Src Phosphorylates Grb2-associated Binder 1 upon Hepatocyte Growth Factor Stimulation*

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Grb2-associated binder 1 (Gab1) is known to play an important role in hepatocyte growth factor (HGF) signaling, which rapidly becomes tyrosine-phosphorylated upon HGF stimulation. In this study, we found that the tyrosine phosphorylation of Gab1 in the cells derived from Src/Yes/Fyn null mouse embryos was ~40% lower than that in their wild type counterparts upon HGF stimulation. Increased expression of wild-type Src enhanced HGF-induced phosphorylation of Gab1, and, in contrast, expression of the Src kinase-deficient mutant or treatment of the specific Src inhibitor PP1 suppressed it. Expression of a constitutively active Src mutant (Y527F) or oncogenic v-Src led to a prominent increase in Gab1 phosphorylation independent of HGF stimulation. Moreover, Src interacted with Gab1 via both its Src homology 2 and 3 domains and was capable of phosphorylating purified Gab1 in vitro. Finally, the increased phosphorylation of Gab1 by Src selectively potentiated HGF-induced activation of ERK and AKT.

Taken together, our results establish a new role for Src in HGF-induced Gab1 phosphorylation.

Hepatocyte growth factor (HGF),† also known as scatter factor, is a mesenchymally derived factor that elicits mitogenic, motogenic, and morphogenic activities on various cell types (1). The diverse biological effects of HGF are transmitted through activation of its transmembrane receptor encoded by the c-met proto-oncogene (2, 3). In vivo, HGF/c-Met signaling plays critical roles in embryogenesis (4, 5), angiogenesis (6), organ regeneration (7), and tumorigenesis (8). The Met receptor is a heterodimer composed of a 45-kDa chain (9–11). Upon HGF binding, the intrinsic tyrosine kinase of the receptor is activated, resulting in autophosphorylation on specific tyrosine residues in the β chain (12, 13). Two tyrosine residues in the COOH terminus of the β chain (Tyr1349 and Tyr1356) are required for all biological activities of the receptor (14, 15) and serve as docking sites for the Grb2-associated binder-1 (Gab1) docking protein (16–18) and multiple Src homology 2 (SH2) and phosphotyrosine binding domain-containing proteins, including phosphatidylinositol 3-kinase, Src, Grb2, Shc, and STAT3 (19–21).

Gab1 is the major phosphorylated protein downstream of the Met receptor in epithelial cells (18), which belongs to a family of docking proteins, including Gab2 and Gab3 in mammals, Dos in Drosophila melanogaster, and SOC-1 in Caenorhabditis elegans (22). Gab1 contains an NH2-terminal pleckstrin homology domain, a Met-binding domain, six proline-rich sequences, and 16 potential tyrosine phosphorylation sites that represent SH2-binding motifs (23, 24). Upon HGF stimulation, Gab1 associates with the p85 regulatory subunit of phosphatidylinositol 3-kinase, phospholipase Cγ, the tyrosine phosphatase SHP-2, and the adaptor protein Crk and acts to recruit these signaling proteins to the Met receptor (25–28). Overexpression of Gab1 in epithelial cells induces ligand-independent morphogenesis characteristic of c-Met activation (23). In addition, Gab1 is also phosphorylated after stimulation of cells by several other growth factors, such as epidermal growth factor (29, 30), insulin (31), nerve growth factor (32), and basic fibroblast growth factor (33) as well as by different cytokines and antigen receptors (34). The gab1 gene knockout is lethal to mouse embryos, and the fibroblasts derived from Gab1−/− mouse embryos are defective in extracellular signal-regulated kinase (ERK) activation in response to multiple growth factors and the cytokine IL-6 (35, 36).

Gab1 is recruited to the Met receptor by both direct and indirect mechanism. The minimal amino acid sequence in the Met-binding domain of Gab1 sufficient for direct binding to the Tyr1349 and to a lesser extent to the Tyr1356 of the Met, called the Met-binding sequence, comprises 13 amino acids (positions 487–499) and is absent in other Gab proteins (37). Because the Met-binding sequence lacks obvious sequence similarity to other SH2 and phosphotyrosine binding domains, it may represent a novel type of phosphotyrosine binding domain. However, most Gab1-Met interaction is mediated indirectly via the adaptor protein Grb2. Two of the proline-rich motifs of Gab1 mediate the binding of the COOH-terminal SH3 domain of Grb2 (18). One of these is a classical Grb2 SH3 binding site (342PXPXK347), but the other (513PXXRXXK352) fails to conform to this motif (24). Grb2 also contains an SH2 domain, which targets the constitutive Grb2-Gab1 complex to the phosphorylated Tyr1356 of the Met receptor (18).
The tyrosine kinase c-Src is activated in response to HGF (19, 38) and plays a crucial role in HGF-induced cell motility and anchorage-independent growth (39, 40). The SH2 domain of c-Src binds either Tyr3145 or Tyr3156 of the Met receptor with fast association and dissociation rates (19). In this study, we set out to examine the role of Src in HGF-induced Gab1 phosphorylation. Our study reveals that Src binds to and phosphorylates Gab1 in response to HGF, which contributes to HGF-induced activation of ERK and AKT.

EXPERIMENTAL PROCEDURES

Materials—Recombinant human HGF, Protein A-Sepharose beads, and glutathione-agarose beads were purchased from Sigma. Lipofectamine™ was purchased from Invitrogen. The specific Src inhibitor PP1 was purchased from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA). G418 sulfate was purchased from Calbiochem. The rabbit polyclonal anti-Gab (H-190), anti-Met (C-28), and anti-Src (N-16), anti-C-terminal Src kinase (C-20), and anti-ERK (K-23) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The rabbit polyclonal anti-phospho-Met (Tyr1234/Tyr1235) was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). The rabbit polyclonal anti-phospho-ERK (Thr202/Tyr204), anti-AKT, and anti-phospho-AKT (Thr308) were purchased from Cell Signaling Technology, Inc. (Beverly, MA). The rabbit polyclonal anti-phospho-Met (Tyr105) was purchased from BIOSOURCE International, Inc. (Camarillo, CA). The monoclonal anti-hemagglutinin (HA) epitope was purchased from Roche Applied Science. The monoclonal anti-phosphotyrosine (PY20) was purchased from BD Transduction Laboratories (Lexington, KY). The mouse ascites produced by the hybridoma (CRL-2651) was purchased from American Type Culture Collection. The monoclonal anti-Met, anti-Src, or anti-C-terminal Src kinase were washed from BD Transduction Laboratories (Lexington, KY). The mouse ascites produced by the hybridoma (CRL-2651) was purchased from American Type Culture Collection.

Plasmids—The expression plasmid pcDNA3-HA-Gab1 was kindly provided by Dr. Toshi Hirano (Osaka University). The plasmids pEVX-cSrc, pEVX-Src, pEVX-SrcY527F, and pM-vSrc were obtained from Dr. Paolo M. Comoglio (University of Torino, Italy). The expression plasmid pcDNA3-HA-Gab1 was kindly provided by Dr. Toshi Hirano (Osaka University). The plasmids pEVX-cSrc, pEVX-Src, pEVX-SrcY527F, and pM-vSrc were obtained from Dr. Paolo M. Comoglio (University of Torino, Italy). The plasmids encoding GST fusion proteins containing the SH2 domains of Src, phosphotyrosinol 3-kinase, and Grb2 and the SH3 domains of Src and Grb2 were described previously (41, 42).

To construct pGEX2T-Gab1, the cDNA encoding Gab1 was amplified by polymerase chain reaction with primers 5'GATGAGCGGTGGTGAAG-3' and 5'H11032-TCATTTCACACTCTTCGCTG-3'. The underlines indicate the positions of substituted bases. The flanking primers were 5'CCTGGAAACTTCTTGGACCTC-3' and 5'CTTCTAGATGGATCCGACGT-3'; the underlines indicate the positions of substituted bases. The flanking primers were 5'CCTGGAAACTTCTTGGACCTC-3' and 5'CTTCTAGATGGATCCGACGT-3'; the underlines indicate the positions of substituted bases. The plasmid was sequenced using pcDNA3-HA-Gab1 as a template, which was then purified and cloned in frame to the Smal site of pGEX2T vector (Amersham Biosciences). The Gab1 p449A/p452A mutant was generated using site-directed mutagenesis by overlap extension using the polymerase chain reaction with mutagenic primers. The mutagenic primers were 5'TCGGCAATGACTGCACCATT-3' and 5'ATTGCGAGTCAATGCGACC-3'; the underlines indicate the positions of substituted bases. The flanking primers were 5'CCTGGAAACTTCTTGGACCTC-3' and 5'CTTCTAGATGGATCCGACGT-3'; the underlines indicate the positions of substituted bases. The plasmid was sequenced using pcDNA3-HA-Gab1 as a template, which was then purified and cloned in frame to the Smal site of pGEX2T vector (Amersham Biosciences). The Gab1 p449A/p452A mutant was generated using site-directed mutagenesis by overlap extension using the polymerase chain reaction with mutagenic primers. The mutagenic primers were 5'TCGGCAATGACTGCACCATT-3' and 5'ATTGCGAGTCAATGCGACC-3'; the underlines indicate the positions of substituted bases. The flanking primers were 5'CCTGGAAACTTCTTGGACCTC-3' and 5'CTTCTAGATGGATCCGACGT-3'; the underlines indicate the positions of substituted bases. The plasmid was sequenced using pcDNA3-HA-Gab1 as a template, which was then purified and cloned in frame to the Smal site of pGEX2T vector (Amersham Biosciences). The Gab1 p449A/p452A mutant was generated using site-directed mutagenesis by overlap extension using the polymerase chain reaction with mutagenic primers. The mutagenic primers were 5'TCGGCAATGACTGCACCATT-3' and 5'ATTGCGAGTCAATGCGACC-3'; the underlines indicate the positions of substituted bases. The flanking primers were 5'CCTGGAAACTTCTTGGACCTC-3' and 5'CTTCTAGATGGATCCGACGT-3'; the underlines indicate the positions of substituted bases.

Cell Culture and Transfections—Cells used in this study were all maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. SYF (yes™; funny™) cells, wild-type control cells, and the SYF cells stably reexpressing chicken c-Src were described previously (43). Madin-Darby canine kidney (MDCK) cells stably expressing the Src kd mutant were established by co-transfection of pcDNA3 with pEVX-Src kd in a 1:1 ratio and selection in the medium containing 0.5 mg/ml G418. NIH/3T3 cells stably expressing c-Src Y527F mutant, or v-Src were established by co-transfection of pcDNA3 with pEVX-cSrc, pEVX-SrcY527F, or pM-vSrc and selection in the medium containing 0.5 mg/ml G418. For transient transfections, human embryonic kidney (HEK) 293 cells (5 x 10⁵) were seeded on a 6-cm culture dish. 18 h later, the cells were incubated with the mixture of plasmid (1--2 µg) and LipofectAMINE (10 µl) for 5 h and lysed in 1% Nonidet P-40 lysis buffer 24 h later. For HGF stimulation, cells were serum-starved for 18 h and treated with 10 ng/ml HGF for 15 min.

Immunoprecipitations, Immunoblotting, and in Vitro Kinase Assays—Cells were lysed in 1% Nonidet P-40 lysis buffer (1% Nonidet P-40, 20 mM Tris-HCl, pH 8.0, 137 mM NaCl, 10% glycerol, and 1 mM Na₂VO₃) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 0.2 mM trypsin inhibitor unit/ml apoprotein, and 20 µg/ml leupeptin). The lysates were centrifuged for 10 min at 4 °C to remove debris, and the protein concentrations were determined using the Bio-Rad protein assay. For immunoprecipitation, aliquots of lysates were incubated with 1 µg of polyclonal anti-Gab1, anti-Met, or anti-Src (N-16) or monoclonal anti-HA for 1.5 h at 4 °C. Immunocomplexes were collected on Protein-A-Sepharose beads. For monoclonal antibodies, Protein A- Sepharose beads were coupled with rabbit anti-mouse IgG (1 µg) before use. The beads were washed three times with 1% Nonidet P-40 lysis buffer, boiled for 3 min in SDS sample buffer, subjected to SDS-polyacrylamide gel electrophoresis, and transferred to nitrocellulose (Schleicher and Schuell). Immunoblotting was performed with appropriate antibodies using the Amersham Biosciences enhanced chemiluminescence system for detection.

RESULTS

Deficiency in the Src Family Kinases Decreases HGF-induced Gab1 Phosphorylation—Although Gab1 associates with activated Met and is phosphorylated on specific tyrosine residues in response to HGF stimulation, it is not clear whether Met is the only kinase responsible for HGF-induced Gab1 phosphorylation. To explore the role of the Src family kinases in this event, the tyrosine phosphorylation of Gab1 was first compared between Src/Yes/Fyn (SYF) null cells and their wild type counterparts. When the cells were grown in the medium supplemented with 10% serum, the tyrosine phosphorylation of Gab1 in SYF cells was only 20% of that in the control cells (Fig. 1A). To evaluate the relative contribution of the Src family kinases to HGF-induced Gab1 phosphorylation, SYF cells and the control cells were serum-starved and then treated with HGF for 15 min. Although HGF stimulation increased the phosphorylation of Gab1 in both SYF cells and the control cells, the Gab1 phosphorylation in SYF cells was 40% lower than that in the control cells (Fig. 1B). Reexpression of c-Src into SYF cells enhanced the ability of HGF to stimulate Gab1 phosphorylation (Fig. 1C). These results together suggest that in addition to Met, Src family kinases account partially for Gab1 phosphorylation upon HGF stimulation.

Increased Expression of c-Src Enhances HGF-induced Gab1 Phosphorylation, and, in Contrast, Expression of Src Kinase-deficient Mutant Suppresses It—To examine the effect of c-Src overexpression on HGF-induced Gab1 phosphorylation, HA-epitope-tagged Gab1 (HA-Gab1) was transiently co-expressed with c-Src or its kd mutant in HEK293 cells (Fig. 2A). In the absence of HGF stimulation, increased expression of c-Src by itself was not sufficient to induce Gab1 phosphorylation. Upon HGF stimulation, elevated expression of c-Src substantially promoted HGF-induced phosphorylation of Gab1, and, in contrast, expression of the Src kd mutant suppressed it. To exclude the possibility that the effect of c-Src or its kd mutant on Gab1 phosphorylation was indirectly through its modulation on the activity of Met, thereby leading to alteration on Gab1 phosphorylation, the activation of Met was measured by immunoblotting.
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Oncogenic Src Persistently Induces Gab1 Phosphorylation—To examine the effect of oncogenic Src on Gab1 phosphorylation, HA-Gab1 was co-expressed with the activated c-Src mutant (Y527F) or v-Src in HEK293 cells (Fig. 2A). Consistently, stable expression of Src kd mutant in MDCK cells significantly suppressed the total Src activity and Gab1 phosphorylation without an effect on Met activation (Fig. 2B). Accordingly, HGF-induced Gab1 phosphorylation was ~60% inhibited by the specific Src inhibitor PP1 in MDCK cells (Fig. 2C). Notably, PP1 substantially inhibited the activating phosphorylation of Src at Tyr416 but had no effect on Met activation induced by HGF stimulation (Fig. 2C).

Fig. 1. Deficiency in the Src family kinases decreases HGF-induced Gab1 phosphorylation. A, SYF cells and their wild type counterparts (control) were grown in the medium supplemented with 10% serum. To analyze the tyrosine phosphorylation of Gab1, Gab1 proteins were immunoprecipitated (IP) by anti-Gab1 and subjected to immunoblotting (IB) with anti-phosphotyrosine (PY) or anti-Gab1. The tyrosine phosphorylation of Gab1 was quantified and expressed as -fold relative to that of the control cells. Whole cell lysates (WCL) were immunoprecipitated with anti-Gab1 and subjected to immunoblotting with anti-Src to verify the expression of Src. B, the cells as described for A were serum-starved and treated with (+) or without (-) 20 ng/ml HGF for 15 min. The tyrosine phosphorylation of Gab1 was analyzed as described for A and expressed as -fold relative to that of the serum-starved control cells. C, the SYF cells reexpressing c-Src (SYF/Src) and the control SYF cells were serum-starved and stimulated with HGF for 15 min. The tyrosine phosphorylation of Gab1 was analyzed as described for A.

Src Directly Phosphorylates and Associates with Gab1—Experiments to this point clearly indicate that Src is involved in HGF-induced Gab1 phosphorylation. To examine whether Src directly phosphorylates Gab1, GST-Gab1 fusion proteins were purified and added to in vitro kinase assays as a substrate (Fig. 4A). The result showed that GST-Gab1, but not GST alone, was phosphorylated by Met and c-Src. C-terminal Src kinase was used as a negative control to demonstrate the specificity of the assay, which did not phosphorylate Gab1. Next, we performed co-immunoprecipitation to demonstrate the in vivo association of Src with Gab1. In the SYF cells stably reexpressing Src, the Src-Gab1 association was enhanced upon HGF stimulation (Fig. 4B). In vitro, the SH2 domain of Src bound to HA-Gab1 transiently expressed in HEK293 cells in a manner dependent upon HGF (Fig. 4C), in accord with the above findings that HGF promotes the in vivo association of Src with Gab1. To examine whether the Met-mediated Gab1 phosphorylation provides docking site(s) for the SH2 domain of Src, Gab1 in SYF cells was examined for its binding to the SH2 domain of Src (Fig. 4D). The result showed that more Gab1 from HGF-treated SYF cells bound to the SH2 domain of Src than that from serum-starved SYF cells, supporting the possibility that Met-mediated phosphorylation of Gab1 may initially provide binding site(s) for the SH2 domain of Src.

It is already known that the COOH-terminal SH3 domain of Grb2 binds to Gab1 with high affinity (18, 24). Our result showed that the SH3 domain of Src bound to Gab1 as efficiently as the SH3 domain of Grb2 in vitro. Moreover, although the SH2 and SH3 domains of Src could bind to Gab1, the SH3 domain of Src appeared to be more efficient than its SH2 domain in this aspect (Fig. 4C). Gab1 contains six proline-rich sequences, two of which have been demonstrated to serve as the binding sites for the COOH-terminal SH3 domain of Grb2 (24). Because the preferential binding sequences for the SH3 domains of Src and Grb2 were suggested to be different (44), we reasoned that the SH3 domain of Src might bind to proline-rich sequences of Gab1, to which the SH3 domain of Grb2 does not bind. On this basis, a Gab1 mutant (P449A/P452A) was generated by substitutions of two proline residues with alanine in the proline-rich sequence of Gab1, 445PMNPNPSP457, which displays the class II SH3 ligand PXXPXR motif. The Gab1 P449A/P452A mutant had a marked (~50%) decrease in association with Src (Fig. 4E), supporting the significance of the SH3 domain-mediated interaction between Src and Gab1 in vivo.

Src-stimulated Gab1 Phosphorylation Potentiates HGF-induced Activation of ERK and AKT—Gab1 is known to be important for HGF-induced activation of ERK and AKT (36). We found that, in HEK293 cells, increased expression of Gab1 alone only slightly increased HGF-induced activation of ERK and AKT (Fig. 5A). However, simultaneous expression of Gab1 and Src selectively potentiated HGF-induced activation of ERK.
FIG. 2. Effect of c-Src wild type (wt), its kd mutant, or the specific Src inhibitor PP1 on HGF-induced Gab1 phosphorylation. A, HA epitope-tagged Gab1 (HA-Gab1) was co-expressed with wild type (wt) Src or its kinase-deficient (kd) mutant in HEK293 cells. 24 h after transfection, the cells were serum-starved and treated with (+) or without (−) HGF for 15 min. To analyze the tyrosine phosphorylation of HA-Gab1, HA-Gab1 proteins were immunoprecipitated by anti-HA and analyzed by immunoblotting with anti-PY or anti-HA. The tyrosine phosphorylation of HA-Gab1 was measured and expressed as fold relative to that of the serum-starved cells. Whole cell lysates were analyzed by immunoblotting with anti-Src, anti-phospho-Met, or anti-Met. B, MDCK cells stably expressing the Src kd mutant were serum-starved and treated with or without HGF for 15 min. The tyrosine phosphorylation of Gab1 was measured as described in the legend to Fig. 1A. Whole cell lysates were analyzed by immunoblotting with anti-Src or anti-phospho-Met. The activity of Src was measured by an in vitro kinase assay as described under “Experimental Procedures.” C, MDCK cells were treated with or without HGF for 15 min in the presence or absence of the specific Src inhibitor PP1 at 10 μM. The tyrosine phosphorylation of Gab1 was analyzed as described in the legend to Fig. 1A. Whole cell lysates were analyzed by immunoblotting with anti-phospho-Src, anti-Src, or anti-phospho-Met.
and AKT, but not c-Jun N-terminal kinase, concomitant with increased tyrosine phosphorylation of Gab1. To further confirm the contribution of Src-mediated Gab1 phosphorylation to ERK activation, Src was transiently expressed in SYF cells, and its effects on Gab1 phosphorylation and ERK activation were measured (Fig. 5B). Our result showed that HGF-induced phosphorylation of Gab1 was enhanced along with increased expression of Src, correlated with increased activation of ERK upon HGF stimulation. These results together suggest that Src may play a role in HGF-induced activation of ERK and AKT through its phosphorylation on Gab1.

DISCUSSION
Although it is generally accepted that Met is the kinase responsible for HGF-induced Gab1 phosphorylation, it remains possible that other kinase(s) may participate in this event. Because both Src and Gab1 are recruited by activated Met, we speculated that Src might be able to phosphorylate Gab1 upon

![Fig. 3. Oncogenic Src persistently induces Gab1 phosphorylation independent of HGF stimulation. A, HA-Gab1 was transiently co-expressed with constitutively active SrcY527F mutant, v-Src, or Tpr-Met in HEK293 cells. 24 h after transfection, the cells were serum-starved and treated with (+) or without (−) HGF for 15 min. The tyrosine phosphorylation of HA-Gab1 was analyzed as described in the legend to Fig. 2A. Whole cell lysates were analyzed by immunoblotting with anti-phospho-Met, anti-Met, or anti-Src. The expression of Tpr-Met was verified by immunoblotting with anti-Met. The activity of Met was measured by an in vitro kinase assay using GST-Gab1 as a substrate. B, NIH3T3 cells stably expressing Src wild type (wt), SrcY527F mutant, or v-Src were serum-starved and subjected to analysis for the tyrosine phosphorylation of Gab1. Whole cell lysates were analyzed by immunoblotting with anti-phospho-Src, anti-Src, or anti-phospho-Met.](image-url)
HGF stimulation. In this study, we provide several lines of evidence to support a role for Src to phosphorylate Gab1 in response to HGF stimulation. First, the extent of HGF-induced Gab1 phosphorylation in the Src family kinase-deficient cells is 40% lower than that in the wild type control cells (Fig. 1B). Second, increased expression of Src enhances HGF-induced Gab1 phosphorylation (Fig. 2A). Third, the expression of Src kd mutant, which serves as a dominant negative version of Src, significantly (~60%) inhibits HGF-induced Gab1 phosphorylation (Fig. 2A and B). Fourth, the ability of HGF to stimulate Gab1 phosphorylation is partially (~60%) inhibited by the specific Src inhibitor PP1 (Fig. 2C). Fifth, Src directly phosphorylates purified Gab1 in vitro (Fig. 4A). These data together strongly suggest that Src plays a crucial role in HGF-induced Gab1 phosphorylation. However, no matter what approaches we employed in this study, involving the use of SYF cells, Src kd mutant, and the Src inhibitor PP1, the HGF-induced Gab1 phosphorylation could only be partially inhibited by 40–60%, suggesting that in addition to Src, other kinase(s), most likely Met, is important for HGF-induced Gab1 phosphorylation.

We show in this study that in the absence of HGF stimulation, an elevated level of c-Src by itself is not sufficient to induce Gab1 phosphorylation (Figs. 2A and 3B), and, conversely, expression of oncogenic v-Src leads to persistent phosphorylation of Gab1 independent of HGF stimulation (Fig. 3). In addition, we found that the expression of c-Src or its kd mutant has no effect on HGF-stimulated activation of Met (Fig. 2), indicating that the effects of c-Src and its kd mutant on
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Gab1 becomes tyrosine-phosphorylated in response to diverse extracellular stimuli, including various cytokines and growth factors (29–34). However, so far only four protein-tyrosine kinases have been reported to directly phosphorylate Gab1, including epidermal growth factor (EGF) receptor (30), insulin receptor (31), Met (18) (this study), and Src (this study). The in vitro phosphorylation sites of Gab1 for EGF receptor and insulin receptor have been determined (30, 31), which reveals a very similar array of phosphorylation sites for these two receptors. However, among the identified phosphorylation sites on Gab1, EGF receptor has a predominant (∼50%) phosphorylation of Gab1 at Tyr657 (30), a binding site for the SH2 domain of SHP2 phosphatase (45). On the other hand, insulin receptor predominantly (75%) phosphorylates Gab1 at Tyr447, Tyr772, and Tyr641, which are in YXXM motifs potentially for phosphatidylinositol 3-kinase binding (31). Because Met-phosphorylated sites of Gab1 have not been determined, it is not clear whether the phosphorylation sites for Met are similar to those for EGF receptor and insulin receptor. A previous study (25) using in vivo 32P labeling and phosphopeptide mapping demonstrated that the in vivo phosphorylation sites of Gab1 in response to EGF stimulation are the same with those in response to HGF stimulation, suggesting that the phosphorylation sites of Gab1 for EGF receptor and Met may be similar, if not the same. Again, even if this is the case, Met and EGF receptor may have their own preferential phosphorylation sites among all possible ones, as discussed above for EGF receptor and insulin receptor. In this study, we demonstrate that in addition to Met, Src is another kinase responsible for HGF-induced Gab1 phosphorylation. It is not clear whether Src and Met predominantly phosphorylate Gab1 at different tyrosine residues. To clarify this, mass spectrometry will be employed to determine the phosphorylation sites of Gab1 for Src and Met, from which the relative phosphorylation intensity among phosphorylated tyrosine residues will be measured.

The association of Src with Gab1 is another novel aspect of our findings. We demonstrate that both of the SH2 and SH3 domains of Src are involved in this association (Fig. 4C). Because Src and Gab1 could be co-precipitated from the cells in the absence of HGF stimulation (Fig. 4B), rendering it possible that Src is constitutively associated with Gab1 in resting cells. Mutation that disrupts one of the proline-rich sequences of Gab1 partially (∼50%) decreases the binding of Src to Gab1 in serum-starved cells (Fig. 4E), supporting the existence of SH3-mediated interaction in vivo and suggesting that the SH3 domain of Src may bind to more than one proline-rich sequences of Gab1. Experiments are in progress to determine the proline-rich sequences of Gab1 bound to the SH3 domain of Src. Moreover, the association of Src with Gab1 is increased upon HGF stimulation, concomitant with increased Gab1 phosphorylation (Fig. 4B). It is possible that activated Met may initially phosphorylate Gab1 at certain tyrosine residues, which in turn recruit the binding of the SH2 domain of Src, leading to activation of Src and subsequent phosphorylation of Gab1 by Src. Alternatively, Src may be initially recruited and activated by Met, which in turn phosphorylates Gab1, thereby creating binding sites for its own SH2 domain. These two possibilities are not mutually exclusive; however, the former one was demonstrated in Fig. 4D, which shows that the SH2 domain of Src binds to Gab1 from HGF-treated SYF cells, in which the Gab1 phosphorylation is caused by activated Met.

Recently, the Src family kinases were reported to phosphorylate Gab2, another Gab family protein, in response to EGF stimulation (46). Interestingly, the association of Gab2 with amplified downstream signals may account at least partially for the ability of v-Src to cause cell transformation.
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Src seems to be constitutive and requires the proline-rich sequences of Gab2. Simultaneous mutations of two proline-rich sequences of Gab2 completely abolishes its association with Src and significantly (more than 70%) reduces its phosphorylation in response to EGF stimulation, suggesting that direct binding between Src and Gab2 is important for Src-mediated Gab2 phosphorylation. Whether direct binding to Gab1 is required for Src to phosphorylate Gab1 remains to be examined. Moreover, Gab1 and Gab2 are expressed ubiquitously, both of which interact with activated Met (47). It is not known whether Src also participates in HGF-induced Gab2 phosphorylation. If it is the case, it will be of importance to determine whether Src-mediated phosphorylation of Gab1 and Gab2 relays unique signals that trigger MAPK activation in response to extracellular stimuli. In this study, we found that the Src-mediated phosphorylation of Gab1 selectively augments the activation of ERK and AKT upon HGF stimulation (Fig. 5).

In summary, we demonstrate in this study that Src plays a crucial role in HGF-induced Gab1 phosphorylation not only by its direct act to phosphorylate Gab1 but also by its stable association with Gab1, which may facilitate Gab1 phosphorylation by Met or Src. Our findings add a new and potentially important substrate to the list of proteins phosphorylated by Src. Because many growth factors and cytokines known to induce Gab1 phosphorylation often activate Src, it is possible that Src or its family members are generally involved in Gab1 phosphorylation in response to various extracellular stimuli. In accordance with this assumption, we have found that Src is also important for increased phosphorylation of Gab1 in response to EGF stimulation.²

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