Regulation of the Mitogen-activated Protein Kinase Signaling Pathway by SHP2*

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Gab1-SHP2 association is required for Erk mitogen-activated protein kinase activation by several growth factors. Gab1-SHP2 interaction activates SHP2. However, an activated SHP2 still needs to associate with Gab1 to mediate Erk activation. It was unclear whether SHP2 is required to dephosphorylate a negative phosphorylation site on Gab1 or whether SHP2 needs the Gab1 pleckstrin homology (PH) domain to target it to the plasma membrane. We found that expression of a fusion protein consisting of the Gab1 PH domain and an active SHP2 (Gab1PH-SHP2ΔN) induced constitutive Mek1 and Erk2 activation. Linking the active SHP2ΔN to the PDK1 PH domain or the FRS2β myristoylation sequence also induced Mek1 activation. Mek1 activation by Gab1PH-SHP2ΔN was inhibited by an Src inhibitor and by Csk. Significantly, Gab1PH-SHP2ΔN induced Src activation. Gab1PH-SHP2ΔN expression activated Ras, and the Gab1PH-SHP2ΔN-induced Mek1 activation was blocked by RasN17. These findings suggest that Gab1PH-SHP2ΔN activated a signaling step upstream of Src and Ras. The SHP2 tyrosine phosphatase activity is essential for the function of the fusion protein. Together, these data show that the Gab1 sequence, besides the PH domain and SHP2 binding sites, is dispensable for Erk activation, suggesting that the primary role of Gab1 association with an activated SHP2 is to target it to the membrane.

In response to epidermal growth factor (EGF),1 EGF receptor autophosphorylates on tyrosine residues and recruits the Grb2-Sos1 complex to turn on the membrane-associated Ras, which then activates the Raf-Mek1/2-Erk2 kinase cascade (1). Although this EGF signaling pathway is well known, it does not completely account for the mechanism of Erk activation by EGF. Several laboratories have reported that the protein tyrosine phosphatase (PTPase) activity of SHP2 is required for Erk activation by EGF and other growth factors (2–6). However, because the SHP2 substrate that controls Erk activation has not been identified, the mechanism for the requirement of SHP2 PTPase activity in Erk activation remains unexplained.

SHP2 is a PTPase with two Src homology-2 (SH2) domains (N-SH2 and C-SH2) located in the N-terminal region and two potential Grb2 SH2 domain binding sites located in the C-terminal region (see Fig. 1) (7). SHP2 is basally inactive due to auto-inhibition by its N-SH2 domain. It can be activated by binding to tyrosine-based activation motifs or by deletion of the N-SH2 domain (8). A major SHP2-binding protein in growth factor-stimulated cells is Gab1 (9–13). Gab1 is a multisite docking protein that contains an N-terminal pleckstrin homology (PH) domain, multiple tyrosine phosphorylation sites, and two Grb2 SH3 domain binding sites (see Fig. 1). Gab1, as well as SHP2, becomes tyrosine-phosphorylated in cells stimulated with EGF and other growth factors (5, 9, 11, 13). Tyrosine-phosphorylated Gab1 binds SHP2, phosphoinositide 3-kinase, Shc, CrkL, and possibly other signaling proteins (9–14). We recently found that Tyr-627 and Tyr-659 of Gab1 constitute a biphosphoryl tyrosine-based activation motif that mediates binding and activation of SHP2 in EGF-stimulated cells (15). Furthermore, an activated SHP2 must also associate with Gab1 in EGF-stimulated cells to permit Erk activation (15).

However, it was unclear why physical association of an activated SHP2 with Gab1 is required for Erk activation by EGF. Interestingly, expression of a chimeric protein consisting of an SHP2-binding-defective Gab1 and an N-SH2 domain deletion mutant of SHP2 (Gab1FF-SHP2ΔN) induced Erk2 activation (15). This observation suggests either that SHP2 dephosphorylates a negative phosphorylation site on Gab1 to activate Erk MAP kinase or that SHP2 needs Gab1 to bring the activated PTPase to the proximity of its substrate.

In this study, we demonstrate that Gab1 sequence, besides the PH domain, is dispensable for Mek1-Erk2 activation by the Gab1-SHP2 chimera. Furthermore, linking the active SHP2 to the PDK1 PH domain or to the FRS2β/SNT-2 myristoylation sequence can also cause constitutive activation of the Erk MAP kinase signaling pathway. Significantly, expression of the Gab1 PH domain-active SHP2 chimera (Gab1PH-SHP2ΔN) activates c-Src and Ras, and inhibition of c-Src or Ras blocks Mek1 activation by Gab1PH-SHP2ΔN. This finding suggests that the primary role of Gab1 association with an activated SHP2 in the Erk activation pathway is to target the PTPase to the membrane to activate a signaling step upstream of Src and Ras.

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DNA Constructs—The following constructs have been described (13, 15): HA-tagged Erk2 and N-SH2 domain deletion mutant of SHP2 (SHP2ΔN); FLAG-tagged Gab1, Gab1 with Tyr-to-Phe mutations at amino acids 627 and 659 (Gab1FF), and Gab1FF-SHP2ΔN fusion protein. A plasmid for HA-tagged Mek1 was provided by Dr. Michael Weber (see Ref. 16). H-RasN17 was provided by Dr. Richard Jove (see Ref. 17). HA-tagged H-Ras, H-RasN17, and H-RasV12 were obtained from Guthrie cDNA Resource Center. GST-Raf-RBD fusion protein was prepared as described (18). The Grb2 expression vector was constructed by subcloning a 2.3-kb EcoRI-XhoI fragment from pKL12 (19) into pCDNA3.1. A Ser-17 to Asn mutant of Rap1A (Rap1AN17) was made by polymerase chain reaction from a human Rap1A cDNA (American Type Culture Collection) and cloned into pCDNA3.1. A HA-tagged c-Src expression vector was made by subcloning the human c-src coding sequence into a pCDNA3-HA vector.

A plasmid for expression of a fusion protein consisting of the Gab1 PH domain and SHP2ΔN (Gab1PH-SHP2ΔN) was made from Gab1FF-SHP2ΔN by deleting the coding sequence for amino acids 177–625 of Gab1 (see Fig. 1). A plasmid for the PTPase-inactive Gab1PH-SHP2ΔN-CS was made from Gab1PH-SHP2ΔN by mutating Cys-463 of SHP2 to Ser. Gab1PH-SHP2ΔN-acat was made by replacing the SHP2ΔN coding sequence in Gab1PH-SHP2ΔN with the corresponding sequence from SHP2-acat (13), which has a deletion of a 29-amino acid coding sequence in the SHP2 catalytic site. Similarly, Gab1PH-SHP2ΔN-FF was prepared from Gab1PH-SHP2ΔN by replacing the SHP2ΔN coding sequence with the corresponding sequence from an SHP2ΔN with double Tyr-to-Phe mutations at Tyr-546 and Tyr-584 (provided by Dr. Lin Mei).

The coding sequence for amino acids 423–559 of PDK1 was amplified from mouse PDK1 cDNA (American Type Culture Collection) (20, 21) with codons for PDK1 Asp-221 and Leu-422 (GATTGG) changed to ACCATG to create an optimal translation start site. The DNA fragment was used to replace the Gab1 PH domain coding sequence in Gab1PH-SHP2ΔN to generate a plasmid for PDK1PH-SHP2ΔN (see Fig. 1). A plasmid for myristoylated SHP2ΔN (myr-SHP2ΔN) was made by replacing the Gab1 PH domain coding sequence in Gab1PH-SHP2ΔN with the corresponding sequence for amino acids 1–26 of FR35β (22), which includes a coding sequence for the N-terminal myristoylation site (MG-SCCSC) (see Fig. 1). All constructs were confirmed by DNA sequencing.

Cell Culture and Transfection—HEK293 and COS-7 cells were grown in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (FCS). Transfection was performed with LipofectAMINE (Invitrogen). Typically, cells grown in 60-mm plates were incubated with DNA (2 μg of total)-LipofectAMINE (12 μl) complexes in serum-free medium for 5 h. The DNA-Lipofectamine complexes were then added to the medium. The cells were starved in DMEM/0.1% FCS for 20 h and then used for experiments.

The PathDetect in vivo signal transduction pathway trans-reporting systems for Elk1, c-Jun, and CHOP were obtained from Stratagene (23). For PathDetect trans-reporting assays, cells in 6-well plates were transfected with 0.525 μg of DNA for Gab1PH-SHP2ΔN or empty vector along with 0.0325 μg of trans-activator construct (pFA2-Elk1, pFA2-c-Jun, or pFA-CHOP), 0.325 μg of pFR-luc reporter, and 0.05 μg of pCMV-βgal. pCDNA3.1FG was used to equalize the DNA content in each transfection to 1 μg of total DNA. After transfection, cells were incubated in DMEM/0.1% FCS for 20 h and then processed for determination of luciferase and β-galactosidase activity (24). The luciferase assay was then normalized to β-galactosidase activity as an internal control for transfection efficiency.

Immunoprecipitation and Immunoblotting—Cells were lysed in buffer A (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 25 mM EGTA, 25 mM NaF, 5 mM Na3P04, 1% Triton X-100, 1 mM Na3VO4, 2 μg/ml leupeptin, 2 μg/ml aprotinin, and 100 μg/ml phenylmethylsulfonyl fluoride). Immunoprecipitation and immunoblotting were performed essentially as described previously (13, 15, 24).

Kinase Assays—Cells were lysed in buffer A containing 1 mM dithiothreitol and 20 mM p-nitrophenyl phosphate. HA-Mek1, HA-Erk2, or HA-Src were immunoprecipitated with a monoclonal antibody against the HA tag (Covance). Mek1 kinase activity was determined by an immune complex kinase assay with [γ-32P]ATP using a recombinant, kinase-defective Erk2 (KR) as substrate. Erk2 activity was determined in the immune complex with [γ-32P]ATP using myelin basic protein as substrate. Src kinase activity was determined in the presence of [γ-32P]ATP by autophosphorylation and by the use of acid-denatured enolase as exogenous substrate. Detailed conditions for these kinase assays have been given (15, 24, 25). Phosphorylation was quantified with a PhosphoImager.

Ras Activation Assay—Active Ras in transfected cells was detected by means of Ras-GTP bound to a GST fusion protein of the Ras-GTP binding domain of Raf fragment (GST-Raf-RBD) (18) followed by immunoblotting with an anti-HA-tag antibody.

Preparation of Membrane and Cytosolic Fractions—COS-7 cells in 60-mm plates were transfected with the plasmid for Gab1PH-SHP2ΔN, PDKPH-SHP2ΔN, or myr-SHP2ΔN. Transfected cells were starved for 20 h in 0.1% FCS. After washing twice with phosphate-buffered saline, cells were scraped into 1 ml of hypotonic buffer (20 mM HEPES, pH 7.4, 10 mM NaCl, 1.5 mM MgCl2, 0.5 mM Na3VO4, 2 μg/ml leupeptin, and 100 μg/ml phenylmethylsulfonyl fluoride) and incubated on ice for 15 min. The cells were homogenized by passage through a 21-gauge needle. Nuclei and whole cells were removed by centrifugation at 1,000 × g for 3 min. Membranes were isolated from the cleared lysates by centrifugation at 100,000 × g for 60 min at 4 °C in a tabletop ultracentrifuge (Beckman). The supernatants were retained (cytosolic fraction), and the pellets were resuspended in hypotonic buffer (membrane fraction) (26).

RESULTS

Constitutive Activation of Mek1 and Erk2 by a Gab1 PH Domain-active SHP2 Fusion Protein—SHP2 PTPase activity is required for Erk MAP kinase activation by EGF and several other growth factors. We found previously that Gab1-SHP2 association resulted in activation of SHP2 PTPase (15). How- ever, an activated SHP2 still must associate with Gab1 in order to mediate activation of the Erk MAP kinase pathway (15). It was found that expression of a fusion protein consisting of an SHP2-binding-defective Gab1 (mutations of Tyr-627 and Tyr-659 to Phe) and an active SHP2 (Gab1FF-SHP2ΔN) induced Erk2 activation in COS-7 cells (15). Gab1 contains several known tyrosine phosphorylation sites and Grb2 SH2 domain binding sites between the PH domain and Tyr-627 (Fig. 1) (27, 28). To determine whether these docking sites are essential for Erk activation by the fusion protein, we deleted the region between the Gab1 PH domain and Phe-627 in Gab1FF-SHP2ΔN to generate Gab1PH-SHP2ΔN (Fig. 1). Gab1PH-SHP2ΔN and HA-Erk2 were co-expressed in HEK293 and COS-7 cells, and Erk2 activity was determined (Fig. 2). Consistent with previous observations, Gab1FF-SHP2ΔN activated SHP2-mediated Erk activation in this study. For Gab1, the PH domain, eight known phosphorylation sites by EGF receptor tyrosine kinase, two Pro-based Grb2 C-SH3 domain binding sites, and the binding sites for phosphoinositide 3-kinase (PI3K) and SHP2 are shown. For SHP2, the tandem SH2 domains, PTPase domain, and two potential tyrosine phosphorylation sites/Grb2 binding sites are shown. All three chimeric proteins contain Phe-627/Phe-659 of Gab1 in the Gab1-derived sequence. G, a three-residue glycine linker; M, myristoylation signal; F, FLAG tag; Numbers in italics indicate Gab1 amino acid residue numbers. Proteins are depicted to scale.
Erk2 in these cells (Fig. 2, B–E). Interestingly, expression of Gab1PH-SHP2ΔN resulted in constitutive Erk2 activation to a higher extent than Gab1FF-SHP2ΔN. Similarly, comparison of Mek1 activity in COS-7 cells transfected with Gab1PH-SHP2ΔN and Gab1FF-SHP2ΔN showed that the constitutive Mek1 activity was about 2-fold higher in cells transfected with the Gab1 PH domain fusion protein than the full-length Gab1 fusion protein (Fig. 2, F and G). Therefore, the Gab1 sequence between the PH domain and Tyr-627 is not required for Erk2 or Mek1 activation by the fusion protein.

Because constitutive Erk2 activation appeared to up-regulate expression of the exogenous HA-Erk2 but not HA-Mek1 and because Mek1 activation correlates with Erk2 activation in the cells, we used Mek1 activity as a measure for activation of the Mek1-Erk2 signaling pathway in subsequent experiments. To determine whether constitutive Mek1 activation by Gab1PH-SHP2ΔN is affected by the amount of serum in the medium, transfected cells were incubated for 20 h in DMEM containing 0, 0.1, or 10% FCS. A, averages and ranges of Mek1 activity obtained from 2 independent experiments. Cells were left untreated (lanes 1–6) or stimulated with 10% FCS for 5 min (lanes 7–8). HA-Mek1 was immunoprecipitated. Mek1 kinase activity (B) and protein (C) in the immunoprecipitates were then determined.

**Linking an Active SHP2 to the PDK1 PH Domain or the FRS2β Myristoylation Sequence Also Induces Constitutive Activation of Mek1**—Data presented in Figs. 2 and 3 indicate that linking an active SHP2 to the Gab1 PH domain is sufficient to induce constitutive Mek1-Erk2 activation. The Gab1 PH domain is known to target Gab1 to the plasma membrane (29). We therefore tested whether targeting an active SHP2 to the plasma membrane through other sequences could induce Mek1 activation. For this purpose, we replaced the Gab1 PH domain sequence in Gab1PH-SHP2ΔN with the PDK1 PH domain sequence to produce the myr-SHP2ΔN construct (Fig. 1). The PDK1 PH domain binds phosphoinositides with high affinity (21). FRS2 is a fibroblast growth factor receptor substrate that binds SHP2 in response to fibroblast growth factor and nerve growth factor (30).

Like Gab1PH-SHP2ΔN, PDK1PH-SHP2ΔN and myr-SHP2ΔN induced constitutive Mek1 activation, whereas SHP2ΔN had no effect (Fig. 4, A–C). These data indicate that the Gab1 PH domain can be replaced with the PDK1 PH domain or the FRS2β myristoylation sequence for Mek1 activation. Fig. 4D shows that Gab1PH-SHP2ΔN and PDK1PH-SHP2ΔN were found in both membrane and cytosolic fractions,
and protein (C) were then determined from the immunoprecipitates.

COS-7 cells were transfected with plasmids for Gab1PH-SHP2N, PDK1PH-SHP2 (G-PH), and P-PH, whereas co-transfection of cells with Csk and Gab1PH-SHP2N plasmid. Fig. 5, shows that incubation of cells with PP1 resulted in Src activation, but expression of Gab1PH-SHP2N in COS-7 cells was transfected with Gab1PH-SHP2N (0.2 μg) and Gab1PH-SHP2AN (0.6 μg) together with Csk (0.6 μg) or pcDNA3.1FG as indicated and then incubated in DMEM/0.1% FCS in the presence or absence of the Src inhibitor PP1 (10 μM) for 20 h. HA-Mek1 activity (A and B) and protein (C and E) were then determined after immunoprecipitation with an anti-HA antibody. D-G, COS-7 cells were transfected with the indicated amounts of Gab1PH-SHP2AN, Gab1PH-SHP2AN-CS, and Src. Total DNA in each transfection was kept at 2 μg by the use of pcDNA3.1FG. Twenty-four h after transfection, cells were serum-starved in DMEM/0.1% FCS for 20 h. HA-Src was immunoprecipitated. One-half of each immunoprecipitate was used for Src kinase assay (D and F), and the other half of each immunoprecipitate was used for immunoblotting analysis of Src protein using a monoclonal anti-Src antibody (N2–17) (E and G).

![Fig. 5. Involvement of Src tyrosine kinase in Gab1PH-SHP2AN-induced Mek1 activation.](image)

Expression of Gab1PH-SHP2AN Activates Src Tyrosine Kinase—To assess the possibility that Src tyrosine kinase may be involved in the Gab1PH-SHP2AN-induced Mek1 activation pathway, we incubated the transfected cells with an Src tyrosine kinase inhibitor, PP1. Alternatively, cells were co-transfected with Csk, which phosphorylates and inactivates c-Src. Fig. 5, A–C, shows that incubation of cells with PP1 resulted in 85% inhibition of Mek1 activation by Gab1PH-SHP2AN, whereas co-transfection of cells with Csk and Gab1PH-SHP2AN led to 61% inhibition of Mek1 activation by Gab1PH-SHP2AN. These results suggest that Src tyrosine kinase activity is involved in the Gab1PH-SHP2AN-induced Mek1 activation pathway.

To determine whether Gab1PH-SHP2AN expression resulted in Src activation, we assayed Src tyrosine kinase activity in cells transfected with different amounts of Gab1PH-SHP2AN plasmid. Fig. 5, D and E, shows that a low Src tyrosine kinase activity was detected in control cells that have been transfected with the empty vector. Both autophosphorylation and substrate phosphorylation activities of Src tyrosine kinase were increased with increasing amounts of Gab1PH-SHP2AN plasmid used in transfection. To evaluate whether the SHP2 PTPase activity is involved in Gab1PH-SHP2AN-induced Src activation, we compared Src tyrosine kinase activity in cells transfected with Gab1PH-SHP2AN and a PTPase-inactive construct of Gab1PH-SHP2AN (Gab1PH-SHP2AN-CS, see below). As shown in Fig. 5, F and G, expression of Gab1PH-SHP2AN resulted in Src activation, but expression of Gab1PH-SHP2AN-CS did not lead to Src activation. Thus, the SHP2 PTPase activity is required for Src activation by Gab1PH-SHP2AN.

Activation of Mek1 by the SHP2AN Fusion Protein Is Ras-dependent—To determine whether Gab1PH-SHP2AN causes Mek1 activation through a Ras-dependent mechanism, we co-expressed dominant negative RasN17 or Rap1AN17 with Gab1PH-SHP2AN and HA-Mek1 in COS-7 cells. As shown in Fig. 6, A and B, RasN17 completely blocked Mek1 activation by Gab1PH-SHP2AN, whereas Rap1AN17 only slightly affected Mek1 activation by Gab1PH-SHP2AN. Therefore, Gab1PH-SHP2AN-mediated Mek1 activation is Ras-dependent.

To measure Ras activation, we employed the GST-Raf-RBD fusion protein pull-down assay (18). Fig. 6, C and D, shows that the constitutively active RasV12 was bound to GST-Raf-RBD, whereas the constitutively inactive RasN17 was not, validating the methodology. Control cells transfected with pcDNA3.1FG (empty vector) had a small amount of GST-Raf-RBD-bound wild-type Ras. Transfection of COS-7 cells with Gab1PH-SHP2AN resulted in an average of a 3-fold (n = 4) increase of the wild-type Ras bound to GST-Raf-RBD (Fig. 6, C and D). This result indicates that expression of Gab1PH-SHP2AN induces Ras activation.

Gab1PH-SHP2AN Selectively Activates the Elk1 trans-Reporter—At least three distinct MAP kinases (Erk, Jnk, and p38) are downstream of Ras (31). Erk, Jnk, and p38 selectively phosphorylate transcriptional factors Elk1, c-Jun, and CHOP, respectively.
expression of Gab1PH-SHP2
N resulted in a 16-fold activation/Hz004
vates one or more of these reporter activities. Fig. 7 shows that c-Jun, and CHOP, we tested whether Gab1PH-SHP2
N acti-
tcation with an anti-HA antibody.

A
B
C
D
- - - - 0.45 0.9 - - - - RasN17
- - - - - - 0.45 0.9 Rap1AN17
- 0.9 0.9 0.9 0.9 0.9 0.9 Gab1PH-SHP2ΔN
1.8 0.9 0.45 - 0.45 - pCDNA3.1FG
0.2 0.2 0.2 0.2 0.2 HA-Mek1

- - - - 1.6 Gab1PH-SHP2ΔN
1.6 1.6 1.6 - pCDNA3.1FG
0.4 - - - HA-RasV12
- 0.4 - HA-RasN17
- - 0.4 0.4 HA-Ras-WT

- - - - HA-Mek1

- - KR

- Active HA-Ras

- HA-Ras

FIG. 6. Gab1PH-SHP2ΔN-induced Mek1 activation is Ras-de-
pendent. A and B, COS-7 cells were transfected with the indicated plasmids and then incubated in DMEM/0.1% FCS for 20 h. HA-Mek1 activity (A) and protein (B) were then determined after immunoprecipitation with an anti-HA antibody. C and D, COS-7 cells were transfected with 2 μg of DNA consisting of the indicated plasmids and pCDNA3.1FG. Transfected cells were serum-starved, and a GST-Raf-RBD pull-down assay was performed followed by immunoblotting detection of HA-tagged Ras (C). D, an aliquot of each cell lysate supernatant was analyzed by immunoblotting with an anti-HA-antibody.

respectively, leading to activation of their transcriptional activity. Using the PathDetect trans-reporting systems for Elk1, c-Jun, and CHOP, we tested whether Gab1PH-SHP2ΔN activates one or more of these reporter activities. Fig. 7 shows that expression of Gab1PH-SHP2ΔN resulted in a 16-fold activation of the Elk1-trans-reporter activity, confirming activation of the Mek1-Erk2 pathway by Gab1PH-SHP2ΔN. The increases in c-Jun-trans-reporter and CHOP-trans-reporter activities by Gab1PH-SHP2ΔN were marginal. Thus, Gab1PH-SHP2ΔN selectivity activates the Erk2-Elk1 signaling pathway.

Roles of Grb2 Binding and PTPase Activities of SHP2 in Mek1 Activation by Gab1PH-SHP2ΔN—To determine the mechanism by which Gab1PH-SHP2ΔN causes Mek1 activation, we prepared three mutants of Gab1PH-SHP2ΔN. Gab1PH-SHP2ΔN-CS has a Cys-to-Ser mutation in the catalytic Cys-463 of rat SHP2 PTPase (5). Gab1PH-SHP2ΔN has two Tyr-to-Phe mutations in the two potential Grb2 binding sequences. These results indicate that the primary function of Gab1PH-SHP2ΔN, but they are not essential. In contrast, SHP2 PTPase activity is required for Mek1 activation by the fusion protein.

To determine whether Grb2 is associated with Gab1PH-

SHP2-2AN and whether Tyr-546 and Tyr-584 of SHP2 mediate this association, COS-7 cells were transfected with plasmids for Gab1PH-SHP2ΔN or Gab1PH-SHP2ΔN-FF together with a Grb2 expression vector (Fig. 9). Grb2 immunoprecipitates from these cells were then analyzed by immunoblotting for the presence of Gab1PH-SHP2ΔN and Gab1PH-SHP2ΔN-FF (Fig. 9). Fig. 9, A and C, shows that Grb2 and the two fusion proteins were expressed in transfected cells. Fig. 9B shows that Gab1PH-SHP2ΔN but not Gab1PH-SHP2ΔN-FF was detected in the Grb2 immunoprecipitates.

Tyrosine phosphorylation of Gab1, Gab1FF-SHP2ΔN, and Gab1PH-SHP2ΔN in COS-7 cells was weak but detectable after long exposure of the film, whereas no tyrosine phosphorylation was found in Gab1PH-SHP2ΔN-FF (Fig. 9D). This result suggests that Tyr-546 and/or Tyr-584 were weakly phosphorylated in the cells and therefore could serve as the Grb2 docking sites.

**DISCUSSION**

SHP2 association with Gab1 is required for Erk activation by EGF (15). The activated SHP2 may dephosphorylate a specific negative phosphorylation site on Gab1 or on a Gab1-associated protein. Alternatively, the activated SHP2 may require the Gab1 PH domain to translocate it to the plasma membrane to dephosphorylate a membrane-associated substrate. The data presented in this study illustrate that physical association of an activated SHP2 with the Gab1 PH domain is sufficient to activate the Erk MAP kinase pathway. Furthermore, the Gab1 PH domain can be replaced with other membrane-targeting sequences. These results indicate that the primary function of Gab1 association with an activated SHP2 in Erk MAP kinase activation is for the Gab1 PH domain to target the activated SHP2 to the membrane rather than for SHP2 to dephosphorylate a specific phosphorylation site on Gab1. In agreement with this conclusion, it was found that SHP2 binding to another...
membrane-anchored docking protein, FRS2, could mediate fibroblast growth factor-stimulated Erk MAP kinase activation (30).

Although the critical SHP2 PTPase substrate remains to be identified, our experiments suggest that it functions upstream of Src and Ras. Mek1 activation by Gab1PH-SHP2ΔN was inhibited by PP1 and Csk, indicating that the Src tyrosine kinase was involved in Mek1 activation by Gab1PH-SHP2ΔN.

Importantly, expression of Gab1PH-SHP2ΔN in COS-7 cells resulted in activation of Src tyrosine kinase in a process that requires the SHP2 PTPase activity. However, it remains to be determined whether Src is a direct substrate of Gab1PH-SHP2ΔN or whether Gab1PH-SHP2ΔN acts on a regulatory step upstream of Src.

Our PathDetect trans-reporting assays suggest that Gab1PH-SHP2ΔN selectively activates the Erk-Elk1 pathway. It is possible that an additional signal is required for Jnk and p38 activation, which is not activated by Gab1PH-SHP2ΔN.

However, a previous study (6) found that SHP2 mutants specifically blocked Erk, but not Jnk, activation by EGF. Thus, selective activation of the Erk-Elk1 signaling pathway by Gab1PH-SHP2ΔN is likely due to specific regulation of the Erk MAP kinase pathway by SHP2.

**Fig. 8.** Effects of SHP2 PTPase and tyrosine phosphorylation site mutations on Mek1 activation by Gab1PH-SHP2ΔN. COS-7 cells were transfected with the indicated constructs (1.8 μg of each) and the HA-Mek1 plasmid (0.2 μg) and then incubated in DMEM/0.1% FCS for 20 h. A, 10 μg of each cell lysate was analyzed by immunoblotting with an anti-FLAG antibody. Arrowhead, a nonspecific reacting band. HA-Mek1 kinase activity (B and D) and protein (C) were determined from HA-Mek1 immunoprecipitates. D, means and standard deviations of Mek1 activity obtained from 3 independent experiments.

**Fig. 9.** Tyr-546 and Tyr-584 of SHP2 mediate Grb2 binding to Gab1PH-SHP2ΔN. A–C, COS-7 cells were transfected with plasmids for Gab1PH-SHP2ΔN plus Grb2 (1 μg of each), Gab1PH-SHP2ΔN-FF plus Grb2 (1 μg of each), or the empty vector (2 μg) and incubated in DMEM/0.1%FCS for 20 h. Grb2 was immunoprecipitated from cellular lysates with a polyclonal anti-Grb2 antibody. The immunoprecipitates were analyzed by immunoblotting (IB) with a monoclonal anti-Grb2 antibody (A) or a monoclonal anti-FLAG antibody (B). The cell lysates were also analyzed by immunoprecipitation with the anti-FLAG antibody (C). D, COS-7 cells were transfected with the indicated plasmids (2 μg of each) and incubated in DMEM/0.1%FCS for 20 h. Cell lysates were subjected to immunoprecipitation with an anti-FLAG antibody. The immunoprecipitates (IP) were then analyzed by immunoblotting with an anti-phosphotyrosine antibody (RC20). Arrowhead, Gab1PH-SHP2ΔN or Gab1PH-SHP2ΔN-FF.

Our SHP2 mutation experiments suggest that targeting Grb2 to the membrane contributes to Erk activation by the Gab1PH-SHP2ΔN fusion protein. However, targeting Grb2 to the membrane by Gab1PH-SHP2ΔN, similar to the recruitment of Grb2 to autophosphorylated EGF receptor in EGF-stimulated cells, does not appear to be sufficient for Erk activation. In both cases, SHP2 PTPase activity is required. This observation reinforces the significance of SHP2 PTPase activity in the regulation of Erk activation. Activation of Mek1 and Erk2 by SHP2ΔN fusion proteins is constitutive, indicating that membrane-associated SHP2ΔN is able to bypass the signal desensitization mechanism. Interestingly, cells grown in 10% FCS, which is able to induce Mek1 activation, do not appear to have constitutive Mek1 activity. Therefore, our observation that membrane-associated SHP2ΔN can cause constitutive Mek1 activation suggests that the SHP2 substrate plays a very important role in preventing constitutive Erk activation, which would predictably have undesirable cellular consequences.

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