A Function for Phosphoinositide 3-Kinase β Lipid Products in Coupling βγ to Ras Activation in Response to Lysophosphatidic Acid*

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Although Gβγ is thought to mediate mitogen-activated protein kinase (MAPK) activation in response to G protein-coupled receptor stimulation, the mechanisms involved in this pathway have not been clearly defined. Phosphoinositide 3-kinase (PI3K) has been proposed as an early intermediate in this process, but its role has remained elusive. We have observed that dominant negative mutants of p110β, but not of p110γ, inhibited PI3K stimulation in response to lysophosphatidic acid (LPA). The role of p110β was located upstream from Ras. To determine which of the lipid or protein kinase activities of p110β were important for Ras activation, we produced a mutant p110β lacking the lipid but not the protein kinase activity. This protein displayed a dominant negative activity similar to a kinase-dead mutant, indicating that p110β lipid kinase activity was essentially involved in Ras activation. In agreement, overexpression of the lipid phosphatase PTEN was found to specifically inhibit Ras stimulation induced by LPA. In addition, we have observed that the PH domain-containing adapter protein Gab1, which is involved in p110β activation during LPA stimulation, is also implicated in this pathway downstream of p110β. Indeed, both membrane redistribution and phosphorylation of Gab1 were reduced in the presence of PI3K inhibitors or dominant negative p110β. Downstream of Gab1, the tyrosine phosphatase SHP2 was found to mediate Ras activation in response to LPA and to be recruited through PI3K and Gab1, because transfection of Gab1 mutant deficient for SHP2 binding inhibited Ras activation without interfering with PI3K activation. We conclude that LPA-induced Ras activation is mediated by a p110β/Gab1/SHP2 pathway. Moreover, we present data indicating that p110β is effectively the target of βγ in this pathway, suggesting that the p110β/Gab1/SHP2 pathway provides a novel link between βγ and Ras by integrating two early events of LPA signaling, i.e. Gγ release and tyrosine kinase receptor transactivation.

Lysophosphatidic acid (LPA)1 is an intercellular lipid mediator potentially involved in tissue regeneration, brain development, tumorigenesis and atherosclerosis, although its precise physiopathological role in vivo remains to be explored (1–5). LPA is produced by activated cells, notably platelets, and promotes the proliferation or survival of a large number of cell types, similarly to a canonical growth factor. It is now well accepted that the biological activity of LPA is mediated by at least three different G protein-coupled receptors (GPCR), namely LPA1, LPA2, and LPA3 (Edg 2, 4 and 7, respectively) which belong to a recently described family of receptors to bioactive lysosphospholipids. These receptors can activate in concert the Gαq, Gα12/13 subfamilies of G proteins, even though the coupling specificity between each receptor and the different G proteins remains to be clearly established (6–8).

A number of studies have started to delineate the intracellular signaling pathways that mediate the biological activity of LPA. This lipid was first found to activate the so-called Ras/mitogen-activated protein kinases (MAPK) pathway, which controls cell proliferation, differentiation and survival in response to numerous extracellular stimuli (see Refs. 1, 9 for recent reviews). Nevertheless, the early mechanisms involved in MAPK activation in response to LPA are still incompletely defined. LPA can potentially activate this pathway through Gq-dependent Ca2+ mobilization leading to stimulation of protein kinase C (PKC) that in turn can activate Raf or MEK (MAP kinase/ERK kinase) (10). However, pertussis toxin that inactivates Gαi was found to inhibit MAPK stimulation induced by LPA or other Gi-coupled receptor agonists (9). In addition, the role of Gi was located upstream from Ras, and the involvement of βγ subunits has been well documented (11, 12). In contrast, the molecular intermediates between βγ and Ras have not been clearly identified (for recent reviews, see (9, 13)). Since Ras is classically activated downstream of tyrosine kinases, various candidates, including Src, Pyk2 and growth factor receptors were shown to mediate MAPK activation in response to GPCR stimulation (14–17). However, there is little evidence for a participation of βγ in the activation of these tyrosine kinases, and, in the case of Pyk2 or EGF receptor (EGFR), their recruitment has been shown to occur through Gq-dependent pathways.
(14, 18, 19). Nevertheless, it has been proposed that βγ could participate in Src activation, leading to EGFR phosphorylation and MAPK stimulation (20). G protein-coupled receptor kinases and β-arrestins might be intermediates between βγ and Src (21), or even actors of the MAPK pathway (22), but these models have been essentially described in cells overexpressing adrenergic receptors and their relevance to LPA signaling remains to be established.

Besides tyrosine kinases, phosphoinositide 3-kinase (PI3K) has been shown to be an early intermediate of MAPK activation in response to LPA (9, 23). PI3K phosphorylates the 3′ position of the inositol ring of phosphoinositides to produce lipids that are now considered as crucial spatio-temporal organizers of various signaling pathways. From a molecular point of view, three classes of PI3Ks have been defined, and mitogen signaling involves essentially class I enzymes that are subdivided into two subclasses (A and B) (24, 25). Class IA PI3Ks include the catalytic subunits p110α, β, and δ associated with a p85 regulatory subunit and activated by phosphorytrosine-containing motifs encountered on receptors or adapter proteins. In the case of p110β, G protein βγ subunits were shown to dramatically increase its activation by phosphorytrosine motifs, even though the molecular determinants of this synergism have to be further defined (26, 27). Class Iκ is represented by the catalytic subunit p110γ that has the unique feature to be directly activated by G protein βγ subunits. p110γ can be associated with a p101 regulatory subunit that has no homology with any known protein but contributes to the sensitivity of p110γ to Gβγ (28, 29). From a mechanistic point of view, processes by which PI3Ks activate signaling pathways have been recently unraveled. Their lipid products interact with a number of signaling proteins, resulting in their membrane targeting and/or modulation of their enzyme activity. For example, phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P3) binds to a conserved protein motif called the pleckstrin homology (PH) domain, thereby inducing the activation of the serine/threonine kinase Akt/protein kinase B (PKB) and its upstream activators, the phosphoinositide-dependent kinases (30, 31).

The hypothesis that PI3K might participate in the MAPK pathway has emerged when blockade of PI3K was found to interfere with MAPK activation in response to LPA (9, 23). Further studies have suggested that p110γ was a strong candidate to mediate this effect (32–34), and to date it is commonly accepted that p110γ can link GPCR stimulation to MAPK activation (13). However, the mechanism of this connection has been incompletely characterized. Although it has been initially reported that p110γ linked Gβγ to MAPK activation through an Shc-Grb2-Sos-Ras pathway (32), the putative tyrosine kinase activated downstream of p110γ remains to be identified. In addition, in a particular CHO cell line displaying a deficient activation of Ras, Takeda et al. (33) have observed that LPA can activate MAPK at the level of MEK through a pathway involving p110γ upstream of PKCζ. PKCζ can be stimulated directly by PtdIns(3,4,5)P3 or by the phosphoinositide-dependent kinases (35, 36). However, this model has been challenged by the demonstration that the protein kinase activity of p110γ, but not its lipid kinase activity, was important for MAPK activation (34). Indeed, PI3K catalytic subunits have an intrinsic protein kinase activity that was thought to simply regulate their lipid kinase activity (37, 38), but, at least in the case of p110γ, the protein kinase activity was shown to be critical for MAPK activation by promoting MEK phosphorylation (34). However, the protein substrate of p110γ remains to be identified. Further studies are certainly required to complete these different models and define their physiological relevance, but the fact that p110γ seems essentially expressed in blood cells suggests that its role between Gβγ and MAPK might be that of a tissue-specific function.

We and others have previously reported (39–41) that LPA can activate p110β in different non-hematopoietic cell lines. The mechanism is thought to implicate cooperation between Gβγ and a transactivation pathway involving the EGFR and the adapter protein Gab1 that provides the consensus phosphorytrosine motifs. Activation of p110β is essential for cell cycle progression of NIH-3T3 cells stimulated with LPA (39), which suggested that this PI3K was an important actor of the signaling pathways involved in the mitogenic activity of LPA. We have thus searched for a role of p110β in the MAPK pathway in a non-transformed cell line stimulated through endogenous LPA receptors. By using a strategy based on the transfection of dominant negative mutants, p110β was found to contribute to MAPK activation upstream from Ras. In addition, the lipid kinase activity of p110β was found to be essential, pointing out a key element of Ras activation that involves sequentially PtdIns(3,4,5)P3, the PH domain-containing adapter Gab1, and the tyrosine phosphatase SHP2. Moreover, we present data indicating that this pathway provides a novel connection between βγ and Ras.

**EXPERIMENTAL PROCEDURES**

**Materials**—LPA, pertussis toxin, and wortmannin were from Sigma Chemical Co. LY294002 and AG1478 were from BIOMOL. Ro-31-8220 was from Calbiochem. Monoclonal anti-phosphoErk and anti-GST antibodies were from Sigma. Polyclonal antibodies against ERK2, Grb2, SHP2, and EGFR and monoclonal anti-myec were from Santa Cruz Biotechnology. Polyclonal antibody against Gab1 was from Upstate Biotechnology Inc. Polyclonal antibody against phosphoAkt/PKB (Ser-473) was from Cell Signaling. Monoclonal anti-pan Ras was from Oncogene Research. Monoclonal anti-His tag antibody was from Invitrogen, anti-HA tag was from Roche Molecular Biochemicals, and anti-T7 tag was from Novagen. Cell culture reagents were from Invitrogen.

**Expression Plasmids**—A construct encoding C-terminal Myc/Histagged ERK1 was obtained by subcloning ERK1 (kindly provided by Dr. E. van Obberghen, Nice, France) into pcDNA3.1-MycHis (Invitrogen). The pRK5 plasmid encoding wild type HA-tagged p110β was kindly provided by Drs. P. Hsu and J. Schlessinger (New York University, New York, New York) (44). The kinaseinactive K805R mutant of p110γ was obtained by site-directed mutagenesis (QuikChange, Stratagene) with the following mutagenic primer: 5′-TTGGGATGGATTATTATAAGATGTTGATT TACG-3′ (the changed nucleotide is underlined). p110β “protein kinase only” mutant was obtained by deleting amino acids 948 to 955 using the following mutagenic primer: 5′-ATTCTTGGAAATTTCTAGGCTT TACG-3′. The mutations were verified by sequencing, and the integrity of the encoded proteins was controlled by immunoblotting and kinase assays following expression in COS cells. A construct encoding full-length p110β in antisense orientation was obtained by excising p110β from the pRK5 construct using EcoRI and HindIII. The insert was then subcloned through the same sites into pH6M (Roche Molecular Biochemicals, Meylan, France), and the construction was verified by restriction mapping. The plasmids encoding p110γ, βARK, PTEN, and Gab1 mutants have been previously described (32, 45–47). The p85 and SHP2 constructs were kindly provided by Drs. W. Ogawa (University of Kobe, Japan) and N. Rivard (Sherbrooke University, Canada), respectively.

**Cell Culture, Transfection, and Stimulations**—Vero cells (ATCC CCL 81) were maintained in Dulbecco’s modified Eagle medium supplemented with 7.5% fetal bovine serum and antibiotics. For transfection experiments, cells in 60-mm plates were incubated 3 h with 2 ml of Dulbecco’s modified Eagle medium containing 2 μg of total DNA and 6 μg of each LipofectAMINE and Plus reagents (Invitrogen). Cells were incubated overnight in serum-free medium before stimulation with LPA (10 μM, 5 min). When indicated, cells were incubated with 100 nM wortmannin or 25 μM LY294002 15 min before stimulation.

**Cell Lysis, Immunoprecipitation, and Immunoblotting**—Cells were scrapped off in lysis buffer containing 20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1% Nonidet P-40, 10 μg/ml of each aprotinin and leupeptin, 1 mM orthovanadate. After shaking for 15 min at 4 °C, soluble material was incubated with the appropriate antibody for 2 h at 4 °C. The antigen-antibody complexes were incubated with protein A- or
protein G-Sepharose (Sigma) for 1 h, then collected by centrifugation and washed with lysis buffer containing 0.1% Nonidet P-40, 1 μg/ml of each aprotinin and leupeptin, 0.1 mM orthovanadate. Pellets were then processed for in vitro kinase assays or resuspended in electrophoresis sample buffer and analyzed by immunoblotting. Blots were developed using chemiluminescence (Amersham Biosciences, Inc.) and semi-quantitative analysis following protein kinase buffer: 50 mM Tris, pH 7.4, 100 mM NaCl, 1 mM dithiothreitol, 0.5 mM EDTA.

Measurements of ERK Phosphorylation in Transfected Cells—Cells were transfected with 1 μg of each plasmid encoding ERK1-His and the indicated effector protein. After stimulation, cells were harvested in lysis buffer supplemented with 300 mM NaCl. Soluble material was incubated with 30 μl of Protein A-Sepharose (Sigma) for 1 h at 4 °C then washed twice with lysis buffer supplemented with 5 mM imidazole. The pellets were then resuspended in electrophoresis sample buffer and processed for anti-phosphoERK and anti-His immunoblotting.

In Vitro Akt/PKB Kinase Assay—Vero cells were cotransfected with 0.5 μg of DNA encoding HA-tagged Akt/PKB and 1.5 μg of plasmid encoding the indicated dominant negative protein. In the case of Δp85, only 1 μg was used to avoid nonspecific effects, supplemented with 0.5 μg of empty vector. After stimulation, cells were scrapped off in lysis buffer then subjected to anti-HA immunoprecipitation. Immunoprecipitates were washed twice with lysis buffer, then twice with kinase buffer containing 50 mM Tris, pH 7.5, 10 mM MgCl2, 1 mM dithiorthreitol. The reaction was performed in 25 μl of kinase buffer containing 10 μg of histone 2B (Roche Molecular Biochemicals), 50 μM ATP, and 3 μCi of [γ-32P]ATP. The reaction was incubated during 30 min at 25 °C, then stopped by addition of electrophoresis sample buffer and analyzed by SDS-PAGE. Phosphorylation of histones was quantified using a PhosphorImager and the software ImageQuant (Molecular Dynamos).

In Vitro Lipid and Protein Kinase Assays—COS cells were transfected with the constructs encoding HA-tagged Akt/PKB and 1.5 μg of plasmid encoding the indicated dominant negative protein. In the case of Δp85, only 1 μg was used to avoid nonspecific effects, supplemented with 0.5 μg of empty vector. After stimulation, cells were scrapped off in lysis buffer then subjected to anti-HA immunoprecipitation. Immunoprecipitates were washed twice with lysis buffer, then twice with kinase buffer containing 50 mM Tris, pH 7.5, 10 mM MgCl2, 1 mM dithiorthreitol, 0.5 mM EDTA. Pellets were then resuspended in 100 μl of kinase buffer supplemented with 150 μM phosphatidylserine, 75 μM phosphatidylinositol, 20 μM ATP and 5 μCi of [γ-32P]ATP. The reaction was performed at 37 °C for 30 min, then stopped by addition of 100 μl of HCl (1.5 s), followed by lipid extraction (40). Lipid extracts were then separated by thin layer chromatography and analyzed using a PhosphorImager. To perform the protein kinase assay, immunoprecipitation pellets were washed twice in the following protein kinase buffer: 50 mM Tris, pH 7.4, 100 mM NaCl, 1 mM MnCl2. Pellets were then resuspended in 50 μl of protein kinase buffer supplemented with 40 μM ATP and 3 μCi of [γ-32P]ATP. The reaction was incubated at 37 °C for 30 min, then stopped by adding electrophoresis sample buffer and analyzed by SDS-PAGE. Autophosphorylated p110γ was detected using a PhosphorImager.

Activated Ras Affinity Precipitation Assay—The assay was performed essentially as described previously (43). The recombining Ras binding domain (RBD) of Raf1 (kindly provided by Dr. F. R. McKenzie, Nice, France) was expressed as a GST fusion protein in Escherichia coli and extracted using glutathione-Sepharose beads. To measure Ras activation in stimulated cells, Vero cells were scrapped off in 1 ml of lysis buffer containing 50 mM Tris, pH 8.0, 150 mM NaCl, 10 mM MgCl2, 0.5% deoxycholate, 1% Nonidet P-40, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 1 μM of each aprotinin and leupeptin. Cleared lysates were incubated at 4 °C for 30 min with 30 μg of GST-RBD bound to glutathione-Sepharose beads. Beads were washed three times in lysis buffer then boiled, and proteins were resolved by SDS-PAGE. Immunoblotting was performed with anti-Ras antibodies. To study Ras activation in transfected cells, the cells were cotransfected with 0.5 μg of plasmid encoding HA-tagged wild type Ras (kindly provided by Dr. B. M. Burgering, Utrecht, The Netherlands) and 0.5 μg of the indicated effector, unless otherwise indicated. The GST-RBD pull-down assay was performed as above, except that immunoblots were revealed with anti-HA antibodies.

Membrane Fractions—Membrane fractions were prepared essentially as described (48). Cells were scrapped off in lysis buffer containing 20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 10 mM EGTA, 2 mM MgCl2, 10 μg/ml of each aprotinin and leupeptin, 1 mM orthovanadate, then Dounce-homogenized. The homogenate was centrifuged at 100,000 x g for 1 h. The pellet was washed once in lysis buffer then dissolved in lysis buffer supplemented with 1% Triton X-100. The insoluble material was spun out, and the supernatant was taken as the solubilized membrane fraction.

RT-PCR Analysis—This study was performed essentially as described (42). Total RNA was extracted from cultured cells by the TRIzol method (Invitrogen) followed by purification on NucleoSpin RNA II columns (Amersham-Pharmacia Biotech). The content of the genomic DNA was monitored by PCR amplification of β-actin. Pure RNA preparations were then reverse-transcribed with SuperScript II (Invitrogen) using oligo-dT or random hexamer primers as indicated. PCR reactions were carried out in 50 μl containing 5 μl of cDNA, 200 μM dNTP, 500 μl primers, PCR buffer (Invitrogen) supplemented with 1.5 mM MgCl2, and 0.5 μl of Taq DNA polymerase (Invitrogen). Without otherwise indicated, PCR conditions were: 95 °C for 15 s followed by 35 cycles (95 °C for 30 s, 55 °C for 45 s, 72 °C for 30 s) and final extension (72 °C for 7 min). PCR products were separated on 2% agarose and stained with ethidium bromide. The primers for β-actin were: 5′-CTG-GAACGGTGAAAGTGCA-3′ (1275–1294) and 5′-GGTCTCAGACTTGTCA-3′ (1676–1696); LPA; 5′-CCGAGACGCATGCTGTTGAC-3′ (286–305) and 5′-GCTCAGAAGATCTGCGAGA-3′ (664–683); LPA; 5′-CCCAACAGAGCTAAGC-3′ (1123–1147) and 5′-GAGGCTTATCTTCCTCACCA-3′ (1401–1420); LPA; 5′-GGAGACCATGAAGTAAAG-3′ (695–714) and 5′-CTGTTGTCTTCTCGAGA-3′ (931–950).

RESULTS

MAPK Activation by LPA Requires p110γ Rather Than p110α—To examine the role of PI3K in MAPK activation, we have used a non-transformed monkey kidney cell line (Vero) that displays a strong increase of phosphorylated ERK during LPA stimulation (Fig. 1A). The involvement of PI3K was first approached using the conventional inhibitors wortmannin and LY294002. As shown in Fig. 1A, each drug inhibited by approximately 60% ERK phosphorylation in response to LPA. In contrast, each compound abolished Akt/PKB phosphorylation, indicating that they fully blocked PI3K activation in our experimental conditions. These results suggested that MAPK activation in Vero cells stimulated with LPA was partially dependent on PI3K. Because the function of PI3K in the MAPK pathway is poorly understood, we have studied the involvement of different PI3K isozymes using a dominant negative strategy. Cells were then transiently cotransfected with distinct PI3K mutants and His-tagged ERK1. Following cell stimulation, ERK1-His was extracted and its phosphorylation was analyzed by immunoblotting. We have first examined the role of p110γ, because this enzyme was shown to participate in MAPK stimulation mediated by βγ subunits (32). This was achieved by cotransficting ERK1-His with the dominant negative p110γ-K832R mutant. Fig. 1B shows that expression of this mutant did not reduce ERK1-His phosphorylation induced by LPA. As a control, we have verified that p110γ-K832R acted as a dominant negative molecule by blocking ERK activation induced by βγ overexpression (Fig. 1C), in agreement with previous reports (32, 40). This suggested that p110γ did not significantly participate in MAPK activation in response to LPA. In contrast, ERK1-His phosphorylation was strongly inhibited in cells transfected with a form of p85 lacking the p110 binding site (Δp85) (Fig. 1B). This protein is a widely used dominant negative mutant for class Iγ PI3K, which suggested the involvement of p110γ because other class Iγ enzymes are not activated by GPCRs. The role of p110γ was further studied using a kinase-inactive mutant of the catalytic subunit (p110γ-K805R). This protein significantly reduced ERK1-His phosphorylation induced by LPA, although the effect was less pronounced than that of p110γ. To understand this difference, we have compared the capacity of the two mutants to interfere with PI3K activation in response to LPA. For convenience, the activation of Akt/PKB was used as readout of PI3K stimulation, by cotransficting HA-tagged Akt/PKB with Δp85 or p110γ-K805R, followed by an in vitro kinase assay. As shown in Fig. 1D, whereas Δp85 abolished the stimulation of HA-Akt/
PKB induced by LPA, p110β-K805R produced only a partial inhibition, consistent with the respective capacity of the two mutants to interfere with MAPK activation. In addition, p110β-K805R did not significantly interfere with HA-Akt/PKB stimulation induced by LPA, further suggesting that in Vero cells p110β does not significantly participate in LPA signaling. Altogether, these data strongly suggested that p110β was involved in MAPK activation in response to LPA.

Role of p110β Upstream from Ras—To study p110β function in the MAPK pathway, we have first determined at which level of the cascade p110β was involved. Because Ras is located in a central position, we have determined whether p110β was necessary for Ras activation. This was achieved by using the precipitation assay for activated Ras that takes advantage of a GST fusion protein containing the Ras-binding domain of Raf (RBD) to extract selectively activated GTP-bound Ras (43). The amount of activated Ras in the pull-down assays was determined by anti-Ras immunoblotting. As shown in Fig. 2A, LPA strongly stimulated endogenous Ras in Vero cells. Pretreatment of the cells with wortmannin or LY294002 significantly reduced this activation, suggesting an involvement of p110β upstream of Ras. To test this hypothesis, the activation of HA-tagged wild type Ras was studied in cotransfection experiments with PI3K mutants. As shown in Fig. 2B, the Δp85 and p110β-K805R mutants significantly inhibited HA-Ras activation induced by LPA, with a relative efficiency corresponding to their ability to block Akt/PKB activation (Fig. 1D). In addition, dominant negative p110γ mutant did not prevent Ras activation induced by LPA (Fig. 2B), further excluding a role for this PI3K in this pathway. Finally, we have used an antisense
strategy to confirm the involvement of p110β in Ras activation. We have constructed a plasmid encoding full-length p110β in antisense orientation. Transfection of this plasmid in Vero cells produced a partial reduction of endogenous p110β, which might be due to the low percentage of transfected cells (Fig. 2C). In these conditions, we have tested whether this construct prevented Ras activation in cotransfection experiments to circumvent the low percentage of transfection. As shown in Fig. 2C, transfection of antisense p110β led to a partial but significant inhibition of Ras activation. Taken together, these data indicated that p110β contributed to Ras stimulation in response to LPA.

Role of the Lipid and Protein Kinase Activities of p110β—Because each of the lipid or protein kinase activities of PI3K can be involved in the MAPK pathway, we have determined for p110β the respective participation of each activity in Ras activation. We have thus prepared a protein kinase only (PKO) mutant by deleting the region that putatively interacts with the phosphoinositide polar head, according to the sequence alignment between p110β and p110γ (34). The effect of this deletion on p110β was verified using immune complex kinase assays following expression in COS cells. The lipid kinase assay was performed using phosphatidylinositol as a substrate, and autophosphorylation was used as a protein kinase assay. As controls, the assays were also performed with wild type (wt) and kinase-inactive p110β (K805R). Fig. 3A shows that, as expected, the PKO protein has lost the ability to phosphorylate phosphatidylinositol. In contrast, its protein kinase activity was conserved and was even higher than that of wild type p110β (Fig. 3B), which supported the notion that the two catalytic activities of PI3K are antagonistic (37). Therefore, the above deletion did convert p110β into a protein kinase only mutant. To determine whether this mutant could exhibit dominant negative effects, we have tested its ability to interfere with HA-Akt/PKB activation in cotransfection experiments. As shown in Fig. 3C, the PKO mutant inhibited HA-Akt/PKB stimulation as efficiently as the kinase dead K805R mutant, demonstrating that the loss of p110β lipid kinase activity had turned the PKO protein into a dominant negative mutant for D3-phosphoinositol-dependent processes. We have thus tested this mutant in cotransfection experiments with HA-Ras to determine whether p110β lipid kinase activity was dispensable for Ras activation. However, Fig. 3D shows that the PKO protein inhibited HA-Ras stimulation as efficiently as the kinase-dead mutant, which suggested that p110β lipid kinase activity was essential to Ras activation. To strengthen this result, Ras activation was measured in cells overexpressing PTEN, a phosphatase that degrades PI3K lipid products (46). As shown in Fig. 3E, overexpression of wild type PTEN significantly inhibited the stimulation of HA-Ras induced by LPA. Because PTEN has also a protein phosphatase activity that might interfere with Ras activation, we have used as a control
the "protein phosphatase only" mutant of PTEN (G129E) that does not interfere with PI3K signaling (46). Unlike wild type PTEN, this mutant did not modify HA-Ras activation induced by LPA, which indicated that the lipid phosphatase activity of PTEN was essentially responsible for Ras inhibition. Taken together, these results showed that the lipid products of p110\(\beta\)/H9252 were critical for Ras stimulation in response to LPA.

Gab1 and SHP2 Link p110\(\beta\) to Ras

To define p110\(\beta\) function upstream from Ras, we have studied the proteins that associate with Grb2 in a PI3K-dependent manner. This was achieved by immunoprecipitating Grb2 from LPA-treated cells, preincubated or not with wortmannin. Immunoprecipitates were then analyzed by antiphosphotyrosine immunoblotting. As shown in Fig. 4A, LPA increased the phosphorylation of six different proteins, five of which were insensitive to wortmannin: The 180-kDa protein comigrated with the epidermal growth factor receptor (EGFR), the 66-, 52-, and 46-kDa proteins comigrated with Shc, and we failed to identify the 95-kDa protein (data not shown). In contrast, wortmannin inhibited the phosphorylation of the 115-kDa protein (Fig. 4A). This protein comigrated with Gab1 upon reblotting the samples with anti-Gab1 antibody (data not shown). Although Gab1 is an essential activator of p110\(\beta\) by providing p85-binding motifs (40), this result, and the fact that Gab1 contains a PH domain (49), suggested that Gab1 might also be the downstream effector of p110\(\beta\) lipid products. To assess this hypothesis, we have first studied the effect of PI3K inhibitors on Gab1 phosphorylation. This was achieved by immunoprecipitating Gab1 from control or LPA-treated cells, followed by antiphosphotyrosine immunoblotting. As shown in Fig. 4B, Gab1 phosphorylation in response to LPA was strongly reduced when cells were incubated with PI3K inhibitors. In addition, Gab1 recruitment was
These data showed that PI3K was essential for Gab1 recruitment in LPA signaling, suggesting that Gab1 was the downstream effector of p110β lipid products. To further establish the respective role of p110β and p110γ in this pathway, we have studied the effect of their dominant negative mutants on Gab1-myc phosphorylation. As shown in Fig. 4D, the p110β, but not p110γ, mutant inhibited LPA-induced Gab1 phosphorylation, further suggesting that Gab1 was the downstream target of p110β lipid products.

Gab1 is a multifunctional adapter protein that contains binding sites for numerous signaling proteins, including Grb2, p85, and the tyrosine phosphatase SHP2 that can be involved in Ras stimulation (47, 50). This suggested that SHP2 might be a downstream effector of p110β and Gab1 required for Ras activation in response to LPA. To get further insight on the role of Gab1 and its partners in this pathway, we have studied the activation of both Ras and Akt/PKB in cells expressing Gab1 mutated on its SHP2 binding site (Gab1-Y627F) or on its three p85 binding sites (Gab1-YF3). These mutants were previously characterized using coimmunoprecipitation experiment as unable to associate with SHP2 or p85, respectively (47). As expected, Gab1-YF3 that prevents PI3K activation, significantly reduced HA-Ras stimulation induced by LPA (Fig. 5A). Interestingly, Gab1-Y627F strongly inhibited HA-Ras activation, suggesting that the association between Gab1 and SHP2 was important for Ras stimulation. To determine whether this interaction was required upstream or downstream from PI3K we have studied the ability of Gab1-Y627F to interfere with p110β activation using Akt/PKB as readout. As shown in Fig. 5B, Gab1-Y627F slightly increased Akt/PKB stimulation induced by LPA compared with Gab1-wt, whereas, as a control, Gab1-YF3 nearly abolished this activation. These results suggested that the association between Gab1 and SHP2 was important for activation of Ras, but not of PI3K, implying that SHP2 was a downstream effector of PI3K and Gab1 in this pathway. To assess this hypothesis, we have studied the role of PI3K in the recruitment of SHP2 in Ras activation complex. This was achieved by examining its association with Grb2 in coimmunoprecipitation experiments. As shown in Fig. 5C, LPA induced the association of SHP2 with Grb2, and this interaction was strongly inhibited by wortmannin. This showed that SHP2 was recruited downstream of PI3K, and supported the idea that the phosphatase was an intermediate between p110β and Ras. To further explore this pathway, we have determined whether SHP2 was required for Ras activation in response to LPA. This was achieved by studying HA-Ras activation in cotransfection experiments with a catalytically inactive mutant of SHP2 (C/S), or wild type SHP2 as control. Fig. 5D shows that SHP2-C/S, but not wt-SHP2, inhibited HA-Ras activation, which indicated that SHP2 catalytic activity was necessary for Ras activation induced by LPA. Taken together, these data showed that Gab1 and SHP2 mediated the participation of p110β to Ras stimulation in response to LPA.

The results also suggested that the pathway described herein might provide a novel link between βγ and Ras, because βγ is thought to participate in p110β activation. On the other hand, this pathway could be simply activated downstream of Gαi, because it requires EGFR transactivation. To gain insight on the G protein specificity of this pathway, we have first studied Ras activation in cells treated with pertussis toxin or transfected with the C-terminal domain of the β-adrnergic receptor kinase (βARK-CT) that acts as a βγ-scavenger molecule (51). As shown in Fig. 6 (A and B), pertussis toxin and βARK-CT strongly inhibited Ras activation in response to LPA, which indicated that Gα-derived βγ played an essential role in this pathway. Although p110β might be the downstream effec-

![Fig. 4. Role of PI3K in Gab1 recruitment.](https://www.jbc.org/)

**FIG. 4.** Role of PI3K in Gab1 recruitment. A, anti-Grb2 immunoprecipitates were obtained from control (ctrl) or LPA-treated cells, preincubated or not with wortmannin (+W), as indicated. Samples were analyzed by immunoblotting with antiphosphotyrosine (IB P-Tyr) and anti-Grb2 (IB Grb2) antibodies. As a control, the immunoprecipitation was performed without adding Grb2 antibody (−Ab). B, before stimulation with LPA, cells were incubated with PI3K inhibitors when indicated (+W, +LY). Cells were then subjected to Gab1 immunoprecipitation, followed by antiphosphotyrosine (IP P-Tyr) and anti-Gab1 (IB Gab1) immunoblotting. C, membrane fractions from cells treated as in A were prepared by ultracentrifugation. Membrane fractions were then analyzed by anti-Gab1 immunoblotting (upper panel), followed by anti-EGFR immunoblotting to control gel loading (lower panel). D, cells were cotransfected with Gab1-myc and the indicated constructs: empty vector (V); kinase-dead p110β (βKR); kinase-dead p110γ (γKR). After stimulation, cells were subjected to Gab1-myc immunoprecipitation followed by antiphosphotyrosine (upper panel) and anti-myc (lower panel) immunoblotting. The results displayed are representative of at least two independent experiments performed in duplicate.
Fig. 5. Role of Gab1 and SHP2 in Ras and Akt activation. A, cells were cotransfected with HA-tagged Ras and the indicated construct: empty vector (V), wild type Gab1-myc (wt); Gab1-myc mutated on its SHP2 binding site (Y627F); Gab1-myc mutated on its three pp5 binding sites (YF3). Following stimulation, cells were lysed and processed for GST-RBD assay. The amount of precipitated activated HA-Ras was determined by anti-HA immunoblotting (upper panel). Lysates were also directly subjected to anti-myc immunoblotting (lower panel) to control expression of HA-Ras and Gab1-myc constructs. The graph represents a quantification (mean ± S.E.) from three independent experiments. *p, less than empty vector; ns, not significant; p < 0.05, paired t test. B, cells were cotransfected with HA-tagged Akt/PKB and the indicated Gab1 constructs, then processed for HA-Akt/PKB immunoprecipitation followed by in vitro kinase assay. Phosphorylation of histones (H2B) was quantified with a PhosphorImager (upper panel + graph, mean ± S.E. from three experiments) and HA-Akt/PKB was revealed by immunoblotting (lower panel). C, anti-Grb2 immunoprecipitates were performed from cells pretreated or not with wortmannin and stimulated with LPA as indicated. Immunoprecipitates were then analyzed by anti-SHP2 (upper panel) and anti-Grb2 (lower panel) immunoblotting. D, cells were cotransfected with HA-tagged Ras and empty vector (V), catalytically inactive SHP2 (C/S) or wild type SHP2 (wt) as indicated. Cells were then stimulated and subjected to the GST-RBD pull-down assay as in A.

As shown in Fig. 1, the PI3K-dependent pathway described herein seems to account for about half of ERK activation induced by LPA. As a preliminary study of the other half of this activation, we have used Ro-31-8220, an inhibitor of all PKC family members, alone or in combination with wortmannin or pertussis toxin. As shown in Fig. 6, Ro-31-8220 partially reduced ERK phosphorylation induced by LPA. In addition, when cells were treated with both Ro-31-8220 and wortmannin, ERK phosphorylation was nearly abolished, suggesting that PKC and PI3K acted on distinct pathways leading to MAPK activation. In agreement with this, Ro-31-8220 displayed also an additive effect with pertussis toxin that was shown to block the PI3K pathway at the level of Gi (see Fig. 6C). Moreover, Ro-31-8220 did not interfere with LPA-induced activation of both Akt/PKB and Ras (Fig. 6, A and C), further suggesting that PKC and the Gp110β/Ras pathway provide two distinct routes leading to ERK activation in response to LPA.

The ability of LPA to stimulate signaling pathways is thought to be mediated through at least three different GPCR, namely LPA₁ (Edg2), LPA₂ (Edg4), and LPA₃ (Edg7). As a first approach to define their role in the pathway described herein, we have studied their expression using RT-PCR analysis. As
shown in Fig. 7, both LPA$_1$ and LPA$_3$ could be amplified from Vero cell cDNA. However, LPA$_1$ was produced more readily, suggesting that it was expressed to a higher level. Because LPA$_1$ and LPA$_3$ preferentially activate G$_i$ and G$_q$, respectively (6–8), these results suggested that LPA$_1$ might be involved in the G/p110$\beta$/Ras pathway whereas LPA$_3$ could be responsible for the PKC-dependent pathway leading to MAPK activation.

**DISCUSSION**

Although PI3Ks are important effectors of LPA signaling, their role in the MAPK pathway has remained somewhat elusive. p110$\gamma$ appeared to be the best candidate to participate in this pathway, but the mechanisms connecting p110$\gamma$ to MAPK activation have become incompletely characterized. We report here a novel mechanism involving p110$\beta$, a widely expressed...
P110 is a major component of the complex signaling pathway leading to Ras activation in response to LPA.

The involvement of p110 in this pathway is based on the dominant negative effects of three different P110 mutants. First, Δp85, the standard inhibitor of class IA P110s, inhibited MAPK activation induced by LPA, which strongly suggested the involvement of p110, because other class IA enzymes are known to be activated by GPCR stimulation. In agreement with this, a kinase-inactive mutant of p110 catalytic subunit was found to interfere with the stimulation of both Ras and MAPK, although less efficiently than Δp85. This difference seems to be due to the differential ability of p85 and p110 mutants to interfere with P110 stimulation, as shown by studying Akt/PKB activation. An explanation for this latter observation could be that p110 mutants must displace a pre-existing complex between endogenous p85 and p110 to produce dominant negative effects, whereas Δp85 can act on its own. Moreover, through a different approach using antisense cDNA of p110, we have also observed that this protein was important for Ras activation in response to LPA. Finally, the fact that the PKO mutant also produced a dominant negative effect on Ras activation further demonstrated the involvement of p110.

The PKO mutant was produced to distinguish which of the protein kinase or lipid kinase activities of p110 were involved in this pathway, considering that each activity can potentially participate in MAPK activation. However this mutant displayed a dominant negative activity on Ras activation, suggesting that the lipid kinase activity of p110 was essentially involved. In addition, overexpression of the phosphatase PTP that degrades P110 lipid products was found to specifically interfere with Ras activation, further supporting a major role for p110 lipid products in this pathway. Therefore, the functional link between P110 protein kinase activity and the MAPK pathway remains thus far restricted to p110, although its protein substrate remains to be identified.

Moreover, it seems that the notion that p110 is a major intermediate between GPCR stimulation and the MAPK pathway must be somewhat moderated. Although it has been reported that dominant negative p110 interfered with MAPK activation in COS or CHO cells stimulated with LPA, we failed to reproduce this result in Vero cells. However, the study in COS cells was performed with a very low dose of LPA (40 nM), suggesting that the role of p110 is limited to weak stimulations of the MAPK pathway (34). A common theme may also be evoked to explain the results in CHO cells, because the involvement of p110 was essentially demonstrated in a particular cell line where Ras activation had been compromised (33). This suggests that p110 provides in these cell lines a limited contribution to MAPK activation that has been masked under our experimental conditions. Indeed, we have studied the response of normal cells stimulated with a mitogenic dose of LPA (10 μM) that engages at least two pathways, i.e., PEC and Ras, leading to MAPK activation. In addition, although p110 is abundant in neutrophils or platelets, its level of expression is much lower in Vero and other adherent cell lines (COS, Rat1, and NIH-3T3) (data not shown). We therefore assume that the capacity of p110 to contribute significantly to the MAPK pathway, and other cellular responses, might be restricted to hematopoietic cells. In agreement with this, the phenotypes described to date in p110 knockout mice are associated with blood cell functions (52–55), taking into account that the role of p110 in colorectal cancer has been recently challenged (56). Nevertheless, additional studies are necessary to determine whether p110 is physiologically important for MAPK activation in hematopoietic cells.

Several biochemical mechanisms have suggested a role for P110 in the MAPK pathway at the level of Raf or MEK, because these enzymes are potential targets of kinases activated through P110-dependent processes (10, 36, 57–59). However, we have observed that P110 can play an important role upstream from Ras in cells stimulated with LPA. Indeed, the lipid products of p110 were found to facilitate the recruitment of the docking protein Gab1 that participates in Ras activation. Gab1 was thought to provide the phosphotyrosine-binding motifs required for p110 activation, but we have observed that it also plays a downstream target of p110. We thus propose the following model connecting LPA to Ras where Gab1 is involved at two different steps (Fig. 8). Following LPA stimulation, Gab1 is recruited to phosphorylated EGFR through Grb2 or direct binding to the EGFR (49, 60). This induces the phosphorylation of Gab1 on p85 and SHP2 binding sites, leading to p110 activation in synergy with βγ subunits released during stimulation of LPA receptors. Activation of p110 produces PtdIns(3,4,5)P3 that will recruit additional Gab1 molecules in the vicinity of the EGFR through binding to its PH domain. We assume that this mechanism provides a way to amplify the recruitment of p85 and SHP2, because the physical association of Gab1 with the EGFR is limited by the low number of EGFR molecules that are phosphorylated in response to LPA (data not shown). Downstream of Gab1, we have observed that SHP2 could provide a link with Ras, although the nature of this connection remains obscure. It has been proposed that SHP2 can function as an adapter protein in platelet-derived growth factor signaling, because it can bind to both the receptor and Grb2, and therefore contributes to the recruitment of Grb2-Sos (61). However, we have observed that the catalytic activity of SHP2 was important for Ras activation in response to LPA, in agreement with a recent report showing that this phosphatase was necessary for MAPK activation in HEK293 cells stimulated through LPA, (62). This suggests that SHP2 activates by dephosphorylation a protein involved in Ras activation, or down-regulates an inhibitor of Ras. One possible candidate...
could be the GTPase-activating proteins that promote Ras deactivation. Interestingly, in U937 cells, PI3K was found to play a permissive role in basal activation of Ras through inhibition of GTPase-activating proteins (63). However, the mechanism of this regulation is not known, and additional studies are required to define whether Ras GTPase-activating proteins are a downstream target of SHP2.

One important aspect of the pathway described herein is that it provides a substantial connection between βγ and the MAPK pathway. Although it is well established that βγ is important for Ras activation, the effectors of this pathway have not been clearly identified (9, 13). Interesting models have suggested that βγ might be involved in EGFR recruitment through β-arrestin and Src (20, 21), but we have observed that βγ was required at the level of p110β, but not EGFR transactivation in Vero cells stimulated with LPA. Considering that EGFR stimulation per se can stimulate PI3K, one may wonder why βγ and p110β are particularly required in this pathway. We assume that the direct recruitment of class I PI3Ks to transactivated EGFR produces only a marginal PI3K stimulation corresponding to the strength of this transactivation. Therefore, the “boosting” effect of the interaction between βγ and p110β (26, 27) is probably important to initiate a significant activation of PI3K that will be subsequently amplified through Gab1.

In conclusion, p110β appears to be a pivotal link between βγ and Ras, due to its unique ability to integrate two separate signals arising from GPCR stimulation, i.e. the release of βγ and EGFR transactivation. We presume that the mechanism described here will apply to other cellular systems and other GPCR agonists, considering the relative ubiquity of p110β expression and EGFR transactivation.

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A Function for Phosphoinositide 3-Kinase β Lipid Products in Coupling βγ to Ras Activation in Response to Lysophosphatidic Acid

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