Epidermal Growth Factor-induced DNA Synthesis

KEY ROLE FOR Src PHOSPHORYLATION OF THE DOCKING PROTEIN Gab2

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We have previously demonstrated that phosphatidylinositol 3-kinase (PI3-kinase) is necessary and sufficient to account for epidermal growth factor (EGF)-induced mitogenesis in rat primary hepatocytes. A cytosolic Gab2-containing complex accounts for >80% of the total EGF-induced PI3-kinase activity (Kong, M., Mounier, C., Wu, J., and Posner, B. L. (2000) J. Biol. Chem. 275, 36035–36042), suggesting a key role for Gab2 in EGF-induced mitogenesis. Here, we demonstrate that PP1, a selective inhibitor of Src family kinases, blocks the EGF-induced Gab2 tyrosine phosphorylation without inhibiting EGF-induced phosphorylation of the EGF receptor, ErbB3, or Shc. We also show that Gab2 phosphorylation is increased in Csk knockout cells in which Src family kinases are constitutively activated. Furthermore, PP1 blocks Gab2-associated downstream events including EGF-induced PI3-kinase activation, Akt phosphorylation, and DNA synthesis. We demonstrate that Gab2 and Src are constitutively associated. Since this association involves the proline-rich sequences of Gab2, it probably involves the Src homology 3 domain of Src kinase. Mutation of the proline-rich sequences in Gab2 prevented EGF-induced Gab2 phosphorylation, PI3-kinase/Akt activation, and DNA synthesis, demonstrating that Gab2 phosphorylation is critical for EGF-induced mitogenesis and is not complemented by ErbB3 or Shc phosphorylation. We also found that overexpression of a Gab2 mutant lacking SHP2 binding sites increased EGF-induced Gab2 phosphorylation and the activation of PI3-kinase but blocked activation of MAPK. In addition, we demonstrated that the Src-induced response was down-regulated by Gab2-associated SHP2. In summary, our results have defined the role for Src activation in EGF-induced hepatic mitogenesis through the phosphorylation of Gab2 and the activation of the PI3-kinase cascade.

Upon ligand binding, the activated epidermal growth factor receptor (EGFR) mediates a number of important biological responses, including the stimulation of cell proliferation, migration, and differentiation (1–3). Src family kinases are non-receptor tyrosine kinases and have been described as essential mediators of EGF signaling (4). Like most nonreceptor tyrosine kinases, Src family kinases contain an Src homology 2 (SH2) domain, which binds phosphotyrosine residues, and an Src homology 3 (SH3) domain, which binds proline rich sequences (reviewed in Ref. 5). Several studies have established that Src kinases are required for growth factor-induced mitogenesis such as that effected via receptors for EGF (6–8), the platelet-derived growth factor (9, 10), and colony stimulation factor-1 (6). To date, the manner in which Src family kinases participate in effecting the mitogenic response is unclear.

Several reports have identified Gab (Grb2-associated binder) family proteins as key molecules for EGF-induced mitogenesis (11, 12). Gab proteins, which include mammalian Gab1, Gab2, and Gab3, the Drosophila homolog DOS (daughter of sevenless), and the Caenorhabditis elegans homolog Soc1 (Suppressor of clear), belong to a family of scaffolding proteins closely related to insulin receptor substrates (IRS-1, IRS-2, and IRS-3), FRs2 (fibroblast growth factor substrate), LAT (linker of T cell), and Dok (downstream of kinase) (reviewed in Refs. 13–15). They have in common a central proline-rich domain and multiple potential binding sites for the SH2 domains of p85, SHP2 (Src homology 2 domain-containing protein-tyrosine phosphatase-2), phospholipase Cγ, or Crk. Gab2 is tyrosine-phosphorylated upon stimulation of hepatocytes by EGF (12) and T cells by cytokines (16) and following activation of T- and B-cell antigen receptors (17, 18). Phosphorylated Gab2 has been shown to bind PI3-kinase via its 85-kDa (p85) regulatory subunit (19) as well as Grb2 (growth factor receptor-bound 2) and SHP2.

In previous studies, we demonstrated that the activation of PI3-kinase and not mitogen-activated protein kinase (MAPK), is necessary and sufficient to account for EGF-induced mitogenesis (12, 20). Although activated PI3-kinase was shown to associate with three phosphotyrosine-phosphorylated proteins (ErbB3, Shc, and Gab2), over 80% was found in a multimeric complex consisting of Gab2-p85-SHP2-Grb2 (12). Confirming the key role of Gab2 was our finding that overexpression of wild type Gab2 (WTGab2) augmented EGF-induced PI3-kinase activity and DNA synthesis, whereas the Gab2 mutant (Gab2Δp85) lacking p85 binding sites (pYXXX motifs as reviewed in Ref. 21, where pY represents phosphotyrosine) affected no such augmentation. Furthermore, we showed that following EGF treatment, the phosphorylated multimeric Gab2 complex was exclusively cytosolic and did not associate with membranes. Nor did overexpression of the pleckstrin homology domain of Gab2 interfere with EGF-induced Gab2 phosphorylation or mitogenesis.2

1 The abbreviations used are: EGF, epidermal growth factor receptor; EGFR, epidermal growth factor; PI3-kinase, phosphatidylinositol 3-kinase; MAPK, mitogen-activated protein kinase; MOI, multiplicity of infection; SHF and -3, Src homology 2 and 3, respectively; IRS, insulin receptor substrate; WTBab2, wild type Gab2; IP, immunoprecipitation; aPY, anti-phosphotyrosine.

2 M. Kong, C. Mounier, A. Balbis, G. Baquiran, and B. I. Posner, submitted for publication.
In the present study, we considered the possibility that Gab2 is phosphorylated by a tyrosine kinase other than the EGFR and thus sought to elucidate a link between Src family kinases and Gab2 in the regulation of EGF-induced mitogenesis in primary hepatocytes. Our study demonstrates that Src kinase(s) promote EGF-induced PI3-kinase activation and DNA synthesis through effecting the tyrosine phosphorylation of Gab2. We found that the wild-type and Gab2 mutants are essential for constitutive Src association with and tyrosine phosphorylation of Gab2. Finally, we demonstrate that these Src-dependent responses are down-regulated by the association of SHP2 with Gab2 and that this latter association is critical to EGF-induced MAPK activation.

**EXPERIMENTAL PROCEDURES**

**Materials—**Mouse EGF was obtained from Collaborative Biomedical Products (Bedford, MA). Collagenase was from Worthington. Cell culture medium and antibiotics were from Invitrogen. Fetal bovine serum (FBS), streptomycin, and penicillin, and 500 IU/ml penicillin, and 500 μg/ml streptomycin. Wild-type and Csk-deficient embryos exhibit an order of magnitude increase in activity of Src and the related Lyn kinase (22, 25). To further confirm the role of Src family kinases on Gab2 phosphorylation, we inflicted Csk-deficient and wild type mouse embryo fibroblasts with recombinant Gab2 adenovirus and starved the cells for 48 h. Western blot analysis, using a specific phospho-Src antibody (p-Src416) (26), demonstrated that Src kinase is constitutively activated in Csk-deficient mice. Gab2 tyrosine phosphorylation was then investigated in the absence of EGF stimulation. Immunoprecipitation with a Gab2 antibody and Western blotting with anti-phosphotyrosine demonstrated that Gab2 phosphorylation (Fig. 2A, middle panels) is increased 1.8-fold in Csk−/− compared with wild type fibroblasts (Fig. 2B). This study supports the view that Gab2 is a substrate for Src family kinases in vivo.

**RESULTS**

A Key Role for Src Family Kinases in EGF-induced Gab2 Phosphorylation—In the liver, tyrosine-phosphorylated Gab2 is largely localized in the cytosol, could not be demonstrated to associate with the EGFR, and is phosphorylated in a pleckstrin homology domain-independent manner. EGF has been shown to activate tyrosine kinases of the Src family (4), and studies have linked their activation to cell proliferation (6–10). We tested the hypothesis that Gab2 is a Src substrate by examining the effect of PP1, a selective inhibitor of Src family kinases (23), on EGF-induced Gab2 tyrosine phosphorylation in rat primary hepatocytes. As shown in Fig. 1A (top panels), PP1 reduced EGF-induced tyrosine phosphorylation of Gab2 in a dose-dependent manner. Compared with cells incubated with vehicle only, 1 μM PP1 decreased Gab2 phosphorylation by 50% and 20 μM PP1 by more than 90%. In contrast, the same doses of PP1 had no effect on EGF-induced tyrosine phosphorylation of EGFR (Fig. 1A, middle panels), ErbB3, or Shc (Fig. 1A, bottom panels). Nor did these doses of PP1 affect insulin-induced tyrosine phosphorylation of IRS-1 or IRS-2 (Fig. 1B). PP2, another selective inhibitor of Src family kinases, also inhibited the stimulatory effect of EGF on Gab2 tyrosine phosphorylation (data not shown). The specificity of inhibition by PP1 and PP2 of EGF-induced tyrosine phosphorylation of Gab2 suggests that, in rat hepatocytes, this molecule, but not other tyrosine-phosphorylated docking proteins, is a substrate for Src family kinase(s).

All Src family tyrosine kinases are negatively regulated by phosphorylation at a carboxyl-terminal tyrosine (Tyr-527 in Src kinase) carried out by another nonreceptor tyrosine kinase, Csk (C-terminal Src kinase) (24). Cells derived from Csk-deficient embryos exhibit an order of magnitude increase in activity of Src and the related Fyn kinase (22, 25). To further confirm the role of Src family kinases on Gab2 phosphorylation, we inflicted Csk-deficient and wild type mouse embryo fibroblasts with recombinant Gab2 adenovirus and starved the cells for 48 h. Western blot analysis, using a specific phospho-Src antibody (p-Src416) (26), demonstrated that Src kinase is constitutively activated in Csk−/− cells (Fig. 2A, top panel). Gab2 tyrosine phosphorylation was then investigated in the absence of EGF stimulation. Immunoprecipitation with a Gab2 antibody and Western blotting with anti-phosphotyrosine demonstrated that Gab2 phosphorylation (Fig. 2A, middle panel) is increased 1.8-fold in Csk−/− compared with wild type fibroblasts (Fig. 2B). This study supports the view that Gab2 is a substrate for Src family kinases in vivo.
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Fig. 1. Src kinase inhibitor PP1 specifically inhibits Gab2 phosphorylation. Serum-deprived hepatocytes were preincubated with either vehicle (Me2SO (DMSO)) or PP1 (1 or 20 μM) for 30 min followed by treatment with (+) or without (−) 100 ng/ml EGF (A) or 100 nm insulin (B) for 1 min. Cell lysates were incubated with different antibodies, and the immunoprecipitated proteins were subjected to SDS-PAGE (7.5% gel) followed by immunoblotting with the indicated antibodies as described under “Experimental Procedures.”

Gab2 Tyrosine Phosphorylation by Src Family Kinases Is Essential for EGF-dependent Downstream Signaling Events—In previous work, we showed that over 80% of PI3-kinase, activated following EGF treatment, was associated with tyrosine-phosphorylated Gab2 (12) and that overexpression of Gab2 was sufficient to augment EGF-induced DNA synthesis.2 In this study, we pretreated hepatocytes with either vehicle (Me2SO) or 20 μM PP1 for 30 min followed by a stimulation with 100 ng/ml EGF. As shown in Fig. 3A, EGF induced a 20-fold increase in Gab2-associated PI3-kinase activity in control cells. Pretreatment with PP1 abolished this activation, that of Akt, as reflected in Akt-Ser-473 phosphorylation (Fig. 3B), and DNA synthesis (Fig. 3C). Similar results were obtained by treatment of the cells with PP2 (data not shown). Our results demonstrate that EGF-induced tyrosine phosphorylation of Gab2, mediated by Src family kinases, is necessary for EGF-induced DNA synthesis in primary rat hepatocytes and that the tyrosine phosphorylation of ErbB3 and Shc, which is unaffected by PP1 (Fig. 1), cannot supplant the critical requirement for tyrosine-phosphorylated Gab2 for this response.

Association of Src with Gab2: Significance for Gab2 Tyrosine Phosphorylation and Downstream Signaling—In our previous experiments, demonstrating that Gab2 is a substrate for Src family kinases, we observed a 60-kDa band in anti-Gab2 immunoprecipitates (data not shown). We therefore examined the possibility that Src kinase was associated with its substrate, Gab2. As shown in Fig. 4A (top panel), Src family kinases are

Fig. 2. Increased tyrosine phosphorylation of Gab2 in Csk knockout (Csk−/−) cells. A, wild type (WT) or Csk knockout (Csk−/−) cells were infected with 10 MOI of recombinant Gab2 adenovirus for 3 h and then incubated for 48 h in serum-free medium. Cell lysates were prepared, and aliquots (50 μg of protein) were subjected to 7.5% SDS-PAGE followed by immunoblot analysis with anti-phospho-Src416 antibody (top panel). Other aliquots were incubated with anti-Gab2 antibody, and the immunoprecipitates were subjected to 7.5% SDS-PAGE followed by immunoblot analysis with anti-phosphotyrosine antibody (middle panel) or anti-Gab2 antibody (bottom panel). B, the level of Gab2 tyrosine phosphorylation, quantified as described under “Experimental Procedures,” is expressed in Csk−/− cells (solid bar) as -fold over wild type cells (hatched bar) (mean ± S.E.; n = 3; *, p < 0.01).

Fig. 3. Src family kinases are required for Gab2-mediated downstream events. A, serum-deprived hepatocytes were preincubated with 20 μM PP1 or vehicle (Me2SO (DMSO)) for 30 min followed by treatment with (+) or without (−) EGF (100 ng/ml) for 1 min. Cell lysates were incubated with anti-Gab2 antibody, and immunoprecipitated proteins were either assayed for PI3-kinase activity (top panel) or subjected to SDS-PAGE (7.5% gel) followed by immunoblotting with anti-Akt antibody (bottom panel). B, serum-deprived hepatocytes were preincubated with 20 μM PP1 or vehicle (Me2SO) for 30 min and then treated with (+) or without (−) EGF (100 ng/ml) for 5 min. Cell lysates were subjected to SDS-PAGE (10% gel) followed by immunoblotting with either anti-phospho-Akt473 (top panel) or Akt antibody (bottom panel). C, hepatocytes were incubated in serum-free medium for 24 h followed by incubation with either vehicle (Me2SO) (open and hatched bars) or PP1 (20 μM) (solid bar). After 30 min, 5 μCi of [3H]methylymethionine was added, and the incubation was continued with (hatched and solid bars) or without (open bar) EGF (100 ng/ml) for 18 h in serum-free medium as described under “Experimental Procedures.” Results are expressed as -fold over control (non-EGF-treated cells). The inhibitory effect of PP1 on EGF stimulation of DNA synthesis was assessed in three separate experiments (mean ± S.E.; *, p < 0.01).
constitutively associated with endogenous Gab2, and EGF treatment did not appear to augment this association. This suggested that the association may result from the binding of the SH3 domain of Src with proline-rich sequences in Gab2. To test this hypothesis, we generated a recombinant adenovirus containing a Gab2 mutant in which key proline-rich sequences were mutated (ΔProGab2) (see "Experimental Procedures"). Hepatocytes were infected with either WTGab2 or ΔProGab2 recombinant adenovirus and starved for 48 h before EGF treatment. In contrast to WTGab2, overexpressed ΔProGab2 manifested no association with Src kinase (Fig. 4B, top panel, lanes 1 and 2 versus lanes 3 and 4), indicating that the proline-rich sequences are essential for mediating the binding of Src family kinases to Gab2. Src kinase phosphorylation was analyzed in anti-Gab2 immunoprecipitates. A tyrosine-phosphorylated band migrating at the same position as Src kinase (60 kDa) was detected in EGF-treated cells infected with WTGab2 but not in EGF-treated cells infected with ΔProGab2 (Fig. 4C, top panel). These results suggest that Src kinase is activated upon EGF treatment, although it is constitutively associated with Gab2.

We sought to determine the extent to which the loss of constitutive Src binding influenced Gab2 phosphorylation by Src family kinases. Primary hepatocytes were infected with recombinant WTGab2 or ΔProGab2 adenoviruses. As shown in Fig. 5 (top panel), EGF treatment resulted in substantial tyrosine phosphorylation of WTGab2, whereas the phosphorylation of ΔProGab2 is reduced by more than 70% (Fig. 5, bottom panel). These results demonstrate that, in hepatocytes, the proline-rich sequences of Gab2 are important for Src kinase-mediated Gab2 phosphorylation.

Since overexpressing ΔProGab2 dramatically reduced EGF-induced Gab2 tyrosine phosphorylation, we examined whether this resulted in a decrease of Gab2-dependent downstream signaling. Cells were infected with either control recombinant adenovirus (LacZ) or Gab2 constructs (WTGab2 or ΔProGab2). As previously observed, overexpression of WTGab2 potentiated EGF-induced PI3-kinase activation by 25-fold (Fig. 6A, top panel, lane 2 versus lane 4), whereas overexpression of ΔProGab2 reduced EGF-induced PI3-kinase activity by more than 70% (Fig. 6A, top panel, lane 4 versus lane 6). This parallels the association of Gab2 with p85 (Fig. 6A, middle panel). Interestingly, ΔProGab2 did not exert a dominant negative effect in that it did not reduce PI3 kinase activity to less than control levels despite being expressed at 20-fold the level of endogenous Gab2. Corresponding to the PI3-kinase activity, we found that EGF treatment augmented Akt serine 473 phosphorylation in cells overexpressing WTGab2 but not in those overexpressing ΔProGab2 (WTGab2- versus ΔProGab2-infected cells, expressed as a percentage of LacZ-infected cells was 146 + 4.7 versus 110 ± 4.1 (n = 3, mean ± S.E., p < 0.005) (Fig. 6B). Parallel results were obtained when we measured EGF-induced DNA synthesis (Fig. 6C). The observations in Fig. 6 are consistent with a greater sensitivity to EGF of PI3-kinase activation and DNA synthesis. This could explain the closer correlation between Akt activation and the extent of stimulation of DNA synthesis. These findings indicate that the proline-rich sequences in Gab2, which mediate Src binding, are important for EGF-induced PI3-kinase activation and DNA synthesis.

We then examined the effect of WTGab2 and ΔProGab2 on ERK phosphorylation. As shown in Fig. 6D, ERK phosphorylation was barely observed with 0.2 ng/ml EGF in LacZ-infected cells, whereas, at this dose, there was clear phosphorylation of ERK in cells infected with WTGab2. We also demonstrated that ERK1/2 phosphorylation at higher doses of EGF (10 or 100 ng/ml) is much greater than that at 0.2 and 1.0 ng/ml. At these concentrations, we did not observe any effect of overexpressed Gab2 (data not shown). This suggests that overexpressed Gab2 increases the sensitivity of ERK to EGF, which correlated with previous studies showing that at a low dose of EGF (0.25 ng/ml), overexpression of Gab1 potentiated EGF-induced ERK activation in HEK293 cells (27). These results may indicate that, at higher doses of EGF, the influences of Gab2 could be obscured by input(s) from Gab2-independent signaling pathways. Alternatively, a high sensitivity of ERK phosphorylation to EGF may result in its saturation at the 10 ng/ml dose of...
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Fig. 6. Influence of Gab2 proline-rich sequences on EGF-induced activation of PI3-kinase/Akt, MAPK, and DNA synthesis. Hepatocytes were infected with 10 MOI of either recombinant LacZ, WTGab2, or ΔProGab2 adenovirus for 3 h followed by incubation in serum-free medium for 48 h. A, serum-deprived hepatocytes were treated with (+) or without (−) EGF (100 ng/ml) for 1 min, and cell lysates were prepared, equally divided, and incubated with anti-Gab2 antibody. Immunoprecipitated proteins were assayed for PI3-kinase activity (top panel) or subjected to SDS-PAGE (7.5% gel) followed by immunoblotting with anti-p85 antibody (middle panel). The membrane was stripped and reblotted with an anti-Gab2 antibody (bottom panel). B, serum-deprived hepatocytes were treated with (+) or without (−) EGF (100 ng/ml) for 5 min. Cell lysates were subjected to SDS-PAGE (10% gel) followed by immunoblotting with an anti-phospho-Akt antibody (top panel), anti-Akt (middle panel), or anti-Gab2 (bottom panel) antibodies. C, virus-infected hepatocytes were incubated in serum-free medium for 24 h followed by the addition of 5 µCi of [3H]methylthymidine without (hatched bars) or with (solid bars) EGF (100 ng/ml) for 18 h. Incorporation of [3H]methylthymidine into DNA was determined as described under “Experimental Procedures.” Results are expressed as -fold over control (LacZ adenovirus-infected cells without EGF) cells. The difference between EGF-treated hepatocytes infected with WTGab2 versus ΔProGab2 was determined in three separate experiments (mean ± S.E., *p < 0.005). D, hepatocytes were treated with the indicated doses of EGF for 5 min, and cell lysates were prepared and subjected to SDS-PAGE (10% gel) followed by immunoblotting with anti-phospho-Erk1/2 (top panel), anti-Erk1/2 (middle panel), or anti-Gab2 (bottom panel) antibodies.

EGF. As shown in Fig. 6D, ΔProGab2 comparably potentiated EGF-induced ERK1/2 activation as WTGab2 at the low dose of EGF (i.e., following EGF (0.2 ng/ml) treatment, ERK activation in WTGab2 and ΔProGab2 was 218 ± 4.2 and 236 ± 12, respectively, when expressed as a percentage of values in LacZ-infected cells (n = 3, mean ± S.E., *p < 0.05 LacZ versus either construct)). Thus, the reduced level of tyrosine phosphorylation of ΔProGab2 would appear to be sufficient to activate ERK1/2 but not PI3-kinase.

SHP2 Is a Negative Effector for PI3-kinase/Akt Activation but Positive for MAPK Activation—Upon EGF treatment, Src activation leads to substantial tyrosine phosphorylation of Gab2 and activation of DNA synthesis. We previously demonstrated that phosphorylated Gab2 associates with the SH2-containing protein-tyrosine phosphatase, SHP2 (12). We considered that this binding might effect dephosphorylation of Gab2 and therefore generated a Gab2 mutant lacking the SHP2 binding sites (ΔSHP2Gab2; see “Experimental Procedures”). Overexpression of ΔSHP2Gab2 in hepatocytes totally abolished SHP2 binding to Gab2 (Fig. 7A, top panel, lane 2 versus lane 4) and was tyrosine-phosphorylated (Fig. 7B, lane 4 versus lane 6) to an extent 25% greater than WTGab2 (Fig. 7C).

Of interest is the finding that overexpression of ΔSHP2Gab2 potentiated EGF-induced PI3-kinase activation 2.6-fold compared with that observed in cells overexpressing comparable levels of WTGab2 (Fig. 8A, top panel, lane 4 versus lane 6). As expected, this correlated with augmented p85-Gab2 association (Fig. 8A, middle panel, lane 4 versus lane 6). In parallel, we found that following EGF treatment, Akt serine 473 phosphorylation, expressed as a percentage of LacZ-infected cells, was 146 ± 4.7 versus 201 ± 25, in WTGab2 versus ΔSHP2Gab2 (n = 3, mean ± S.E., *p < 0.01) (Fig. 8B, top panel, lane 4 versus lane 6). Similar results were observed when we looked at the EGF-induced DNA synthesis by -fold (Fig. 8C). Thus, SHP2 binding to tyrosine-phosphorylated Gab2 acts as a negative regulator of EGF-induced PI3-kinase activation and DNA synthesis.

In contrast to these findings, whereas overexpression of WT-Gab2 increased the sensitivity of ERK1/2 activation to EGF (Figs. 6D and 8D, lanes 4–6), overexpression of ΔSHP2Gab2 had no capacity to effect EGF-induced activation of ERK1/2 (Fig. 8D, lanes 7–9). Interestingly, whereas following EGF treatment (0.2 ng/ml), ERK activity in WTGab2-versus ΔSHP2Gab2-infected cells, expressed as percentage of LacZ-infected cells, was 218 ± 4.2 versus 130 ± 9 (n = 3, mean ± S.E., *p < 0.05)) (Fig. 8D). These data indicate that SHP2 binding to tyrosine-phosphorylated Gab2 influences EGF downstream signaling by negatively affecting EGF-dependent PI3-kinase activation and positively affecting activation of the MAPK pathway.

The above observations identify the mechanism by which Src regulates EGF-induced mitogenesis in hepatocytes. Upon EGF binding, Gab2-associated Src is activated, leading to activation of the PI3-kinase cascade and eventually DNA synthesis. This signal appears to be attenuated by SHP2 through dephosphorylation of Gab2.

DISCUSSION

In response to the binding of EGF to its receptor, Gab proteins become tyrosine-phosphorylated and bind SH2 domain-containing proteins, including p85, the PI3-kinase subunit, and SHP2 (12, 27–30). The mechanism by which EGF induces the phosphorylation of Gab docking proteins is key to understanding EGF-induced signaling. Several reports indicate that
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EGFR is the kinase that phosphorylates Gab1 (31–33). Furthermore, studies have demonstrated that the pleckstrin homology domain of Gab1 is required for its localization to membranes, thus facilitating its phosphorylation by the EGFR kinase (31, 34, 35). However, the process involved in phosphorylating Gab2 appears to be different. Thus, as we have previously found, Gab2 is phosphorylated in a pleckstrin homology domain-independent manner; nor was it observed to associate with the EGFR. Moreover, the major multimeric Gab2 complex was found exclusively in the cytosol of rat liver (32). For these reasons, we examined the possibility that Gab2 is tyrosine-phosphorylated by a cytosolic kinase and not the EGFR.

Although previous reports have established that Src family kinases are required for EGF-induced mitogenesis (6–8), the mechanism by which Src family kinases act has remained unclear. In the present study, we demonstrate that EGF treatment of rat hepatocytes activates Src, leading to Gab2 tyrosine phosphorylation, after which Gab2 recruits SHP2, Grb2, and p85, thus forming a multimeric cytosolic complex (3). The evidence that Src family kinases are involved in Gab2 tyrosine phosphorylation is based on the inhibitory effects of PP1 and PP2, two widely used specific inhibitors of Src family kinases (36, 37). Thus, in the present study, we observed that the inhibition of Gab2 tyrosine phosphorylation was dose-dependent and occurred in the absence of inhibition of tyrosine phosphorylation of EGFR, ErbB3, or the IRS proteins (Fig. 1). Furthermore, using CSK−/− mouse embryonic cells, in which Src family kinases are constitutively activated, we confirmed a key role for these kinase(s) in the phosphorylation of Gab2. Consistent with our results are previous studies demonstrating that the target involved in Src-dependent mitogenic activity is a cytosolic molecule accessible to unmyristoylated Src. Thus, Src deletion mutants, lacking the amino-terminal one-third of the molecule, including the membrane binding domain, were shown to still induce cell proliferation (38). It has been known for some time that EGF signaling involves c-Src substrates with molecular sizes of 120–130, 100, and 75 kDa (39). Further studies have identified p75 as cortactin (40) and p120–130 as comprising several proteins, including p125FAK (41) and p130CAS (42). However, the identity of p100 has hitherto remained unknown. Our results appear to establish the p100 Src kinase substrate as Gab2.

To elucidate the mechanism by which Gab2 is phosphorylated by Src family kinases, we analyzed the association between these two proteins. In most cases, substrate recognition is dictated primarily by interactions with noncatalytic regions of the Src family kinases such as SH2 and SH3 domains (reviewed in Ref. 5). A constitutive association of Src family kinases with Gab2 was observed, and this association disappeared when the two key proline-rich regions on Gab2 were mutated (Fig. 4), indicating that the SH3 domain of Src is probably involved in this interaction. This is consistent with two previous reports demonstrating that the SH3 domain of Src is required for EGF mitogenic signaling (9, 10) and that this domain can bind to Gab2 in an in vitro binding assay (43). The SH3 domains of Src family kinases recognize proline-rich sequences found in a large number of substrates of Src family kinases (44–46). The SH2 domains of Src family kinase selectively recognize the sequence pYEEI, with a hydrophobic residue at position +3 being an important determinant of binding (47). Interestingly, the Gab2 sequence does not contain a pYEEI motif, suggesting that Src does not bind to Gab2 through its SH2 domain. Although Src family kinases are constitutively associated with Gab2, the activation of Src kinase is only observed after EGF treatment (Fig. 4C). The mechanism by which Src kinase activation is effected by the binding of EGF to the EGFR remains to be elucidated. In hepatocytes, Src, Fyn, and Yes are the three Src family members expressed (48). However, in the present analysis, we could not identify which isoform(s) phosphorylate Gab2. Indeed, the dose of PP1 we...
It was also found that SHP2 association with Gab2 is essential for macrophage colony-stimulating factor-induced macrophage differentiation (52). In the present study, we identified a positive role for SHP2 in Gab2-potentiated EGF-induced MAPK activation (Fig. 6D). Recent reports have also identified a negative effect of SHP2 binding on Gab protein tyrosine phosphorylation. Thus, both Gab1 and Gab2 have been identified as substrates for SHP2 using an in vitro phosphatase assay (18). More recently, two studies have shown that inactive forms of SHP2 markedly increased EGF-stimulated Gab1 tyrosine phosphorylation and PI3-kinase activation (28, 53). The negative regulation of EGF-dependent PI3-kinase activation by SHP2 appears to be through the dephosphorylation of Gab1 p85 binding sites (53). In agreement with this study, we have found that, in rat hepatocytes, a relatively minor increase of ΔSHP2Gab2 phosphorylation compared with WTGab2 (Fig. 7B) was accompanied by a more noticeable augmentation of PI3-kinase activity and DNA synthesis, suggesting that SHP2 is involved in the dephosphorylation of specific p85 binding sites on Gab2 (Fig. 8, A–C). Moreover, the fact that the ΔSHP2Gab2 mutant blocks EGF-induced MAPK activation and augments PI3-kinase and DNA synthesis further confirms, in primary hepatocytes, the key role of PI3-kinase and not MAPK in EGF-induced DNA synthesis.

In summary, we have found that, in rat hepatocytes, Src family kinases regulate EGF-induced mitogenesis through association to and phosphorylation of the major cytosolic docking protein, Gab2. Furthermore, we demonstrate that the association of SHP2 with tyrosine-phosphorylated Gab2 leads to dephosphorylation of the latter and a corresponding decrease in EGF-induced DNA synthesis.
