Requirement of SHP2 Binding to Grb2-associated Binder-1 for Mitogen-activated Protein Kinase Activation in Response to Lysophosphatic Acid and Epidermal Growth Factor*

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Grb2-associated binder-1 (Gab1) is a multisite docking protein containing a pleckstrin homology (PH) domain, multiple potential tyrosine phosphorylation sites, and several proline-rich sequences. Gab1 becomes tyrosine-phosphorylated in cells stimulated with growth factors, cytokines, and ligands for G protein-coupled receptors. A major Gab1-binding protein detected in cells treated with extracellular stimuli is the tyrosine phosphatase, SHP2. Although the role of SHP2-Gab1 interaction in cell signaling has not yet been characterized, SHP2 is known to mediate mitogen-activated protein (MAP) kinase activation induced by the epidermal growth factor (EGF). However, the mechanism by which the SHP2 phosphatase exerts a positive signaling role remains obscure. In this study, we prepared Gab1 mutants lacking the SHP2 binding site (Gab1Δ274), the phosphatidylinositol 3-kinase (PI3K) binding sites (Gab1ΔPI3K), and the PH domain (Gab1ΔPH). Expression of Gab1Δ274 blocked the extracellular signal-regulated kinase-2 (ERK2) activation by lysophosphatic acid (LPA) and EGF. Conversely, expression of the wild-type Gab1 in HEK293 cells augmented the LPA receptor Edg2-mediated ERK2 activation. Whereas the PH domain was required for Gab1 mediation of ERK2 activation by LPA, it was not essential for EGF-induced ERK2 activation. Expression of Gab1ΔPI3K had no apparent effect on ERK2 activation by LPA and EGF in the cells that we have examined. These results establish a role for Gab1 in the LPA-induced MAP kinase pathway and clearly demonstrate that Gab1-GSHP2 interaction is essential for ERK2 activation by LPA and EGF. These findings also suggest that the positive role of SHP2 in the MAP kinase pathway depends on its interaction with Gab1.

Protein-tyrosine kinases participate in the transduction of a variety of cellular signals including activation signals from growth factors, cytokines, and agonists of G protein-coupled receptors. Besides modulating the activities of substrate proteins, a common role of protein tyrosine phosphorylation is to create binding sites for the assembly of specific protein complexes (1). The formation of specific protein complexes mediates cell signaling by targeting enzymes to substrates or by altering enzymatic activities through protein-protein interactions.

Gab1 is a multisite docking protein that contains 694 amino acids and migrates with an apparent molecular mass of 115 kDa in SDS-polyacrylamide gels (1, 2). Gab1 is structurally similar to the Drosophila daughter of sevenless protein and to the insulin receptor substrate family of multisite docking proteins with an amino-terminal PH domain, several proline-rich sequences, and multiple potential tyrosine phosphorylation sites. Gab1 is rapidly phosphorylated on tyrosine residues upon stimulation of cells with EGF, insulin, hepatocyte growth factor (HGF), nerve growth factor, interleukin-6, and erythropoietin (2–6). Overexpression of Gab1 augments interleukin-6-induced ERK2 (the 42-kDa MAP kinase, p42MAPK) activation (5), increases anchorage-independent growth of mouse fibroblasts (2), and induces HGF receptor specific activities in epithelial cells (3).

The major binding partner of Gab1 in cells stimulated with various growth factors and cytokines is the protein-tyrosine phosphatase SHP2, a ubiquitously expressed protein-tyrosine phosphatase with two Src homology 2 (SH2) domains (2, 4, 5, 7). No study describing a role for Gab1-SHP2 interaction in signal transduction has been reported previously. Several groups have shown that the phosphatase activity of SHP2 has a positive role in the activation of the ERK members of the MAP kinase family and in mitogenic signaling by insulin, EGF, and interleukin-6 (5, 8–12). However, the mechanism by which SHP2 phosphatase exerts a positive regulatory role in mitogenic signaling remains obscure.

Other signaling molecules such as the adapter protein Grb2, PI3K, and phospholipase Cγ have also been found to associate with Gab1 in various cell lines (2, 4, 5). HGF induces Gab1 binding to tyrosine-phosphorylated HGF receptor in a Grb2-dependent manner (7), but the role of Grb2-Gab1 interaction in EGF and cytokine signaling remains to be determined. Binding of PI3K to Gab1 has been reported to be involved in the regulation of cell survival by nerve growth factor in PC12 cells (4). It is unclear, however, whether binding of PI3K to Gab1 is involved in the MAP kinase pathway.

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1 The abbreviations used are: Gab1, Grb2-associated binder-1; MAP kinase, mitogen-activated protein kinase; ERK2, extracellular signal-regulated kinase-2 (p42MAPK); LPA, lysophosphatic acid; EGF, epidermal growth factor; PI3K, phosphatidylinositol 3-kinase; PH, pleckstrin homology; PtdIns, phosphatidylinositol; HA, hemagglutinin; HGF, hepatocyte growth factor; BSA, bovine serum albumin.
Role of Gab1-SHP2 Interaction in MAP Kinase Activation

**Fig. 1. Gab1 protein and Gab1 constructs.** A schematic representation of human Gab1 is shown. A PH domain is located at the aminoterminal region. EGF receptor tyrosine kinase phosphorylates eight tyrosine residues in vitro as shown (15). Three tyrosine residues have been identified as PI3K binding sites, and Tyr-627 has been identified as the SHP2 binding site (13). Also shown are the location of the Met binding domain (MBD), which mediates Gab1 binding to the HGF receptor (c-Met) in a yeast experimental system (3), and the regions with the PX/PX motif, which are potential binding sites for SH3 domains. Gab1FL, Gab1ΔPI3K, Gab1Y627F, and Gab1ΔPH are Gab1 constructs that were prepared in pcDNA3.1 for expression of FLAG-tagged wild-type and mutated Gab1 proteins. Specific changes in each construct are indicated in the figure. Black boxes indicate the FLAG epitope. These Gab1 constructs were transiently expressed in HEK293 cells (in duplicate), immunoprecipitated with the anti-FLAG antibody M2, resolved on an 8.5% SDS-polyacrylamide gel, and immunoblotted with an anti-Gab1 antibody (bottom panel). A similar result was also obtained in COS-7 cells (data not shown).

Tyr-627 in the carboxyl-terminal region of Gab1 has been identified as the SHP2 binding site in insulin-stimulated cells (13). Tyrosine residues 447, 472, and 589 in Gab1 are located in a YVPM sequence motif that is selectively recognized by the 85-kDa subunit of PI3Kα (14). All three residues were reported to contribute to binding of PI3K to Gab1 in response to insulin (13). Recently, it was reported that the EGF receptor tyrosine kinase phosphorylates eight tyrosine residues of Gab1 in vitro (Fig. 1), including the three PI3K binding sites and the SHP2 binding site identified previously (15). Gab1 contains an aminoterminal PH domain (16). It has been demonstrated that some PH domains can bind various phosphoinositides and Gβγ (16, 17). A recent study has suggested that the Gab1-PH domain contains the consensus sequence motif for binding to PI3K products (18). In confluent Madin-Darby canine kidney cells grown in high serum concentrations, Gab1 is located predominantly at sites of cell-cell contact in a PH- and PI3K-dependent manner (19), suggesting that the PH domain may bind membrane 3-phosphoinositides and target Gab1 to the vicinity of the plasma membrane.

Lysophosphatidic acid (LPA) is a mitogenic phospholipid that induces an array of cellular responses, including activation of the Ras-MAP kinase pathway, by binding to G protein-coupled receptors (20–23). Recently, Edg2 and Edg4, two G protein-coupled receptors with different tissue distribution patterns, have been identified as LPA receptors (20, 24–26). In a number of cell lines, LPA weakly induces tyrosine phosphorylation of the EGF receptor (28–31). Precubination of LPA with specific inhibitors of the EGF receptor tyrosine kinase or expression of a truncated EGF receptor blocked LPA-induced EGF receptor tyrosine phosphorylation and activation of the ERK subfamily of the MAP kinases (28–30). Thus, EGF receptor tyrosine kinase mediates a signaling pathway of LPA that activates MAP kinase. However, LPA only weakly increases EGF receptor tyrosine phosphorylation and activates signaling mechanisms that are not EGF receptor-dependent, because EGF markedly activates EGF receptor tyrosine kinase and other members of ErbB tyrosine kinases. Thus, many differences in LPA and EGF signaling are likely to exist.

This laboratory and others (32) observed an approximately 115-kDa tyrosine-phosphorylated protein in LPA-stimulated cells. The protein was later identified as Gab1 (29). However, the role of Gab1 in the signal transduction of LPA or other ligands of G protein-coupled receptors has not been established. To investigate the role of Gab1 in the LPA- and EGF-induced MAP kinase signaling pathways, we prepared Gab1 mutants that lack the SHP2 binding site, the PI3K binding sites, and the PH domain and analyzed the effects of expression of these Gab1 constructs on LPA- and EGF-induced ERK2 activation. We report here that Gab1-SHP2 interaction is necessary for ERK2 activation in response to LPA and EGF. Furthermore, the PH domain is required for Gab1 to mediate LPA-induced ERK2 activation. These findings establish the role of Gab1 in the LPA-induced MAP kinase pathway and suggest that the positive regulatory role of SHP2 in MAP kinase activation relies on its interaction with Gab1.

**EXPERIMENTAL PROCEDURES**

**Materials—**Antibodies were purchased from the following sources: RC20H and anti-SHP2 from Transduction Laboratories; anti-Gab1 and anti-85-kDa subunit of PI3K from Upstate Biotechnology, Inc.; anti-FLAG (M2) from Sigma; and anti-HA from BabCo. The expression plasmid for HA-tagged ERK2 (33) and an anti-ERK antibody (34) were kindly provided by Dr. Michael J. Weber (University of Virginia). LPA, fatty acid-free bovine serum albumin (BSA), myelin basic protein, 1-α-phosphatidylcholinolinositol (PtdIns) and PtdIns-4-P were obtained from Sigma. COS-7 cells were obtained from American Type Culture Collection. HEK293 cells, defective in LPA-induced MAP kinase activation, were obtained from Dr. Joseph Schlessinger (New York University). Expression plasmids for the wild-type SHP2 and a phosphatase-inactive SHP2 (SHP2Δcat) that has a 29-amino acid deletion in the catalytic domain have been described (35). The SHP2Δcat coding sequence was excised from the pCMV vector (35) with EcoRI and subcloned into pcDNA3.1 (Invitrogen). Pfu DNA polymerase was obtained from Stratagene. Oligonucleotides, cell culture medium, EGF, Moloney murine leukemia virus reverse transcriptase, and Lipofectamine were obtained from Life Technologies, Inc. The Silica Gel 60 thin-layer chromatography (TLC) plates were obtained from Fisher Scientific Corp.

**Preparation of the Gab1 Expression Plasmids and Gab1 Mutants—**A plasmid containing HA-tagged human Gab1 (5) was kindly provided by Drs. Toshio Hirano and Masahiko Hibi (Osaka University). Gab1 cDNA from this plasmid was subcloned into pcDNA3.1 by polymerase chain reaction using Pfu DNA polymerase to generate a carboxyl-terminal FLAG (Sigma)-tagged expression plasmid for Gab1 (pGab1FL). The Gab1 DNA sequence was confirmed by DNA sequencing.

Site-specific mutagenesis of Gab1 was performed with the GeneEditor in vitro site-directed mutagenesis system (Promega). The mutagenic primer, 5′-pCAGGTGGAATTCTTAGATCTCGAC (the changed nucleotide is underlined) was used for replacing the Tyr-627 with phenylalana- nine in the mutation of the SHP2 binding site (pGab1Y627F). Three oligonucleotides (5′-pCTGGATGAGAATTTGGTCCCAAGTTGCT, 5′-pCAGGAGCGAAGGAATTTGGTCCCAAGTTGCT, and 5′-pGTTAGAACAAATTTGGTCCCAAGTTGCT) were used simultaneously to produce the triple Y447F/Y472F/Y589F mutations of the Gab1 PI3K binding sites (pGab1ΔPI3K). All mutations were confirmed by DNA sequencing.

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To generate the PH domain deletion mutant of Gab1, the DNA sequence between the AccIII and BamHI sites of Gab1 cDNA in pGab1FL was deleted, and a double-stranded oligonucleotide (5'-CCGGATCCATGATGGATGG and 5'-GGATCCTATGATGGATGCT) was inserted between the AccIII and BamHI sites. This plasmid (pGab1ΔP) lacks the coding sequence for amino acids 10–177. These Gab1 constructs are shown schematically in Fig. 1. An example of expression of these constructs in HEK293 cells is also illustrated in Fig. 1, bottom panel.

Preparation of the Edg2 cDNA and Expression Plasmid—cDNA was prepared from 10 μg of total RNA prepared from mouse fibroblasts by reverse transcription with Moloney murine leukemia virus reverse transcriptase, using random hexamers as primers. A full-length Edg2 cDNA was then isolated from this cDNA preparation by polymerase chain reaction using Pfu DNA polymerase and primers (5'-GGCGATCATGATGGATGG and 5'-GGATCCCATGATGGATGCT) based on the published sequences of mouse and human Edg2 cDNA (24, 25). DNA sequence analysis indicates that the isolated cDNA is identical to the reported cDNA from mouse brain (24) except for two nucleotide changes introduced by the degenerate primer, which does not change the identity of the encoded amino acids. The cDNA was subcloned into the BamHI and EcoRI sites of the eukaryotic expression vector pcDNA3.1 to generate pEdg2.

Cell Culture and Transfection—COS-7 and HEK293 cells were grown in Dulbecco’s modified eagle’s medium supplemented with 10% fetal bovine serum. Cells were seeded in 60-mm plates 24 h before transfection to yield a 50% confluent culture on the day of transfection. Transfection was performed using Lipofectamine, according to the supplier’s instructions. Briefly, cells were incubated with DNA-Lipofectamine (2 μg total/12 μl) complexes in serum-free medium for 5–7 h. The DNA complexes were then removed from the medium, and the cells were incubated with complete growth medium. Twenty-four hours after transfection, the cells were starved overnight in serum-free medium and then used for experiments.

Immunoprecipitation and Immunoblotting—For analysis of Gab1 tyrosine phosphorylation and complex formation with SHP2, cells were lysed in buffer A (25 mM Tris-HCl, pH 7.2, 150 mM NaCl, 25 mM NaF, 1 mM benzamidine, 1% Triton X-100, 1 mM Na3VO4, 20 μg/ml p-nitrophenylphosphate, 2 μg/ml leupeptin, and 100 μg/ml phenylmethylsulfonyl fluoride). Endogenous Gab1 was immunoprecipitated with an anti-Gab1 polyclonal antibody. FLAG-tagged mutant or wild-type Gab1 was immunoprecipitated with the anti-FLAG monoclonal antibody, M2. Collected immune complexes were resolved on 8% SDS-polyacrylamide gels, transferred to nitrocellulose filters, and processed for immunoblotting analysis essentially as described previously (30).

P13K Assay—HEK293 cells were transfected with pGab1FL or pGab1ΔP3K, serum-starved, and then stimulated with EGF or mock treated. Cells were lysed in buffer A. Gab1FL and Gab1ΔP3K were immunoprecipitated with antibody M2 and protein G-agarose. For control experiments, P13K was immunoprecipitated with an anti-P13K (p85) antibody. The immunoprecipitates were washed twice with buffer A, twice with buffer B (100 mM Tris-HCl, pH 7.4, 500 mM LiCl, 0.5 mM Na3VO4), and once with buffer C (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA, 0.5 mM Na3VO4). The immunoprecipitates were then incubated with 50 μl of 0.2 mg/ml PtdIns suspended in 10 mM Hepes, pH 7.4, 10 mM MgCl2, 50 μM ATP, and 10 μCi of [γ-32P]ATP (specific activity, 300 Ci/mmol) for 30 min at room temperature. The reaction was stopped with 100 μl of 1% HCl and immediately extracted with 200 μl of chloroform/methanol (1:1, v/v). The lipid products were dried under nitrogen gas, redissolved in chloroform, and spotted onto TLC plates, which had been coated with oxalate to prevent streaking. The plates were developed in chloroform/methanol/ammonium hydroxide/water (45:35:3.5:6.7, v/v/v/v). The radioactivity on the plates was visualized by autoradiography. PtdIns-4-P, which migrates slightly faster than PtdIns-3-P (36), was used as a marker and was visualized by staining with iodine vapor.

ERK2 Kinase Assay—Cells were lysed in buffer A, and HA-ERK2 was immunoprecipitated with the monoclonal antibody, HA.11. Half of each lysate was used to determine ERK2 activity, while the other half was used for ERK2 protein. For the kinase assay, the lysates were incubated for 10 min at 30 °C in 40 μl of MAP kinase buffer (20 mM Hepes, pH 7.5, 10 mM MgCl2, 1 mM diethiothreitol, 10 mM p-nitrophenylphosphate, 40 μM ATP, and 0.375 μM/ml myelin basic protein) containing 10 μCi of [γ-32P]ATP (3000 Ci/mmol). The reaction was terminated with SDS-containing gel-loading buffer, and the reaction mixtures were resolved on 11% SDS-polyacrylamide gels. The gels were processed by autoradiography. For quantification, the gels were analyzed with a PhosphorImager. Analysis of ERK2 protein in the immunoprecipitates was achieved by immunoblotting with a polyclonal antibody to ERK2.

RESULTS

LPA and EGF Induce Tyrosine Phosphorylation of Gab1—During our previous studies, we observed a 115-kDa tyrosine-phosphorylated protein in cells stimulated with LPA and EGF. To determine whether this protein was Gab1, serum-starved COS-7 cells were treated with LPA, EGF, or BSA (mock). Gab1 was then immunoprecipitated from cell lysates, and the immunoprecipitates were resolved on an SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. The tyrosine phosphorylation of Gab1 and association of SHP2 with Gab1 were analyzed by immunoblotting with antibodies to phosphotyrosine (A) or to SHP2 (B). The remaining immunoprecipitates were used in immunoblotting with an anti-Gab1 antibody to confirm that equal amounts of Gab1 were present in the immunoprecipitates (C). IP, immunoprecipitation; IB, immunoblotting; αPtyr, anti-phosphotyrosine antibody.

Expression of a Y627F Mutant of Gab1 Inhibits ERK2 Activation in Response to LPA and EGF—Tyr-627 of Gab1 has been identified as the SHP2 binding site in insulin-stimulated cells (13). A recent in vitro study demonstrated that Tyr-627 is the major tyrosine residue in Gab1 that is phosphorylated by the EGF receptor tyrosine kinase (15). To evaluate the role of Gab1-SHP2 interaction in LPA and EGF signaling, a Tyr-627 Sipphephaline mutation of Gab1 (Gab1Y627F) was prepared. Gab1FL and Gab1Y627F were then co-expressed with SHP2 in COS-7 cells to determine whether Gab1Y627F loses SHP2 binding activity. A basal SHP2 reactivity was detected in serum-starved cells with no difference between Gab1FL and Gab1Y627F. Stimulation of cells with LPA resulted in a small increase in the amount of SHP2 associated with Gab1FL, whereas EGF stimulation markedly increased the amount of SHP2 associated with Gab1FL. Importantly, mutation of Tyr-627 in Gab1 completely eliminated the increase in the binding of SHP2 in cells stimulated with LPA or EGF (Fig. 3A). These results substantiate Tyr-627 as the LPA- and EGF-induced SHP2 binding site in Gab1. The result shown in Fig. 7 (see below) also demonstrates that Gab1Y627F loses the LPA-in-
duced SHP2 binding activity in HEK293 cells.

We next analyzed the effect of the Y627F mutation on ERK2 activation in response to LPA and EGF. COS-7 cells were co-transfected with expression plasmids for HA-ERK2 and Gab1FL or Gab1Y627F and serum-starved for 20 h. Cells were stimulated for 3 min with LPA (50 μM) or EGF (10 ng/ml) or mock treated with BSA. Gab1FL and Gab1Y627F were immunoprecipitated from cell lysates and probed with antibodies to SHP2 (upper panel) and Gab1 (lower panel). B and C, COS-7 cells in 60-mm plates were co-transfected with 0.2 μg of HA-ERK2 and 1.8 μg of either pcDNA3.1 (vector control) or pGab1FL or pGab1Y627F, serum-starved, and stimulated for 5 min with LPA (0.5 μM) or EGF (0.25 ng/ml) or mock treated with BSA. HA-ERK2 was immunoprecipitated with an anti-HA antibody. A portion of the HA-ERK2 immunoprecipitates was used for the determination of ERK2 kinase activity using MBP as substrate (C, upper panel), and the remaining portion of the immunoprecipitates was analyzed by immunoblotting with an anti-ERK antibody (C, lower panel). B, mean ± S.D. of ERK2 activity from three experiments. IP, immunoprecipitation; IB, immunoblotting; MBP, myelin basic protein.

Expression of Gab1ΔPI3K and Gab1ΔPH do not inhibit EGF-stimulated ERK2 activation in HEK293 cells. A, HEK293 were transfected with 2 μg of pGab1FL or pGab1ΔPI3K. After serum starvation, the cells were treated for 5 min with BSA (control) or EGF. Cell lysates were immunoprecipitated with an anti-FLAG antibody, and the PI3K activity in the immunoprecipitates was determined. As a control, PI3K was immunoprecipitated from HEK293 cells with an anti-PI3K antibody. B–D, HEK293 cells were co-transfected with 0.2 μg of HA-ERK2 and 1.8 μg of each of the expression plasmids indicated in B. Serum-starved cells were treated for 5 min with BSA or EGF (0.5 ng/ml). HA-ERK2 was immunoprecipitated, and the ERK2 kinase activity (B and C) and protein (D) were determined as described under “Experimental Procedures.” B shows the average of ERK2 activity in two independent experiments. IP, immunoprecipitation; IB, immunoblotting; PIP, phosphatidylinositol; MBP, myelin basic protein.
Gab1 is involved in ERK2 activation, we prepared a Gab1 mutant (Gab1ΔP13K) containing triple Y447F/Y472F/Y589F mutations in which these three tyrosine residues were changed to phenylalanine (Fig. 1). Fig. 4A shows that Gab1ΔP13K loses its PI3K binding activity in response to EGF.

The role of PI3K binding to Gab1 in ERK2 activation was then analyzed by co-transfection of HEK293 cells with expression plasmids for HA-ERK2 and pGab1ΔP13K. As shown in Fig. 4, B–D, expression of Gab1Y627F blocked EGF-stimulated ERK2 activation, as was observed above in COS-7 cells. However, no difference in ERK2 activation by EGF in HEK293 cells transfected with pGab1ΔP13K was observed.

Gab1 contains an amino-terminal PH domain. To determine whether the Gab1 PH domain has a role in MAP kinase activation, we prepared a PH domain deletion mutant of Gab1 (Fig. 1). The data in Fig. 4, B–D, show that expression of the Gab1ΔPH did not have an inhibitory effect on EGF-stimulated ERK2 activation. Together, these results suggest that PI3K binding to Gab1 and the Gab1 PH domain is not required for ERK2 activation in response to EGF. Similar results were also obtained in COS-7 cells (data not shown).

Expression of Edg2 in a HEK293 Cell Line Restores LPA-induced ERK2 Activation—An Edg2 cDNA was isolated from mouse fibroblasts. To find a suitable cell line for studying Edg2-mediated ERK2 activation, we screened several cell lines in an attempt to identify a cell line that did not respond to LPA in terms of ERK2 activation. We found that LPA did not activate ERK2 in an HEK293 cell line (Fig. 5). It is unlikely that this is because of a defect in the Ras–MAP kinase cascade, because EGF readily activated ERK2 in these HEK293 cells (Fig. 4B). Transient expression of Edg2 in these cells led to LPA-induced activation of HA-ERK2 in cells co-transfected with the HA-ERK2 plasmid (Fig. 5). In other experiments, we detected an approximate 2-fold activation of ERK2 by 1 μM LPA (data not shown). These results demonstrate that Edg2 can mediate LPA-induced ERK2 activation.

The PH Domain and SHP2 Binding Site in Gab1 Are Required for Gab1 to Transmit the Activation Signal from Edg2 to ERK2—To determine whether Gab1 is involved in Edg2-mediated ERK2 activation in response to LPA, HEK293 cells were co-transfected with expression plasmids for Edg2, HA-ERK2, and one of the Gab1 constructs indicated in Fig. 6. Cells were serum-starved and treated with LPA or mock-treated with BSA. ERK2 kinase activity was determined as above after immunoprecipitation of HA-ERK2. In three independent experiments, LPA activated ERK2 4.5-fold in HEK293 cells transfected with Edg2 (Fig. 6A). Expression of Gab1FL increased LPA-stimulated ERK2 activation to 11.3-fold. Thus, expression of the exogenous Gab1 enhanced Edg2-mediated ERK2 activation by 2.5-fold in these cells, indicating the involvement of Gab1 in signal transduction from Edg2 to ERK2. Removal of the PI3K binding sites in Gab1 (Gab1ΔP13K) did not prevent the potentiating effect of Gab1 on LPA-induced ERK2 activation. In contrast, the effect of Gab1 in enhancing LPA-stimulated ERK2 activation was lost if the SHP2 binding site was mutated (Y627F) or if the PH domain was deleted (Fig. 6). To further confirm that the Y627F mutation in Gab1 eliminates the LPA-stimulated SHP2 binding activity in HEK293 cells, we co-expressed Gab1FL or Gab1Y627F with SHP2 and Edg2 in HEK293 cells and analyzed the association of SHP2 with Gab1FL and Gab1Y627F in cells stimulated with or without LPA. As shown in Fig. 7, LPA induced the binding of SHP2 to Gab1FL but not to Gab1Y627F. Together, these data indicate that Gab1 mediates signal transduction from Edg2 to ERK2 and that this function of Gab1 requires its PH domain and SHP2 binding activity.

The Protein-tyrosine Phosphatase Activity of SHP2 Is Required for LPA-induced ERK2 Activation—Several previous studies have shown that SHP2 phosphatase activity is required for MAP kinase activation by growth factors and cytokines such as EGF, insulin, interleukin-6, and interleukin-3 (5, 8–11, 38). To determine whether the tyrosine phosphatase activity of SHP2 is also required for Edg2-mediated ERK2 activation, we...
were co-transfected with 1.5\mu g of pEdg2 and 0.5\mu g of pGab1FL or pGab1Y627F. After serum starvation, cells were treated for 4 min with LPA (25\muM) or BSA. Gab1FL and Gab1Y627F were immunoprecipitated with an anti-SHP2 antibody. The immunoprecipitates were analyzed by immunoblotting with an anti-SHP2 antibody (A) or an anti-Gab1 antibody (B).

transiently expressed either the wild-type SHP2 or a phosphatase-inactive SHP2 (SHP2\text{cat}) with Edg2 and HA-ERK2 in HEK293 cells (Fig. 8A). Alternatively, COS-7 cells were co-transfected with HA-ERK2 and either SHP2\text{cat} or the empty vector (pcDNA3.1). Transfected cells were serum-starved and treated with LPA, and the activity of HA-ERK2 was assayed. As illustrated in Fig. 8A, expression of the wild-type SHP2 had little effect on Edg2-mediated ERK2 activation in response to LPA, whereas expression of SHP2\text{cat} inhibited LPA-induced ERK2 activation by an average of 72% in two experiments. Fig. 8B shows that the expression of SHP2\text{cat} also blocked LPA-induced ERK2 activation in COS-7 cells.

**DISCUSSION**

The association of SHP2 with Gab1 has been detected in cells treated with various growth factors and cytokines (2, 4, 5, 7). However, the role of the Gab1-SHP2 interaction in cell signaling has not been characterized previously. Tyr-627 in Gab1 has been identified as the SHP2 binding site in insulin-stimulated PI3K. This result clearly indicates that Gab1 is a SHP2 substrate in vivo, and dephosphorylation of a specific phosphotyrosine residue in Gab1 by SHP2 produces a positive signal. Indeed, SHP2 can dephosphorylate Gab1\text{FL} (27). However, comparison of tyrosine phosphorylation of Gab1Y627F with Gab1FL in EGF-stimulated cells by immunoblotting indicated that the amounts of phosphotyrosine in Gab1Y627F were similar or slightly less than that in Gab1FL (data not shown). These observations suggest that SHP2 does not dephosphorylate Gab1\text{FL} as observed in the *in vitro* experiment (27). Therefore, a more laborious phosphopeptide mapping experiment is required to evaluate whether SHP2 dephosphorylates a specific site in Gab1. Nevertheless, the hypothesis that SHP2 dephosphorylates a specific Gab1 tyrosine residue to produce a positive downstream signal would predict that phosphorylation of this tyrosine residue is inhibitory and that removal of this residue will release such inhibition. Interestingly, in a recent study of Gab2 (38), which is a protein similar to Gab1, two tyrosine residues corresponding to Tyr-627 and Tyr-659 of Gab1 (Tyr-604 and Tyr-633 in Gab2) were simultaneously mutated to assess the effect of blocking SHP2 binding to Gab2 in interleukin-3 signaling. It was found that the double mutations not only did not inhibit ERK1 activation but enhanced ERK1 activation in response to interleukin-3. This result is consistent with our hypothesis if Tyr-633 of Gab2 is in fact an inhibitory site.

In contrast to the SHP2 binding site mutant, expression of Gab1\text{FL} enhanced the LPA-induced ERK2 activation (Fig. 6). A similar effect was also observed with Gab1\DeltaP13K. This result clearly illustrates that Gab1-SHP2 interaction exerts a positive signaling role, one possibility is that Gab1 is a SHP2 substrate in vivo, and dephosphorylation of a specific phosphotyrosine residue in Gab1 by SHP2 produces a positive signal. Indeed, SHP2 can dephosphorylate Gab1\text{FL} (27). However, comparison of tyrosine phosphorylation of Gab1Y627F with Gab1FL in EGF-stimulated cells by immunoblotting indicated that the amounts of phosphotyrosine in Gab1Y627F were similar or slightly less than that in Gab1FL (data not shown). These observations suggest that SHP2 does not dephosphorylate Gab1\text{FL} as observed in the *in vitro* experiment (27). Therefore, a more laborious phosphopeptide mapping experiment is required to evaluate whether SHP2 dephosphorylates a specific site in Gab1. Nevertheless, the hypothesis that SHP2 dephosphorylates a specific Gab1 tyrosine residue to produce a positive downstream signal would predict that phosphorylation of this tyrosine residue is inhibitory and that removal of this residue will release such inhibition. Interestingly, in a recent study of Gab2 (38), which is a protein similar to Gab1, two tyrosine residues corresponding to Tyr-627 and Tyr-659 of Gab1 (Tyr-604 and Tyr-633 in Gab2) were simultaneously mutated to assess the effect of blocking SHP2 binding to Gab2 in interleukin-3 signaling. It was found that the double mutations not only did not inhibit ERK1 activation but enhanced ERK1 activation in response to interleukin-3. This result is consistent with our hypothesis if Tyr-633 of Gab2 is in fact an inhibitory site.

In contrast to the SHP2 binding site mutant, expression of Gab1\DeltaP13K had no detectable effect on ERK2 activation by LPA and EGF. In HEK293 cells, expression of Gab1FL enhanced the LPA-induced ERK2 activation (Fig. 6). A similar effect was also observed with Gab1\DeltaP13K. This result clearly illustrates that Gab1-SHP2 interaction exerts a positive signaling role, one possibility is that Gab1 is a SHP2 substrate in vivo, and dephosphorylation of a specific phosphotyrosine residue in Gab1 by SHP2 produces a positive signal. Indeed, SHP2 can dephosphorylate Gab1\text{FL} (27). However, comparison of tyrosine phosphorylation of Gab1Y627F with Gab1FL in EGF-stimulated cells by immunoblotting indicated that the amounts of phosphotyrosine in Gab1Y627F were similar or slightly less than that in Gab1FL (data not shown). These observations suggest that SHP2 does not dephosphorylate Gab1\text{FL} as observed in the *in vitro* experiment (27). Therefore, a more laborious phosphopeptide mapping experiment is required to evaluate whether SHP2 dephosphorylates a specific site in Gab1. Nevertheless, the hypothesis that SHP2 dephosphorylates a specific Gab1 tyrosine residue to produce a positive downstream signal would predict that phosphorylation of this tyrosine residue is inhibitory and that removal of this residue will release such inhibition. Interestingly, in a recent study of Gab2 (38), which is a protein similar to Gab1, two tyrosine residues corresponding to Tyr-627 and Tyr-659 of Gab1 (Tyr-604 and Tyr-633 in Gab2) were simultaneously mutated to assess the effect of blocking SHP2 binding to Gab2 in interleukin-3 signaling. It was found that the double mutations not only did not inhibit ERK1 activation but enhanced ERK1 activation in response to interleukin-3. This result is consistent with our hypothesis if Tyr-633 of Gab2 is in fact an inhibitory site.

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demonstrates that binding of PI3K to Gab1 is not required for LPA-induced ERK2 activation in these cells. On the other hand, two observations suggest that PI3K appears to function upstream of Gab1 in LPA-stimulated cells. First, the effect of Gab1 on the augmentation of LPA-induced ERK2 activation requires its PH domain (Fig. 6). Previous studies have suggested that the Gab1 PH domain can bind PI3K products (18, 19). Second, we have observed that the PI3K inhibitor wortmannin blocks LPA-induced Gab1 tyrosine phosphorylation (data not shown).

We have discovered that LPA does not activate ERK2 in a HEK293 cell line (Fig. 5). The responsible defect is currently unknown but is likely at the receptor level because expression of Edg2 restores the LPA response. Nevertheless, this cell line provides a model for us to evaluate the Edg2-mediated MAP kinase pathway. Significantly, expression of the wild-type Gab1 enhances Edg2-mediated ERK2 activation by LPA by 2.5-fold. The SHP2 binding site mutation and the PH domain deletion mutation of Gab1, but not the PI3K binding site mutation of Gab1, lose the effect of the wild-type Gab1. Together with the observation that Gab1Y627F inhibits LPA-induced ERK2 activation in COS-7 cells, a role for Gab1 in the Edg2-mediated MAP kinase pathway in response to LPA is clearly established. Furthermore, expression of a phosphatase-inactive SHP2 blocks the Edg2-mediated ERK2 activation by LPA (Fig. 8). These results demonstrate a positive signaling role for SHP2 tyrosine phosphatase in the LPA-stimulated MAP kinase pathway.

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