proteins, which are means of duplicates).

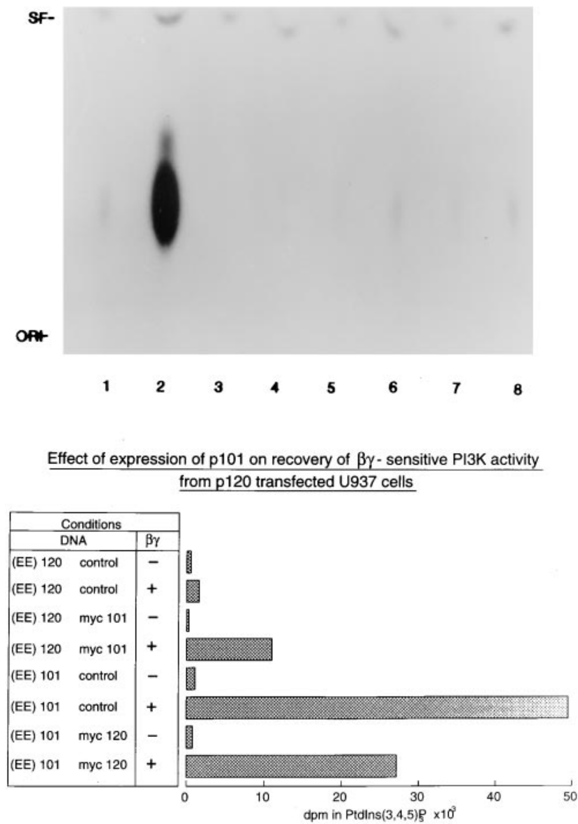

Figure 7. p101 Can Associate with a Gβγ-Stimulated PI3K Activity in U937 Cells

(Upper) U937 cells were transiently transfected with mammalian expression vectors encoding (EE)-p101 (lanes 1–4) or two other, irrelevant, (EE)-tagged proteins (lanes 5–8). Lysates were prepared, precleared, and immunoprecipitated with protein G–Sepharose covalently cross-linked to either α-(EE) (lanes 1,2, and 5–8) or α-(myc) (lanes 3 and 4) monoclonal antibodies. After stringent washing, the beads were assayed for PtdIns(4,5)P_2-directed PI3K activity in the presence and absence of Gβγs (1 μM final concentration), as indicated. The assays were then terminated, and their lipids were extracted, deacylated, and resolved on PEI-TLC plates. An autoradiogram of the TLC plate is shown.

(Lower) U937 cells were transiently transfected with a total of 40 μg of DNA as indicated, then lysed, precleared, and immunoprecipitated with protein G–Sepharose covalently cross-linked to α-(EE) monoclonal antibody. The resulting immunoprecipitates were washed, and PI3K activity was assayed with or without Gβγs (1 μM, final concentration). The PI3K activity detected in immunoprecipitates from cells transfected with irrelevant (EE)-tagged protein, either with or without Gβγs, was subtracted from the data shown (these were means of 1896 dpm and 2862 dpm in the absence and presence of Gβγs, respectively). Data shown are means (n = 2–3); the average range on these means was 5.1%. Parallel transfections labeled with [^{35}S]-methionine showed that the amount of [^{35}S]-p101 and [^{35}S]-p120 in the immunoprecipitates fell by 40% when they were cotransfected (data not shown).

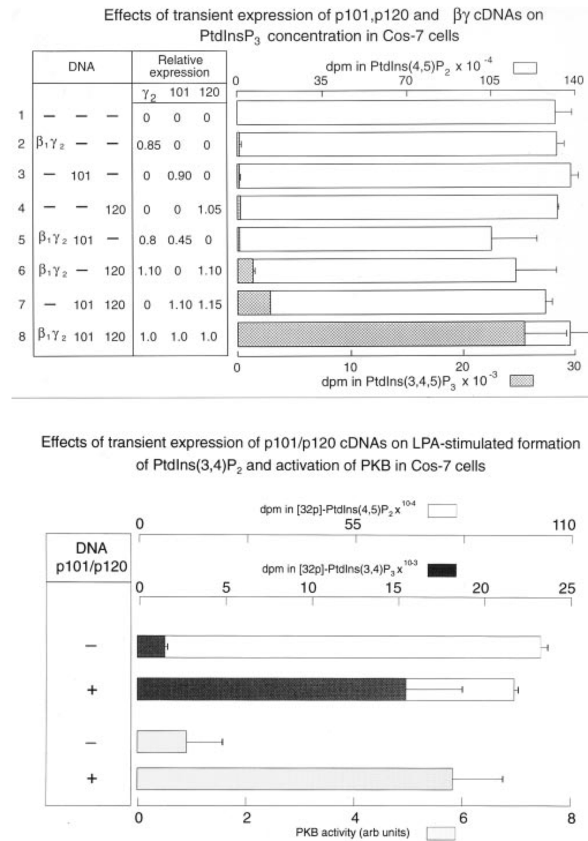

Figure 8. Regulation of p101/p120 by Gβγs In Vivo

(Upper) Cos-7 cells were transfected with mammalian expression vectors as indicated. After 48 hr, cells from each transfection were serum starved. They were then lysed, Western blotted, probed with an α-(myc) monoclonal antibody to quantitate the expression of the various (myc)-tagged proteins, and labeled with [^{32}P]-Pi. Results are given relative to the expression obtained in the presence of all four key vectors (note that the absolute levels of [myc]-p120 and [myc]-p101 were very similar and about 10-fold greater than that of [myc]-[2]). After 90 min, lipids were extracted and deacylated, and water-soluble head groups were resolved by anion-exchange HPLC and quantitated by liquid scintillation counting. Data shown are means ± ranges (n = 2). Data for [^{32}P]-PtdIns(3,4,5)P_3 are above the irrelevant DNA control (972 ± 41 dpm). The pattern of changes in the levels of [^{32}P]-PtdIns(3,4)P_2 was very similar to that of PtdIns(3,4,5)P_3, while the [^{32}P]-PtdIns3P levels did not change significantly.

(Lower) Cos-7 cells were treated as described above, except that some were also transfected with HA-tagged PKB or kinase-dead PKB. After serum starvation, cells (^{32}P-Pi-labeled or not) were stimulated with LPA and either lysed for assay of HA-immunoprecipitated PKB activity or quenched with 1 M HCl; ^{32}P-labeled 3-phosphorylated lipids were quantitated as described. Parallel data describing the LPA-stimulated increase in specific HA immunoprecipitate–associated PKB activity in the presence or absence of p101 and p120 are also shown (means ± range, n = 2). In this case, the LPA-stimulated increase in [^{32}P]-PtdIns(3,4)P_2 and the levels of [^{32}P]-PtdIns(4,5)P_2 in the same samples are shown (means ± range, n = 2) in the presence or absence of p101 and p120. An identical pattern of results was seen with [^{32}P]-PtdIns(3,4,5)P_3. There were no significant changes in the levels of [^{32}P]-PtdIns3P.

To address the issue of whether p101/p120 PI3K can be regulated by cell surface receptors and in turn regulate intracellular signaling pathways, we utilized the LPA receptors present on cos-7 cells that are known to activate pertussis toxin–sensitive (i.e., $G\alpha_i$-linked) cellular responses. Transient transfection of *p101* and *p120* cDNAs amplified the ability of LPA to stimulate PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 accumulation (Figure 8). Cotransfection of mammalian expression vectors encoding p101 and p120 with HA-tagged PKB showed that this enhanced LPA-stimulated accumulation of

PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 was paralleled by a substantial increase in the ability of LPA to stimulate activation of PKB in vivo.

Discussion

It has been proposed that a novel p46/p110 heterodimer underlies Gβγ-sensitive PI3K activity (Kurosu et al., 1995). This proposal is based of the observation that a crude preparation of a 150 kDa (native), Gβγ-sensitive PI3K activity contained both a 46 kDa protein that cross-reacted with an α-p85α antibody and a 100 kDa wortmannin binding protein. Similarly, a Gβγ-sensitive PI3K activity that is apparently recognized by αp85 antibodies has been described in platelets (Thomason et al., 1994). None of these reports has yet led to a complete characterization of the enzyme responsible, but it remains possible that there are several forms of Gβγ-sensitive PI3K activity.

Our data indicate that expression of p101 and p120 (p110γ; we consider p110γ to be the most appropriate name for the catalytic subunit we have described) in mammalian cells can substantially amplify the ability of both transiently transfected Gβγs and endogenous receptors capable of activating $G\alpha_i$ to stimulate PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 accumulation. This suggests that the expression of the p101/p110γ PI3K is critical for the capacity to display this response, that Gα subunits are not necessary for this response, and that expression of p101/p110γ PI3K in a cell could contribute to the ability of heterotrimeric GTPase activating receptors (e.g., ATP, FMLP, etc.) to stimulate PtdIns(3,4,5)P_3 accumulation. A current classification of PI3Ks places p85/p110-type PI3Ks (IA) in the same basic group as p110γ (IB) (Zvelebil et al., 1996). The work described here further emphasizes the parallels between these type IA and IB PI3Ks and, hence, the strength of this scheme by demonstrating the existence of a p110γ-specific adaptor that is necessary for regulation by Gβγs and hence, cell surface receptors.

PKB is becoming accepted as an effector enzyme downstream of PI3K (Franke et al., 1995). We have demonstrated that p101/p110γ PI3K can be regulated by cell surface receptors in a manner that allows activation of PKB. This observation is the first evidence showing that an isoform of PI3K that is distinct to p110α or p85/p110 PI3Ks can activate PKB and, taken with the other results described here, therefore further emphasizes that it is the lipid products of PI3Ks that act as their primary output signal.

Experimental Procedures

Cell Culture

U937 cells were grown in RPMI 1640 with 10% (v/v) HIFBS (heat-inactivated fetal bovine serum) and diluted 4-fold every 2 days. Cos-7 cells were grown in DMEM 10% HIFBS. Sf9 cells were grown in TNM FH with 11% HIFBS in a spinner flask (at 27°C) and were maintained at between 0.5 and 2×10^6 cells/ml.

Transient Expression in Mammalian Cells

Exponentially growing U937 cells (diluted 12 hr previously) were washed twice with PBS and resuspended in 0.45 ml of sterile electroporation medium (EM) containing 30 mM NaCl, 0.12 M KCl, 8.1 mM Na_2HPO_4, 1.46 mM KH_2PO_4, and 5 mM $MgCl_2$ (at 2.8×10^7 cells/ml), mixed with 40 μg of total DNA (in 50 μl of EM and made up to 40 μg with control DNA), and transferred to a cuvette (0.4 cm gap; BioRad). The cells were electroporated (1 pulse at 280 V and 960 μF), then diluted into 35 ml of RPMI, 10% HIFBS, supplemented with penicillin and streptomycin, TPA, and $ZnCl_2$ (final concentrations of 5×10^{-8} M and 200 μM, respectively). If the cells were to be labeled with ^{35}S-methionine (translabel; ICN), then the RPMI used after the electroporation was methionine- and leucine-free and contained 2 mM $NaHCO_3$, 25 mM HEPES, 10% dialyzed HIFBS, and 20 μCi/ml ^{35}S-methionine (plus TPA and $ZnCl_2$, as above). After 12 hr (either with or without ^{35}S) di-isopropylfluorophosphate (1 mM final concentration) was added, then the cells were washed once with PBS and lysed for immunoprecipitation with α-(EE) monoclonal antibody covalently attached to protein G–Sepharose [α-(EE) beads] and subsequent PI3K assay (see below), or SDS–PAGE and autoradiography. Exponentially growing cos-7 cells were trypsinized, washed twice in PBS, and resuspended in EM (2×10^7/ml) mixed with circular plasmid DNA (40 μg total per cuvette, made up of combinations of 10 μg of EXV-(EE)-β_1, 10 μg of EXV-(myc)-γ_2, 10 μg of pCMV-(myc)-p120, 10 μg of pCMV-(myc)-p101, or 10–40 μg of an irrelevant mammalian expression vector (0.5 ml final volume). The cells were electroporated (250 V, 960 μF) and aliquoted into 6 cm dishes (the equivalent of 5 from each cuvette). After 48 hr, dishes were washed into HEPES-buffered DMEM containing 1 mM $NaHCO_3$ and 0.2% fatty acid–free BSA. After another 10 hr, two replicate dishes were harvested for Western blotting with α-(myc) monoclonal antibody as the 1° detection reagent. The remaining dishes were washed into similar but Pi-free medium, then incubated for another 90 min at 37°C with 300 μCi [^{32}P]-Pi per dish (in 4 ml). After stimulation (10 μM LPA for 2 min), the media were aspirated and quenched, and [^{32}P]-phospholipids were quantitated as described (Stephens et al., 1991; Hara et al., 1994). In experiments in which HA-PKB activity was analyzed, transfections contained 2.5 μg each of (myc)-p101 and (myc)-p120 and 20 μg of either pSG5 HA-PKB or pSG5 HA-kinase-dead PKB. Assays were conducted with washed HA-directed immunoprecipitates, using myelin basic protein as a substrate (Burgering and Coffer, 1995).

Cloning of the cDNAs Encoding p120 and p101

We prepared 0.7 mg of total RNA from 4.2×10^9 pig neutrophils (Chomczynski and Sacchi, 1987). This RNA was used by Stratagene to produce Poly(A)-selected mRNA from which they prepared oligo(dT)- and random-primed cDNA libraries in λZAPII (approximately 5.4×10^6 and 3.2×10^6 primary pfu, respectively). Amplified libraries were constructed from approximately 2×10^6 original recombinants, and these were used to screen for *p120* and *p101* cDNAs by standard procedures.

Plaques (2.5×10^6) derived from the oligo(dT)-primed library were screened with a ^{32}P-labeled oligo based on peptide sequence from p120 (CA[T/C] GA[T/C] TT[T/C] ACI CA[A/G] CA[A/G] GTI CA[A/G] GTI AT[T/C/A] GA[T/C] ATG). A total of 12 positive clones were identified, isolated as Bluescript-based plasmids, and sequenced. The inserts of these plasmids represented a series of overlapping clones, two of which defined a full-length ORF encoding all of the peptide sequence derived from p117/p120 tryptic digests (Figure 2).

Plaques (0.9×10^6) derived from the oligo(dT)-primed library were screened using a ^{32}P-labeled oligo based on peptide sequence from p101 (GGI TA[T/C] ATG GA[A/G] GA[T/C] ATI GA[A/G] GA). One positive clone was identified, isolated, and sequenced. The 5′ end of this clone (D11) represented part of the sequence for the tryptic peptide from which the probe was designed, thus identifying it as a partial clone. Plaques (3.5×10^6) from the random-primed library were then screened using a ^{32}P-labeled, 0.35 kb, N-terminal ApaI restriction fragment derived from D11. A total of 98 positive clones were identified. These clones were rescreened (at the stage of primary plaque isolates) by a PCR-based approach, using primers designed against the Bluescript vector (either "forward" or "reverse" primers) and internal D11 sequence. This enabled us to identify (independent of orientation) the longest potential N-terminal extensions encoding the p101 sequence. The clones giving the largest PCR fragment were isolated and sequenced. These represented

overlapping clones that, together with D11, defined a full-length ORF encoding all of the peptide sequence derived from the p101 tryptic digest (Figure 5).

cDNAs were sequenced by generating a series of nested ExoIII/S1 nuclease deletions or using internal primers for the known sequences. Both strands of the total cDNAs of p101 and p120 were independently sequenced.

Construction of Expression Vectors

The use of N-terminal PCR and appropriate restriction sites allowed the *p120* and *p101* ORFs to be manipulated into a form in which they could be inserted in-frame into various expression vectors (in each case, the first amino acid encoded after the N-terminal tag was the start methionine). The entire 3′-untranslated region of the *p120* cDNA was used, and that of the *p101* cDNA was truncated at the most N-terminal BamHI site outside the ORF. The vectors used for BV-driven expression in Sf9 cells were pAcHLT (which encodes an N-terminal 6× His tag followed by a thrombin cleavage site [Pharminogen]; the *p120* ORF was inserted using the XhoI and EcoRI restriction sites) and pAcO-G1 (encodes an N-terminal EE tag [Onyx Pharmaceuticals]; the *p101* and *p120* ORFs were inserted using the EcoRI and NotI restriction sites). The vectors used for CMV-driven expression in mammalian cells were pCMV(EE) (encoding an N-terminal MEEEEFMPMEF or MEEEEFMPMEFSS EE tag for p101 or p120 expression, respectively) and pCMV (myc) (encoding an N-terminal MEQKLISEEDLEF or MEQKLISEEDLEFSS myc tag for p101 or p120 expression, respectively). All vectors were N-terminally sequenced before use.

Sf9 Cell Transfections and Production of Recombinant Proteins

Sf9 cells were transfected using Insectin (Invitrogen) liposomes with linearized baculo-gold DNA (Pharminogen) and the relevant BV transfer vectors. The resulting RBV were plaque-purified and amplified. After infection, cells were harvested into ice-cold 0.41% KCl, 2.66% sucrose, 20 mM $MgCl_2$, and 8 mM NaH2PO4 (pH 6.2, 25°C) containing 1 mM di-isopropylfluorophosphate. The cells were then washed and frozen in liquid N_2 and stored at −80°C.

PI3K Assays

Purified aliquots of Sf9-derived or neutrophil-derived PI3K or immunoprecipitates to be assayed for PI3K activity were diluted in, or washed into, the last wash buffer, which contained ice-cold 0.12 M NaCl, 25 mM HEPES, 1 mM EGTA, 1 mM DTT, 1 mg/ml BSA, 1% betaine (0.02%, w/v), and Tween 20 (pH 7.4, 0°C). Then 30 μl of a mixture of phospholipids with or without Gβγs (see below) and/or Gαs (either GDP-bound or activated) was added to 5 μl fractions or 10 μl of beads and mixed. After 10 min on ice, 5–10 μl of buffer, supplemented with $MgCl_2$ (to give a final concentration of of 3.5 mM), was added and mixed. After 6 min, 5 μl of last wash buffer was added (to give a final assay volume of 50 μl) containing [γ^{32}P]-ATP (typically 10 μCi per assay, Amersham, PB10168) and 3.5 mM $MgCl_2$. The tubes were then mixed and transferred to a 30°C water bath for 15 min. Assays were quenched and extracted, and lipids were deacylated, resolved on PEI-plates, and quantitated (Stephens et al., 1994).

PtdIns(4,5)P_2 (Stephens et al., 1994) and PtdEtn (Sigma) (final concentrations in the assay of 50 μM and 0.5 mM, respectively) were included; in some experiments, PtdIns4P or PtdIns (Sigma) were included. The dried lipid was bath sonicated (at room temperature) into final wash buffer without BSA and supplemented with 0.1% sodium cholate. Sonicated lipids were mixed with a mixture, totaling 1 μl of per assay, of Gβγ storage buffer (1% cholate, 50 mM HEPES [pH 7.5, 4°C], 0.1 M NaCl, 1 mM DTT, and 0.5 mM EDTA), active Gβγ, or an equivalent volume of boiled Gβγ of 3–7 mg/ml stocks in storage buffer. In some experiments, the 1 μl included Gα subunits or their storage buffer, in which case the Gβγs were premixed with the Gα subunits (either GDP-bound or -activated; see below) for 10 min on ice.

Gα subunits (an equimolar mixture of Gα:o, i_1, i_2, and i_3 prepared as described [Sternweis and Pang, 1990] and stored in the same buffer as the Gβγ subunits, but supplemented with 10 μM GDP) were activated by incubation on ice with 10 mM NaF and 30 μM AlCl3 (A/F) for 10 min. Assays into which these subunits were diluted also contained A/F.

Purification of Sf9-Derived Proteins

p120 was purified using a metal-ion chelation column (Talon, Clontech). Cell pellets were thawed and sonicated into 0.1 M NaCl and 50 mM sodium phosphate (pH 8.0, 4°C); 10 mM Tris–HCl (pH 8.0, 4°C), 1 mM $MgCl_2$, and antiproteases (10 μg/ml each of pepstatin A, aprotinin, leupeptin, antipain, and bestatin and 0.1 mM PMSF). A 120,000 × g cytosolic fraction was supplemented with Tween 20 and betaine (0.05%, w/v, and 1%, respectively) and loaded onto Talon resin, which was subsequently washed sequentially with 20 column volumes each of buffers A–D. Buffer (A) contained 50 mM sodium phosphate (pH 8.0, 4°C), 10 mM Tris–HCl (pH 8.0, 4°C), 0.15 M NaCl, 1% betaine, and Tween 20 (0.5%, w/v). Buffer (B) contained (A) plus Triton X-100 (1%, w/v). Buffer (C) contained (A) but was at pH 7.1 and 4°C. Buffer (D) contained (A) but was at pH 7.5 and contained Tween 20 (0.02%, w/v), ethylene glycol (0.05%, v/v), and 1 mM $MgCl_2$. The Talon resin was then washed with 8 column volumes of buffer (E) (which contained [D] supplemented with 10 mM imidazole [pH 7.5]) and buffer F (which contained [D] supplemented with 70 mM imidazole [pH 7.5, final concentration]. Typically, 1 ml fractions were immediately collected and supplemented with 1 mM DTT and 1 mM EGTA (final concentrations). This yielded 4 mg of p120 per liter of Sf9 culture. "p120 blank" preparations were prepared from cells infected with wild-type BV.

(EE)-p101 was purified from frozen pellets of Sf9 cells as follows. Cells were thawed and sonicated into 0.12 M NaCl, 1 mM $MgCl_2$, 25 mM HEPES (pH 7.4, 4°C), and 1 mM EGTA plus antiproteases (above 2 liters of infected Sf9 culture into 50 ml; all subsequent steps deal with this quantity of lysate), after which the cells were centrifuged (120,000 × g, 4°C, 40 min). Then the supernatant was removed (as described above), supplemented with Triton X-100 (1%, w/v), sodium cholate (0.4%), and 0.4 M NaCl (final concentrations), and precleared with 2 ml α-(myc) beads. Next, (EE) proteins were collected with 1 ml of α-(EE) beads. After 2 hr of mixing at 4°C, the beads were washed five times with Triton X-100 (1%, w/v), 0.4% cholate, 0.4 M NaCl, 0.005% SDS, 20 mM HEPES, 1 mM EGTA, and 1 mM DTT and three times with buffer (H), comprised of buffer (G) with Triton X-100 (1%, w/v). Yields of (EE)-p101 were typically only 8%–10% of those of p120. p101 blank (p101C) preparations were from wild-type BV-infected cells. p101/p120 heterodimers formed in vivo by coinfection of Sf9 cells with both forms of RBV were purified as described for (EE)-p101, except that the immunoprecipitates were washed four times with buffer (I) (which contained Triton X-100 [1%, w/v], 0.15 M NaCl; 20 mM HEPES [pH 7.4, 4°C], 1 mM EGTA), twice with buffer (J) (which contained [I] supplemented with 0.4 M NaCl [final concentration], and then three times with buffer (G) before being eluted with 1 bed volume of 150 μg/ml (EE)-peptide in buffer (G) (final concentration; (EE)-peptide, NH_2-T-acetylated EYMPTD, has a very high affinity for the α-(EE) monoclonal antibody; the beads were incubated with (EE)-peptide, on ice, for 40 min, then the supernatant was removed). Aliquots of the eluted proteins were diluted and assayed for PI3K activity (see above) or mixed with SDS sample buffer directly. In in vitro reconstitution experiments, p120 preparations in buffer (G) were supplemented with Triton X-100 (1%, w/v; now equivalent to buffer [H]), mixed with (EE)-p101 (10:1 molar ratio of protein) still bound to the protein (G) matrix, mixed for 2 hr (end on end at 4°C), and then washed and eluted with (EE)-peptide as previously described above for the purification of p101/p120-PI3K reconstituted in vivo. (EE)-p120 was purified from Sf9 cells as described for (EE)-p101. Yields were typically 1.5–2.0 mg per 500 ml of Sf9 culture.

Gβγ Binding Assays

Assays (40–80 μl final volume) contained α-(EE)-beads (2 μl packed volume) that had been preincubated with lysates from Sf9 cells infected with wild-type BV or RBV encoding (EE)-p101 or (EE)-p120 or with the lysis buffer alone. This was arranged such that the beads carried 1.5–4 μg of p101 or 1.8–4.8 μg of p120. Additional α-(EE) beads, preloaded with a lysate from wild-type BV-infected Sf9 cells, were used to make up the total packed volume of beads in each assay to 2 μl. The beads were washed with PBS containing Tween

20 (0.05%, w/v), 1 mM EGTA, and 1 mM DTT. They were then incubated in the same PBS supplemented with 1% BSA (30 mins on ice), washed three more times (in BSA-free buffers), incubated with 500 nM Gβγ (on ice 45 min, 1:1 molar ratio with p101 or p120), and finally, washed three times with the above BSA-free buffer before adding SDS sample buffer.

Purification of Pig Neutrophil Gβγ-Stimulated PI3K Activities

Purifications started with 40–45 g of cytosolic protein (derived from the neutrophils in 750 liters of blood). Two activities (PI3K-A and PI3K-B) were completed resolved.

Final preparations of PI3K were incubated with 100 nM [^{3}H]-17-hydroxy-wortmannin (17.7 Ci/mmol; Amersham, custom made), resolved by SDS–PAGE, stained with Coomassie, and photographed. [^{3}H] was then detected fluorographically.

Generation of Peptides and Peptide Sequencing

Aliquots of protein for sequencing were Western blotted (in a wet blotter) onto nitrocellulose (0.45 μm pore size BA85; Schleicher and Schuell). The filters were stained with 0.1% Ponceau S in 1% acetic acid for 1 min, then destained for 1 min in 1% acetic acid. Approximately 85%–90% of the protein loaded on the gel was recovered on the filter. The bands of interest were excised from the nitrocellulose and processed for internal amino acid sequence analysis (Tempst et al., 1990), with modifications (Lui et al., 1996). An enzyme blank was done on an equally sized strip of nitrocellulose.

HPLC solvents and system configuration were as described (Elicone et al., 1994), except that a 2.1 mm 214 TP54 Vydac C4 (Separations Group, Hesperia, CA) column was used with gradient elution at a flow rate of 100 μl/min. Identification of Trp-containing peptides was done by manual ratio analysis of absorbances at 297 and 277 nm, monitored in real time using an Applied Biosystems (Foster City, CA) model 1000S diodarray detector (Erdjument-Bromage et al., 1994). Fractions were collected by hand, kept on ice for the duration of the run, and then stored at −70°C before analysis.

Purified peptides were analyzed by combination of automated Edman degradation and matrix-assisted laser-desorption ionization time-of-flight (MALDI-TOF) mass spectrometry; details about this combined approach, including mass-aided postchemical sequencing routines can be found elsewhere (Elicone et al., 1994; Erdjument-Bromage et al., 1994; Geromanos et al., 1994). After storage, column fractions were supplemented with neat TFA (to give a final concentration of 10%) before loading onto the sequencer disks and mass spectrometer target. Mass analysis (on 2% aliquots) was carried out using a model Voyager RP MALDI-TOF instrument (Vestec/PerSeptive, Framingham, MA) in the linear mode, with a 337 nm output nitrogen laser, a 1.3 m flight tube, and α-cyano-4-hydroxy cinnamic acid (premade solution obtained from Linear Sci., Reno, NV) as the matrix. A 30 kV ion acceleration voltage (grid voltage at 70%, guide wire voltage at 0.1%) and a −2.0 kV multiplier voltage were used. Laser fluence and number of acquisitions were adjusted as judged from optimal deflections of specific maxima, using a TDS 520 Tektronix (Beaverton, OR) digitizing oscilloscope. Mz (mass to charge) spectra were generated from the time-of-flight files by using GRAMS (Galactic Ind., Salem, NH) data analysis software. Every sample was analyzed twice, in the presence and absence of two calibrants (25 fmol each of APID and P8930), as described (Geromanos et al., 1994). Chemical sequencing (on 95% of the sample) was done using a model 477A instrument from Applied Biosystems (AB). Stepwise liberated phenyl thiohydantoin (PTH) amino acids were identified using an "on-line" 120A HPLC system (AB) equipped with a PTH C18 (2/1 × 220 mm; 5 μm particle size) column (AB). Instruments and procedures were optimized for femtomole level PTH amino acid analysis as described (Erdjument-Bromage et al., 1994; Tempst et al., 1994).

Peptide average isotopic masses were summed from the identified residues (including the presumed ones) by using ProComp version 1.2 software (obtained from Dr. P.C. Andrews, University of Michigan, Ann Arbor, MI).

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EMBL Accession Numbers

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