Phosphotyrosines 627 and 659 of Gab1 Constitute a Bisphosphoryl Tyrosine-based Activation Motif (BTAM) Conferring Binding and Activation of SHP2*

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A major Grb2-associated binder-1 (Gab1) binding partner in epidermal growth factor (EGF)-stimulated cells is protein-tyrosine phosphatase (PTPase) SHP2, which contains tandem SH2 domains. The SHP2 PTPase activity is required for activation of the extracellular signal-regulated kinase (ERK) subfamily of mitogen-activated protein (MAP) kinase by EGF. To investigate the mechanism by which Gab1 and SHP2 mediate ERK activation, we characterized the Gab1-SHP2 interaction. We found that both Tyr-627 and Tyr-659 of Gab1 were required for SHP2 binding to Gab1 and for ERK2 activation by EGF. Far Western blot analysis suggested that the tandem SH2 domains of SHP2 bind to Gab1 in a specific orientation, in which the N-SH2 domain binds to phosphotyrosine (Tyr(P))-627 and the C-SH2 domain binds to Tyr(P)-659. When assayed with peptide substrates, SHP2 PTPase was activated by a bisphosphopeptide containing both Tyr(P)-627 and Tyr(P)-659, but not by monophosphopeptides containing Tyr(P)-627 or Tyr(P)-659 or a mixture of these monophosphopeptides. These results suggest that Tyr(P)-627 and Tyr(P)-659 of Gab1 constitute a bisphosphoryl tyrosine-based activation motif (BTAM) that binds and activates SHP2. Remarkably, while a constitutively active SHP2 (SHP2ΔN) could not rescue the defect of a SHP2-binding defective Gab1 (Gab1FF) in ERK2 activation, expression of a Gab1FF-SHP2ΔN chimera resulted in constitutive activation of ERK2 in transfected cells. Thus, physical association of activated SHP2 with Gab1 is necessary and sufficient to mediate the ERK mitogen-activated protein kinase activation. Phosphopeptides derived from Gab1 were dephosphorylated by active SHP2 in vitro. Consistently, substrate-trapping experiments with a SHP2 catalytic inactive mutant suggested that Gab1 was a SHP2 PTPase substrate in the cells. Therefore, Gab1 not only is a SHP2 activator but also is a target of its PTPase.

SHP2 is a protein-tyrosine phosphatase (PTPase)* with two Src homology-2 (SH2) domains (1, 2). These two SH2 domains, termed N-SH2 and C-SH2 domains, are arranged in tandem at the amino (N)-terminal portion of the protein. SHP2 has a low basal PTPase activity that can be activated by deletion of N-SH2 or both SH2 domains or by specific phosphopeptides that bind to the SH2 domains. The crystal structure of SHP2 shows that the N-SH2 domain is inserted into the catalytic cleft of the phosphatase domain in the absence of a ligand for the N-SH2 domain, thus maintaining the phosphatase in an autoinhibitory state (3). Binding of a specific tyrosine-based activation motif (TAM) to the N-SH2 domain is predicted to induce an allosteric change that disrupts the inhibitory interaction between the N-SH2 and the catalytic domains, leading to phosphatase activation (3). Although there is no direct surface contact between the C-SH2 and the catalytic domains, the C-SH2 domain connects the N-SH2 domain to the PTPase domain and contributes to the selectivity and high affinity binding of the tandem SH2 domains to bisphosphoryl TAMs (4–6).

It has been demonstrated that the SHP2 PTPase activity is required for activation of the extracellular signal-regulated protein kinase (ERK) subfamily of mitogen-activated protein (MAP) kinase by epidermal growth factor (EGF) (7, 8). However, the mechanism by which SHP2 exerts its positive role in ERK MAP kinase activation by EGF is not yet known. The autoinhibitory configuration of the unbound SHP2 necessitates a binding partner for activation of the SHP2 PTPase. Although insulin receptor substrate-1 (IRS-1) and SHP substrate-1 (SHPS-1, also called BIT, SIRPα) can bind and activate SHP2 (5, 6), IRS-1 is not tyrosine phosphorylated in response to EGF (9). Moreover, the interaction between IRS-1 and SHP2 is not required for ERK activation by insulin (10). In fact, interaction between IRS-1 and SHP2 appears to have negative effects on insulin signaling (10). Similarly, it has been reported that SHPS-1 and its interaction with SHP2 play a negative role in the EGF-induced ERK activation pathway (11).

A major SHP2 binding partner in EGF-stimulated cells is Grb2-associated binder-1 (Gab1) (12). Gab1 is a multisite docking protein containing an N-terminal pleckstrin homology (PH) domain and several proline-rich SH3 domain-binding se-

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quencies (12, 13). Upon stimulation of cells with EGF and other growth factors and cytokines, Gab1 becomes phosphorylated on multiple tyrosine residues, which then bind SHP2, phosphatidylinositol 3-kinase, Crkl, and possibly other SH2 domain-containing proteins (12–20). In some cell lines, overexpression of Gab1 enhances ERK MAP kinase activation by EGF (12, 15, 16, 21), lysophosphatidic acid (15), interleukin-6 (14), and hepatocyte growth factor (13). These observations point to Gab1 as a possible physiologically relevant SHP2 binding partner that mediates EGF-stimulated ERK activation.

We previously found that Gab1 with a single mutation at Tyr-627 lost SHP2 binding activity and inhibited EGF-stimulated ERK2 (p42MAPK) activation (15). However, the mechanism by which the Gab1-SHP2 interaction mediates ERK activation has not been elucidated. In the present study, we provide evidence that two tyrosine residues (Tyr-627 and Tyr-659) in the carboxyl (C)-terminal region of Gab1 are both required for SHP2 binding to Gab1 and for EGF-stimulated ERK activation. These two tyrosine residues constitute a bisphosphorylated tyrosine-based activation motif (BTAM) that mediates binding and activation of SHP2 by Gab1. Co-expression of a constitutive active SHP2 (SHP2ΔN) did not rescue the defect of a SHP2 binding-defective Gab1 (Gab1ΔFF), whereas expression of a Gab1FF-SHP2ΔN fusion protein resulted in constitutive activation of ERK2 in COS-7 cells. These data indicate that the activated SHP2 has to be physically associated with Gab1 to mediate EGF-stimulated ERK2 activation and identify Gab1 as the SHP2 activator for the ERK MAP kinase pathway in EGF-stimulated cells. Furthermore, our in vitro dephosphorylation experiments and substrate-trapping study in cultured cells suggest that Gab1 is a substrate of the SHP2 PTase.

EXPERIMENTAL PROCEDURES

Materials—Antibodies were obtained from the following sources: anti-phosphotyrosine (RC20H) from Transduction Laboratories; anti-Gab1 from Upstate Biotechnology; anti-FLAG (M2) from Sigma; anti-HA from Covance; and anti-GST and anti-SHP2 from Santa Cruz Biotechnology. Myelin basic protein and ovalbumin were obtained from Sigma. Pfu DNA polymerase was from Stratagene. GST fusion proteins of SH2 domains (GST-N-SH2 and GST-C-SH2) have been described (22). GST fusion proteins of the full-length (GST-SHP2) and N-SH2 domain deletion (GST-SHP2ΔN) of SHP2 were reported previously (22, 23). The mammalian expression vectors for Gab1 with a Tyr-627 to Phe mutation (Gab1FF) were prepared with the GeneEditor site-directed mutagenesis system (Promega) using pGab1 and the phosphotyrosine-based activation motif (BTAM) that mediates ERK activation. These two tyrosine residues constitute a bisphosphorylated tyrosine-based activation motif (BTAM) that mediates binding and activation of SHP2 by Gab1. Co-expression of a constitutive active SHP2 (SHP2ΔN) did not rescue the defect of a SHP2 binding-defective Gab1 (Gab1ΔFF), whereas expression of a Gab1FF-SHP2ΔN fusion protein resulted in constitutive activation of ERK2 in COS-7 cells. These data indicate that the activated SHP2 has to be physically associated with Gab1 to mediate EGF-stimulated ERK2 activation and identify Gab1 as the SHP2 activator for the ERK MAP kinase pathway in EGF-stimulated cells. Furthermore, our in vitro dephosphorylation experiments and substrate-trapping study in cultured cells suggest that Gab1 is a substrate of the SHP2 PTase.

Preparation of the Gab1 Mutants—Expression vectors for FLAG-tagged Gab1 (pGab1) and pGab1Y627F have been described (15). Expression plasmids for Gab1 with a Tyr-627 to Phe mutation (Gab1ΔFF) and Gab1 with double mutations of both Tyr-627 and Tyr-659 to Phe (Gab1FF) were prepared with the GeneEditor in vitro site-directed mutagenesis system (Promega) using pGab1 and pGab1Y627F, respectively, as templates. The mutagenic primer was 5′-GATGGAGAAGGATGACCTCTGGAAGGATGTCGAC (sense, underlined) and 5′-GATGGAGAAGGATGACCTCTGGAAGGATGTCGAC (antisense). The polymerase chain reaction primers were 5′-GTCCTAGAGGAGGCGGCTGAGGCTCTAGGAAAGGTGTTGCC (sense, un-
of malachite green/Tween 20 solution prepared as described (25). After further incubation for 30 min at room temperature, the absorbance of the mixture at 620 nm (A_{620}) was determined in a half-area, 96-well plate (Costar) with a microplate reader. A standard curve of free phosphate concentrations was prepared using KH$_2$PO$_4$ in conditions identical to the PTPase assay. Although the standard curve was linear between 50 and 4000 pmol of phosphate, a more accurate standard curve was prepared each time in the 25–1000 pmol range.

## RESULTS

### Both Tyrosine 627 and Tyrosine 659 of Gab1 Are Required for SHP2 Binding to Gab1 and for EGF-stimulated ERK2 Activation

We previously showed that Gab1 with a Tyr-627 to Phe mutation (Gab1Y627F), a Tyr-659 to Phe mutation (Gab1Y659F), or double mutations in which these two Tyr residues were changed to Phe (Gab1Y659F) were immunoprecipitated with an anti-FLAG antibody (M2). The immunoprecipitates were analyzed by immunoblotting (IB) with antibodies against SHP2 (A), phosphotyrosine (B), or Gab1 (C).

Little SHP2 was associated with Gab1 in serum-starved cells (Fig. 1A), while expression of Gab1Y659F had a dominant inhibitory effect on EGF-stimulated ERK2 activation (15). Upon EGF stimulation, tyrosine residues in Gab1 became phosphorylated (Fig. 1B) and SHP2 was bound to the wild type Gab1 (Fig. 1A). Tyr to Phe mutation of Gab1 at either Tyr-627 (Gab1Y627F) or Tyr-659 (Gab1Y659F) or both (Gab1Y659F) resulted in loss of the EGF-dependent SHP2 binding activity (Fig. 1A). A control immunoblot showed that equal amounts of Gab1 proteins were present in Gab1 immunoprecipitates (Fig. 1C). This observation indicates that both Tyr-627 and Tyr-659 are required for SHP2 binding to Gab1 in response to EGF.

Immunoblotting analysis with an anti-phosphotyrosine antibody (RC20) indicated that Gab1Y627F, Gab1Y659F, and Gab1FF had lower levels of phosphotyrosine content than that of the wild type Gab1 in EGF-stimulated cells (Fig. 1B). This observation suggests that Tyr-627 and Tyr-659 were phosphorylated in the wild type Gab1 in EGF-stimulated cells, in agreement with a previous report (26). Tyrosine phosphorylation of Gab1FF, although reduced, was still detectable in EGF-stimulated cells, consistent with the observation that tyrosine residues besides Tyr-627 and Tyr-659 were phosphorylated upon EGF stimulation (15, 26).

To determine whether Tyr-659 is required for EGF-stimulated ERK2 activation, Gab1Y659F, Gab1FF, and control constructs (Fig. 2) were co-expressed with HA-tagged ERK2 in COS-7 cells, and EGF-stimulated ERK2 activation was determined. As shown in Fig. 2, EGF treatment resulted in approximately a 12-fold activation of ERK2. Expression of the wild type Gab1 had little effect on EGF-stimulated ERK2 activation, while expression of Gab1Y627F had a dominant inhibitory effect on EGF-stimulated ERK2 activation as reported previously (15). Similar to Gab1Y627F, expression of Gab1Y659F or Gab1FF blocked EGF-stimulated ERK2 activation. Thus, both Tyr-627 and Tyr-659 of Gab1 are necessary for Gab1 to mediate...
the EGF-stimulated ERK2 activation.

**N-SH2 Domain of SHP2 Interacts Selectively with Tyr(P)-627 of Gab1**—To further characterize the interaction between Gab1 and SHP2, we performed Far Western blot analysis using GST fusion proteins containing either the N-SH2 or C-SH2 domain of SHP2. FLAG-tagged Gab1, Gab1Y627F, Gab1Y659F, and Gab1FF were immunoprecipitated from EGF-stimulated COS-7 cells expressing Gab1, Gab1Y627F, Gab1Y659F, or Gab1FF. The immunoprecipitates were resolved on SDS-polyacrylamide gels, transferred to Immobilon-P filters, and probed with GST, GST-N-SH2, GST-C-SH2, GST fusion protein of the C-terminal SH2 domain of SHP2 (GST-C-SH2, D), GST fusion protein of the N-terminal SH2 domain of SHP2 (GST-N-SH2, C), GST fusion protein of the C-terminal SH2 domain of SHP2 (GST-C-SH2, D), followed by an anti-GST antibody. Alternatively, the filters were probed with an anti-FLAG antibody (αFLAG, E) or an anti-phosphotyrosine antibody (αPTyr, F). A, relative band intensities from four experiments.

**Activation of SHP2 PTPase by a Gab1 Bisphosphopeptide Containing Tyr(P)-627 and Tyr(P)-659**—To determine whether phosphopeptides containing Tyr-627 and/or Tyr-659 can activate the PTPase activity of SHP2, we assayed the SHP2 PTPase activity using two phosphopeptides as substrates (Fig. 4). The phosphopeptide SrcPY was used initially as a SHP2 substrate in our experiments. SrcPY contains the autoinhibitory Tyr(P)-527 site of c-Src. Previous reports have identified this peptide as a good substrate of constitutively active SHP2 that does not affect SHP2 activity (6, 27). As shown in Fig. 4A (open bars), under the assay conditions, the SrcPY (100 μM) was dephosphorylated at a slow rate of 17 pmol/min by SHP2 (80 nM). Addition of monophosphopeptides (10 μM) containing either Tyr(P)-627 (PY627) or Tyr(P)-659 (PY659) resulted in a slight increase in the phosphate release rate. This small change in phosphate release rate was due to dephosphorylation of PY627 or PY659 by SHP2, because the slightly higher rate was approximately the sum of the phosphate release rates of SrcPY and PY627 or PY659 when they were incubated separately with SHP2. Therefore, there was only an additive increase in the phosphate release rates, indicating that PY627 and PY659 did not activate the SHP2 PTPase. Similar results were obtained with higher concentrations of PY627 and PY659 that we have tested (up to 100 μM PY627 or PY659, data not shown).

Similarly, a mixture of PY627 and PY659 could not activate SHP2 at all concentrations that we have tested (Fig. 4A, and data not shown). We then tested the effect of a bisphosphopeptide (PY627PY659) on SHP2 activity. PY627PY659 is a 43-amino acid residue phosphosphoryl peptide containing Tyr(P)-627 and Tyr(P)-659 of Gab1 and the naturally occurring amino acid residues between and around Tyr(P)-627 and Tyr(P)-659. As shown in Fig. 4A, addition of PY627PY659 (10 μM) to the reaction mixture resulted in 3-fold activation of SHP2. Activation of SHP2 by PY627PY659 was concentration-dependent (Fig. 4B). The activating activity of PY627PY659 requires phosphorylation of Tyr-627 and Tyr-659, because a non-phosphorylated peptide (Y627Y659) with identical amino acid residues did not have any apparent effect on SHP2 activity (Fig. 4A, B, and data not shown). In fact, the non-phosphorylated Y627Y659 peptide was included as a negative control with SrcPY in our experiments presented in Fig. 4. A, B, and data not shown).

To confirm that PY627PY659 can activate SHP2, we performed the SHP2 PTPase activity assay using a Gab1-derived phosphopeptide, PY589, as substrate. PY589 contains Tyr(P)-589, which is one of the phosphatidylinositol 3-kinase-binding sites in Gab1 (15, 18) and a likely target of SHP2 in the cells. As shown in Fig. 4A (black bars), we found that SHP2 was not activated by monophosphopeptides PY627, PY659, a mixture of PY627 and PY659, or the non-phosphorylated peptide Y627Y659. Again, addition of the phosphopeptide PY627PY659 to the reaction mixture resulted in activation of SHP2 PTPase (Fig. 4A).

To exclude the possibility that the apparent activation of SHP2 by PY627PY659 was due to kinetic differences of the phosphatase toward SrcPY, PY589, PY627, PY659, and
Purified GST-SHP2 was determined using SrcPY (open bars) (nM in the reaction set using SrcPY as substrate and 8.4 nM in the reaction was at 30 °C for 5 min. The GST-SHP2 concentrations were 8024384 phosphate release rate from all phosphopeptides in each reaction. The numbers under the graph indicate the concentrations of peptides (µM) used in each reaction. The reaction was performed as in Panel A using SrcPY as substrate with PY627PY659. The Net increase in PTPase activity was calculated as: phosphate release rate of reaction containing cont. GST-SHP2 only + phosphate release rate of reaction containing SrcPY only + phosphate release rate of reaction containing PY627PY659 only). C, PTPase activity of GST-SHP2ΔN. The reactions were performed using 24 nM GST-SHP2ΔN and either 50 or 100 µM of each phosphopeptide as indicated. The results were mean ± S.D. of three or more experiments.

**Fig. 4. SHP2 PTPase activity assay.** A, the PTPase activity of purified GST-SHP2 was determined using SrcPY (open bars) or PY589 (black bars) peptides as substrate. The numbers under the graph indicate the concentrations of peptides (µM) used in each reaction. The reaction was at 30 °C for 5 min. The GST-SHP2 concentrations were 80 nM in the reaction set using SrcPY as substrate and 8.4 nM in the reaction set using PY589 as substrate. The PTPase activity reflects phosphate release rate from all phosphopeptides in each reaction. B, the reaction was performed as in Panel A using SrcPY as substrate with different concentrations of PY627PY659. The Net increase in PTPase activity was calculated as: phosphate release rate of reaction containing both SrcPY and PY627PY659 – (phosphate release rate of reaction containing SrcPY only + phosphate release rate of reaction containing PY627PY659 only). C, PTPase activity of GST-SHP2ΔN. The reactions were performed using 24 nM GST-SHP2ΔN and either 50 or 100 µM of each phosphopeptide as indicated. The results were mean ± S.D. of three or more experiments.

Association of Activated SHP2 with Gab1 and SHP2

Interaction between Gab1 and SHP2

**Expression of a Chimeric Protein Consisting of SHP2 Binding-defective Gab1 and Active SHP2 PTPase Results in Constitutive Activation of ERK2**—To further test the possibility that association of activated SHP2 PTPase with Gab1 is necessary for ERK2 activation, we constructed a plasmid for expression of a chimeric protein in which SHP2 N (SHP2 N) is fused to the C terminus of Gab1FF-SHP2 N chimera. Expression of the constitutively active SHP2ΔN did not rescue the inhibition of Gab1FF on ERK2 activation (Fig. 5C and D, lane 8). This result suggests that the activated SHP2 must associate with Gab1 in order to mediate ERK2 activation.

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PTPase activity of the full-length SHP2 and an N-SH2 deletion mutant of SHP2 (SHP2ΔN) was measured using SrcPY (100 μM) as substrate. The reaction was performed in the presence or absence of SHP2 activating peptide PY627PY659 (5 μM) as indicated. The results represent means ± S.D. of two experiments performed in duplicate. B-E, COS-7 cells were transfected with plasmids for HA-ERK2 (0.2 μg) and HA-SHP2, HA-SHP2ΔN, Gab1FF, or Gab1FF-SHP2ΔN (0.9 μg/each) as indicated in Panel B. HA-SHP2 (0.9 μg) was included in the transfection of cells represented in lanes 1, 3, 5, 8, and 10 so that equal amounts of HA-tagged HA-ERK2 could be immunoprecipitated from each cell lysate. B, 9 μg of each cell lysate supernatant was analyzed by immunoblotting with an antibody against the C-terminal region of SHP2. SHP2 indicates the endogenous SHP2. Note that only a fraction of cells in each sample was predicted to be transfected with the plasmids and express the exogenous proteins. Therefore, there were higher levels of exogenous proteins than endogenous SHP2 in transfected cells than what appears in Panel B. HA-ERK2 was immunoprecipitated from cell lysate supernatants. One-half of each immunoprecipitate was used to determine ERK2 kinase activity using MBP as substrate (E). The other half of each immunoprecipitate was analyzed by immunoblotting with an anti-ERK antibody (E). C represents the means ± S.D. of ERK2 activity from three independent experiments. D, a representative autoradiograph. Chimera, Gab1FF-SHP2ΔN.

At a ratio of 10:1 for Gab1FF-SHP2ΔN cDNA:HA-ERK2 cDNA, the constitutive ERK2 activity in COS-7 cells transfected with Gab1FF-SHP2ΔN was ~6-fold above the basal level. This level of constitutive ERK2 activity was similar to that detected in COS-7 cells transfected with expression vectors for the Gα1/Gα2 subunits of heterotrimeric G proteins (Fig. 6). Gα1/Gα2 is one of the best combinations of Gα subunits known to activate ERK2 constitutively in COS-7 cells (28, 29).

Catalytic Cysteine Mutant of SHP2 (SHP2CS) Protects Tyrosine Residues in Gab1 from Dephosphorylation—Our in vitro study indicated that phosphopeptides containing Tyr(P)-627 and Tyr(P)-659 are SHP2 substrates (Fig. 4). To evaluate whether Tyr(P)-627 and Tyr(P)-659 of Gab1 are dephosphorylated by SHP2 in the cells, we co-expressed FLAG-tagged Gab1 with the wild type or a catalytically inactive SHP2 (SHP2CS, catalytic Cys to Ser mutation) in COS-7 cells. Following EGF stimulation for various times, tyrosine phosphorylation of Gab1 and the amount of SHP2 retained in the Gab1 complex were compared in cells transfected with SHP2 and SHP2CS. It has been shown that the catalytic Cys to Ser mutations of some PTPases can form a stable complex with their substrates and protect these substrates from dephosphorylation (30–32). Fig. 7, A and D, shows that little tyrosine phosphorylation of Gab1 was detectable in serum-starved cells transfected with the wild type SHP2. EGF stimulation resulted in a rapid increase in Gab1 tyrosine phosphorylation that peaked at 1 min. The Gab1 tyrosine phosphorylation gradually decreased to about half of the maximal at 30 min after EGF stimulation in these cells. This corresponded to the change in the amount of SHP2 in the Gab1 immune complex (Fig. 7B), suggesting that Tyr(P)-627 and/or Tyr(P)-659 of Gab1 were dephosphorylated in these cells.

Cells transfected with SHP2CS had an elevated level of Gab1 tyrosine phosphorylation and Gab1-SHP2 complex in the serum-starved condition (Fig. 7, A and D). After EGF stimulation, there was a continuous rise in Gab1 tyrosine phosphorylation. Importantly, this corresponded to a continuous increase in the amount of SHP2 associated with Gab1 in EGF-stimulated cells. Therefore, SHP2CS was able to trap Gab1 in a tyrosine-phosphorylated state in EGF-stimulated cells, suggesting that Gab1 is a substrate of SHP2 in the cells.

DISCUSSION

Previous experiments have shown that Tyr-627 is required for SHP2 binding to Gab1 in cells stimulated with EGF or insulin (15, 18). The present study provides evidence that Tyr-627, like Tyr-627, is required for SHP2 binding to Gab1 in EGF-stimulated cells and for ERK2 activation by EGF. Because SHP2 contains two SH2 domains that are arranged in tandem in the N-terminal portion of SHP2, the requirement of both Tyr-627 and Tyr-659 for Gab1-SHP2 association suggests that both phosphotyrosines may simultaneously interact with the tandem SH2 domains of SHP2. If this is the case, Tyr(P)-627 and Tyr(P)-659 should be able to bind to different SH2 domains in SHP2. Our Far Western blot binding assay showed that Tyr(P)-627 selectively interacted with the N-SH2 domain...
of SHP2, whereas Tyr(P)-659 preferentially bound to the C-SH2 domain of SHP2. This result suggests that it is possible for the tandem SH2 domains to bind to Tyr(P)-627 and Tyr(P)-659 simultaneously in a specific orientation, in which Tyr(P)-627 binds to the N-SH2 domain and Tyr(P)-659 binds to the C-SH2 domain. In other experiments, we assayed SHP2 PTPase activity using p-nitrophenyl phosphate as substrate. We found that peptide PY627, but not peptide PY659, was sufficient to activate SHP2 PTPase in this assay (data not shown). Because SHP2 activation requires binding of a TAM to its N-SH2 domain, this observation implies that PY627 has a higher affinity than PY659 for the N-SH2 domain of SHP2. Therefore, this observation provides another line of evidence to support the notion that Tyr(P)-627 binds to the N-SH2 domain while Tyr(P)-659 binds to the C-SH2. Interestingly, the same orientation specific binding of the tandem SH2 domains of SHP2 to the BTAM of IRS-1 has also been suggested (35).

Crystal structure analysis indicates that, in the absence of N-SH2 domain binding peptide, the N-SH2 domain of SHP2 interacts with its catalytic domain and thus the enzyme remains in an inactive state (3, 4). Binding of phosphopeptide to the N-SH2 domain induces a conformational change that activates the PTPase (3, 5). We found that a bisphosphopeptide (PY627PY659) derived from Gab1 that contains both the Tyr(P)-627 and Tyr(P)-659 TAMs could activate SHP2 PTPase. In previous studies with IRS-1 BTAM, artificial aminohexanoic acid spacers were used to link the two TAMs that are separated by a long amino acid sequence (3, 5). PY627PY659 used in our study represents the longest BTAM with a natural amino acid sequence that has been tested for SHP2 activation.

In a previous study using artificial IRS-1 BTAM, SHP2 activation was detected at 0.5 μM of optimally spaced Tyr(P)-1172 and Tyr(P)-1222 TAMs, and a maximal activation of about 7-fold was observed (4). In our study, a net increase in phosphate release was detectable at the lowest concentration of PY627PY659 (0.25 μM) that we have tested. PY627PY659 at 1 and 10 μM activates SHP2 about 1- and 3-fold, respectively. In order to keep a low concentration ratio of the activating peptide (PY627PY659) to the designated substrate (SrcPY or PY589), we did not attempt to achieve maximal SHP2 activation. Furthermore, different PTPase assays used in these two studies may contribute to the slight difference in the results.

Our observation that PY627PY659 activates SHP2 PTPase suggests that SHP2 binding to Gab1 would result in SHP2 activation, which is necessary for EGF-stimulated ERK2 activation. Importantly, monophosphopeptides PY627 and PY659...
could not activate SHP2 when SHP2 was assayed using peptide substrates. A mixture of PY627 and PY659 also could not activate SHP2 when peptide substrates were used to assay SHP2. These observations reinforce our finding that both Tyr-627 and Tyr-659 of Gab1 are required for EGF-stimulated ERK2 activation and indicate that the phosphorylated Tyr-627 and Tyr-659 of Gab1 constitute a BTAM for the binding and activation of SHP2. Furthermore, these results suggest that the physical constraint between the two TAMs in the Gab1 BTAM is necessary for SHP2 activation when natural phosphopeptides, rather than p-nitrophenyl phosphate, were its substrates.

We found that expression of a constitutively active SHP2 was insufficient to overcome the inhibition by Gab1FF of EGF-stimulated ERK activation. Interestingly, fibroblasts isolated from mouse embryos with a targeted deletion mutation of the N-SH2 domain of SHP2 were defective in ERK activation (34), suggesting that the activated SHP2 needs to associate with one or more cellular proteins through either its N-SH2 or through both SH2 domains to mediate ERK activation. Importantly, we found that expression of a fusion protein consisting of the constitutively active SHP2AN-linked to the C terminus of the SHP2 binding defective Gab1FF resulted in a constitutative activation of ERK2 in COS-7 cells. These results indicate that the activated SHP2 must associate with Gab1 in order to mediate ERK activation by EGF and that constitutive association of activated SHP2 with Gab1 will result in a constitutively elevated ERK2 kinase activity in the cells.

The reason that the activated SHP2 has to associate with Gab1 in order to mediate ERK activation is unclear at present, but several possibilities may exist. For example, (a) SHP2 may need to dephosphorylate a specific negative phosphorylation site on Gab1; (b) SHP2 may need to dephosphorylate a Gab1-associated phosphoprotein as suggested (17); (c) SHP2 may require Gab1 to translocate it to a specific cellular compartment to dephosphorylate a target that is not associated with Gab1.

Phosphopeptides derived from Tyr(P)-589, Tyr(P)-627, and Tyr(P)-659 of Gab1 were dephosphorylated by SHP2 in vitro. Our subtrate trapping study showed that SHP2CS protected Gab1 from dephosphorylation with a concomitant increase in the amount of Gab1-SHP2CS complex, indicating that SHP2CS was able to trap Gab1 Tyr(P) residues. These experiments demonstrate that Gab1 not only is a SHP2 activator in EGF-stimulated cells but also is a target of the activated SHP2 PTPase in the cells.

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