ERK Regulates the Hepatocyte Growth Factor-mediated Interaction of Gab1 and the Phosphatidylinositol 3-Kinase*

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Based on our previous observations that active ERK associates with and phosphorylates Gab1 in response to HGF, and the prediction that the ERK phosphorylation site is adjacent to one of the phosphatidylinositol 3-kinase (PI3K) SH2 binding motifs, we examined the possibility that ERK phosphorylation can regulate the Gab1/PI3K association. The HGF-mediated association of Gab1 with either full-length GST-p85 or its isolated N- or C-terminal SH2 domains was inhibited by ~50% in the setting of ERK inhibition, a result confirmed by co-immunoprecipitation of the native proteins. A 14- amino acid peptide encoding 472YVPMPT477 (one of the major p85 binding sites in Gab1 and the predicted ERK phosphorylation site) was synthesized with either phosphotyrosine alone (pY), or phosphotyrosine + phosphothreonine (pYT). In both pull-down assays and competition assays, pYT demonstrated a higher affinity for p85 than did pY alone. Finally, examination of the phosphorylation state of Akt after HGF stimulation revealed that ERK inhibition resulted in a decrease in Akt activation at both 5 and 10 min. These results suggest that activated ERK can phosphorylate Gab1 in response to HGF stimulation and thereby potentiate the Gab1/PI3K association and subsequent PI3K activation.

The docking protein Gab1 associates with several receptor tyrosine kinases known to induce cell morphogenesis, including the hepatocyte growth factor (HGF) receptor, c-Met, and the epidermal growth factor (EGF) receptor. Following receptor activation, Gab1 associates and is tyrosine-phosphorylated, in turn recruiting multiple SH2 domain containing signal transducing proteins such as SHC, SHP2, phosphoinositide 3-kinase (PI3K), Crk/CRK-L, and phospholipase Cγ (1–6). These proteins form an intricate signaling complex that regulates cell migration, morphology, and tubule formation. The critical nature of Gab1 signaling in normal cell biology has been demonstrated by the finding that Gab1-deficient mice die in utero (7) and display developmental defects in myotomes, placentas, and liver that recapitulate the loss of HGF and c-Met (8).

The phenotypic effects of Gab1 signaling have been found to be critically dependent on Gab1 interactions with the PI3K. The PI3K is a heterodimer composed of an 85-kDa regulatory subunit (p85) and a 110-kDa catalytic subunit (p110). Two SH2 domains are located in the C-terminal region of the p85 subunit and have been shown to bind to membrane-associated tyrosine-phosphorylated proteins, resulting in recruitment of the PI3K to the membrane and activation of the lipid kinase activity of the 110-kDa subunit. This activity results in the formation of phosphoinositides 3,4,5-trisphosphate (PI3,4,5P3) at the membrane, which in turn serves to recruit and help activate such proteins as protein kinase C (PKC) and Akt (9, 10). The activated PI3K has been demonstrated to result in the regulation of various cellular activities including proliferation (11), differentiation (12), and prevention of apoptosis (13). We have found that PI3K activity is critical for c-Met-mediated cell migration and in vitro tubulogenesis (14), at least in part by activating PKC (15). Another role for PI3K in morphogenic signaling has been demonstrated by the observation that the PH domain of Gab1 binds PI3,4,5P3, which serves to recruit Gab1 to the membrane following PI3K activation (1, 16, 17). Loss of the PH domain of Gab1 results in loss of epithelial morphology following HGF stimulation, despite recruitment of Gab1 to the c-Met receptor and its phosphorylation (16).

We have recently demonstrated that in addition to SH2 and SH3 domain containing proteins, Gab1 also interacts with phosphorylated ERK1 and 2 following HGF or EGF stimulation of epithelial cells (18). The association of ERK with Gab1 results in the phosphorylation of Gab1 on serine and threonine residues, primarily in the Met binding domain (MBD). This domain also includes one of three major consensus sequences for PI3K binding (472YVPMT477), as well as a predicted ERK phosphorylation site immediately adjacent (472YVPMP477). We therefore hypothesized that ERK phosphorylation of this site in vivo might result in secondary regulation of p85 SH2 domain binding to Gab1. In the present study, we confirm that HGF stimulation results in Gab1-p85 association, and demonstrate that this interaction is partly dependent on ERK activation. Furthermore, we show that inhibition of ERK activation not only decreases the association of p85 with Gab1 but also decreases the downstream phosphorylation of Akt.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—Immortalized mIMCD-3 epithelial cells (19) were maintained using standard cell culture techniques in Dulbecco’s modified Eagle’s medium/F-12 containing 10% fetal bovine serum.

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¶ The abbreviations used are: HGF, hepatocyte growth factor; EGF, epidermal growth factor; PI3K, phosphatidylinositol 3-kinase; PI3,4,5P3, phosphoinositide 3,4,5-trisphosphate; PKC, protein kinase C; MBD, Met binding domain; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase/ERK kinase; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; PDGFR, platelet-derived growth factor; PDGFR, PDGFR receptor; PKB/Akt, protein kinase B.
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Growth Factor Stimulation and MEK Inhibition—mIMCD-3 cells were serum-starved for 24 h in Dulbecco's modified Eagle's medium/F-12 and then stimulated with HGF (40 ng/ml) or vehicle control for 20 min. To inhibit ERK activation, cells were pre-treated with 10 μM U0126 (Promega) for 20 min prior to HGF stimulation. This dose was chosen following a dose-response curve in these cells in which 1 μM U0126 was found to inhibit HGF-dependent ERK activation by 90%, whereas 10 μM U0126 inhibited ERK activation completely (22). At doses less than 50 μM, U0126 has been found to be selective for the MEK family of dual-specificity kinases (23), with inhibition of MEK1, MEK2, and MEK5.

Following HGF stimulation, cells were lysed in 800 μl of ice-cold lysis buffer containing 50 mM HEPEs, pH 7.4, 150 mM NaCl, 5 mM dithiothreitol, 5% glycerol, and 1 μg/ml leupeptin until use. Control experiments were performed with GST-Sepharose beads generated by expression of GST fusion proteins. These constructs were kindly provided by Dr. Lucia Rameh (Harvard University). Expression of the fusion proteins was induced in BL21 competent bacteria with isopropyl-β-d-thiogalactoside as previously described (18). Briefly, the bacteria were lysed with sodium deoxycholate, and the supernatant was collected and incubated with a 50% slurry of glutathione-Sepharose 4B (Amersham Pharmacia Biotech). The beads were washed and resuspended in 150 mM HEPEs, pH 7.4, 150 mM NaCl, 5 mM dithiothreitol, 5% glycerol, and 1 μg/ml leupeptin under continuous agitation. Mass of protein loaded onto beads was determined virtually using Coomassie Blue-stained SDS-PAGE with albumin standards. One mg of whole cell lysate protein amounts were estimated virtually using Coomassie Blue-stained SDS-PAGE with albumin standards. One mg of whole cell lysate protein containing ~0.1 μg of endogenous p85 was then incubated with 50 μg of the GST fusion protein of interest for 30 min at 4 °C. The glutathione beads were washed three times with ice-cold lysis buffer followed by resolution via SDS-polyacrylamide gel electrophoresis. Proteins were electrophoretically transferred onto Immobilon-P transfer membrane (Millipore) using a Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad) for 90 min at 18 V. Usually, membranes were blocked for 1 h at room temperature with 5% nonfat dry milk in wash buffer containing 10 mM Tris, pH 7.5, 100 mM NaCl, and 0.1% Tween-20 (TBS-T). After five additional rinses with TBS-T, the membrane was incubated with primary antibody to p85 (Upstate Biotechnology Inc.). After rinsing with TBS-T, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody at 1:5000 dilution in TBS-T for 60 min at room temperature. Blots were visualized by an ECL system (Amersham Pharmacia Biotech).

Co-immunoprecipitation and Western Blotting—600 μg of mIMCD-3 cell lysates were pre-cleared for 1 h at 4 °C with protein A-Sepharose CL 4B (1:1 slurry in phosphate-buffered saline (Amersham Pharmacia Biotech) and centrifuged at 5000 rpm for 2 min at 4 °C. For Gab1 overexpression experiments, lysates from mIMCD-3 cells transiently transfected with FLAG-Gab1 or vector control were utilized. Transfections were performed with LipofectAMINE 2000 (Life Technologies) as previously described (18). Supernatants were incubated with the appropriate antibody (anti-Gab1 or anti-p85 (Upstate Biotechnology Inc.)) overnight followed by addition of protein A-Sepharose CL4B. After incubating for another 2 h at 4 °C, samples were centrifuged at 5000 rpm for 2 min at 4 °C and washed three times with 0.5 ml of lysis buffer prior to resolution via SDS-PAGE. Western blot was performed using the methods mentioned above or following the protocol of the primary antibody manufacturer.

Peptide Synthesis and p55 Pull-down—Three 14-amino acid peptides corresponding to the Gab1 amino acid sequence 466–479 were synthesized at the Tufts University Core Facility (Boston, MA). The peptide sequences are: PIQEPNYVPMTPGT (pY); PIQEPNyPYPMTPT (py); and PIQEPPPYVPMTPPTG (pYpT). The N-terminal of these peptides was conjugated to biotin and 30 nmol of each peptide was bound to 1 ml of SoftLink Soft Release Avidin Resin (Promega). The affinity of these peptides for p85 was examined by incubating 1 mg of mIMCD-3 whole cell lysate with resin-conjugated peptide at the indicated concentration for 60 min, followed by washing three times in lysis buffer and separation by SDS-PAGE. Quantitation of p85 was performed by pooling the data from five peptide pull-down experiments and normalizing to the amount of p85 binding to py peptide at 1 pm using the National Institutes of Health IMAGE program.

For competition experiments, we utilized the bacterial co-expression of the inter-kinase PDGF receptor p85 binding domain and the photophoryrosine kinase Elk to generate a phosphorylated GST-PDGFR fusion protein with a high affinity for p85. These constructs were kindly provided by Saskia Brackmann (Harvard University) and the GST fusion protein was expressed as previously described (see above (24)). One mg of mIMCD-3 whole cell lysates was incubated with the appropriate concentration of non-conjugated synthetic peptide (pY or pYpT) in solution at 4 °C for 60 min, followed by incubation of the mixture for 15 min with the GST-PDGFR receptor fusion protein on glutathione beads. The beads were then washed three times with lysis buffer to remove p85 that was not bound to the GST-PDGFR, and the bound p85 was detected by SDS-PAGE followed by Western blotting.

Statistical Analysis—All experiments were repeated on at least three separate occasions. Quantification of ECL immunoblots was performed using the IMAGE program. Values are expressed as means ± S.E. Results were analyzed using the Student t test. A value of p < 0.05 was considered significant.

RESULTS

Gab1 Is Phosphorylated by Activated ERK in Response to e-Met Engagement—We have previously shown that Gab1 acts as a substrate for ERK2 in vitro and following overexpression of Gab1 and constitutively active MEK1 in HEK cells (18). ERK phosphorylation of Gab1 was determined by both gel retardation of the phosphorylated protein as well as peptide mapping. To determine if growth factor-induced ERK activation could mediate Gab1 phosphorylation, we examined the gel mobility of Gab1 following stimulation with HGF. Prolonged electrophoresis of unstimulated mIMCD-3 cells revealed that Gab1 runs as a doublet at ~110 kDa, with the majority of the protein...
FIG. 3. The HGF-stimulated Gab1-p85 association in intact cells is partially ERK-dependent. A, either FLAG-tagged full-length Gab1 (Flag-Gab1) or empty pFLAG-CMV-II vector (V) was transiently transfected into mIMCD-3 cells followed by HGF (40 ng/ml) stimulation for 20 min or U0126 (10 μM) pretreatment. Anti-FLAG immunoprecipitation and anti-p85 immunoblotting reveal that HGF induces a marked increase in Gab1-p85 association that is partially inhibited by U0126 (upper panel). Immunoprecipitation of either endogenous p85 (B) or Gab1 (C) from untransfected cells stimulated with HGF ± pretreatment with U0126 confirms the marked increase in the co-immunoprecipitation of the two proteins following HGF stimulation and again reveals a substantial inhibition of this increase in the setting of ERK inhibition. Quantitation of the co-immunoprecipitation (D), normalized to 1 for HGF stimulation alone, reveals a 50% decrease in the co-immunoprecipitation of Gab1 and p85 following U0126 treatment (*p < 0.01, n = 3).

The Gab1-PI3K Interaction in Intact Cells Is Partially ERK-dependent—To investigate the role of MAPK phosphorylation on the interaction between Gab1 and PI3K in intact cells, we transiently transfected FLAG-tagged Gab1 into mIMCD-3 cells and examined the interaction between FLAG-Gab1 and p85. We found that FLAG-Gab1 interacted with p85 upon HGF stimulation and that this association was also substantially inhibited by pre-treatment with U0126 (Fig. 3A). We next examined the interaction of native Gab1 with native p85 by co-immunoprecipitation. In both anti-Gab1 immunoprecipitates and anti-p85 immunoprecipitates, we detected association of Gab1 and p85 following stimulation of cells with HGF (Fig. 3, B and C). Again, following inhibition of MAPK activation with U0126, there was a substantial reduction in co-immunoprecipitation of Gab1 and p85. Quantitation of the effect of MAPK inhibition on Gab1-p85 association was performed by pooling the data from three anti-Gab1 immunoprecipitation experiments and normalizing to the amount of p85 immunoprecipitated following HGF stimulation in the absence of U0126. This revealed a 50% decrease in the association of Gab1 and p85 in the setting of MEK inhibition (Fig. 3D, n = 3, p < 0.01). These results confirm that p85 associates with Gab1 in an HGF-dependent manner, presumably due to tyrosine phosphorylation of Gab1 by c-Met, and further demonstrates that the affinity of Gab1 for p85 appears to be additionally modulated by ERK activation.

ERK Inhibition Does Not Appear to Alter c-Met-Gab1 Interactions—Based on our prior work demonstrating that ERK directly phosphorylates Gab1, we predicted that the decrease in p85-Gab1 interaction following U0126 treatment was due to a direct effect of phosphorylation of Gab1 near the p85 SH2 binding site. However, nonspecific effects of U0126 on the ability of HGF to induce c-Met activation and/or c-Met-Gab1 association and phosphorylation were also considered. To determine if treatment with U0126 altered c-Met phosphorylation following HGF stimulation, whole cell lysates from cells ± HGF were immunoprecipitated with anti-phosphotyrosine and c-Met was detected by immunoblotting. Upon HGF stimulation, c-Met was tyrosine phosphorylated equally in the presence and absence of pre-treatment with U0126 (Fig. 4A), dem-
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Phosphorylation at Thr

Blocking ERK Activation

ERK-mediated PKB/Akt Activation Is Partially Inhibited by Blocking ERK Activation—The activation of PKB/Akt is dependent on membrane localization and activation of the PI3K with subsequent PI3,4,5P3 production (28). Because inhibition of ERK activation decreased HGF-mediated Gab1/PI3K association, we postulated that membrane recruitment of the PI3K would be diminished and subsequent Akt activation decreased. To test this we examined the phosphorylation state of PKB/Akt in IMCD cells stimulated with HGF ± U0126. Treatment with

2 L. Cantley, personal communication.
of p85 for pYT was 3-fold, 1.7-fold, and 1.5-fold at 0.5 pM (compared with pY, the relative pull-down of pYT demonstrated greater pull-down of p85 than did pY. B, quantitative analysis of these competition assays reveals that pY decreased the association of p85 with the GST-PDGFR fusion protein by 60% whereas pYT decreased the association by 77% (n = 3; * p = 0.05 for pY versus pYT). Data were normalized to 1 for p85 pull-down by the GST-PDGFR in the absence of competitive peptide.

HGF resulted in a detectable increase in Akt phosphorylation by 2 min, with a further increase at 5 and 10 min (Fig. 7). In the setting of pretreatment with U0126, the initial activation of Akt 2 min after HGF stimulation was unchanged, but the further increases seen at 5 and 10 min were prevented.

**DISCUSSION**

In the present study we demonstrate that the addition of HGF to cells expressing the c-Met receptor results in both tyrosine phosphorylation of Gab1 as well as a decrease in the gel mobility of Gab1. The appearance of a slower mobility on SDS gels is typical of proteins that undergo phosphorylation on serine or threonine residues, particularly in the motif SP or TP. This is apparently because phosphorylation at these sites causes a change in the tertiary structure of the protein by creating a binding site for peptidyl-prolyl cis/trans isomerases such as Pin1 (25, 29). In our previous study, we had found that the serine/threonine kinase ERK interacted with the MBD of Gab1 directly and could phosphorylate Gab1 in vitro and in vivo resulting in an indistinguishable reduction in gel mobility (18). Consistent with this data, we now show that inhibition of ERK activation prevents the HGF-mediated decrease in Gab1 gel mobility, arguing that ERK may directly phosphorylate Gab1 following HGF stimulation. This suggests that ERK phosphorylation of this site might mediate ERK association and to be phosphorylated preferentially by ERK in vitro (18). Interestingly, this ERK phosphorylation site is embedded in the classic PI3K SH2 association site, YVPM, and mutation of the tyrosine residue in this site has been shown to markedly decrease PI3K binding (26, 33). This suggests that ERK phosphorylation of this site might regulate the interaction of Gab1 and the PI3K.

To test the role of endogenous ERK in the Gab1-PI3K association, we examined the ability of the p85 subunit of the PI3K to associate with Gab1 following HGF stimulation in the presence or absence of ERK inhibition. As expected, stimulation...
with HGF resulted in a marked increase in the affinity of p85 for Gab1, both in GST-p85 pull-down experiments and in co-immunoprecipitation of the native proteins. This increased affinity of Gab1 for p85 was reproduced equally for both the N- and C-terminal SH2 domains of p85, demonstrating its requirement for tyrosine phosphorylation of the Gab1 protein. However, pretreatment with the MEK inhibitor U0126 resulted in a substantial decrease in the p85-Gab1 interaction in both the pull-down and co-immunoprecipitation experiments, suggesting that ERK phosphorylation of Gab1 increases the affinity of Gab1 for p85. This result was unexpected, because ERK-mediated serine phosphorylation of the structurally similar docking protein, IRS-1, was found to have an opposite effect (34).

In the case of IRS-1, phosphorylation by several serine/threonine kinases, including ERK, PKC, and Akt, has been shown to decrease the affinity of IRS-1 for p85, at least in some cases by decreasing tyrosine phosphorylation of IRS-1 (34–36). Because the predicted ERK phosphorylation site in Gab1 is in the Met binding domain, we examined the possibility that inhibition of ERK activation was altering the association of c-Met and Gab1 and subsequently the tyrosine phosphorylation state of Gab1. We did not detect a difference in the association between Gab1 and its c-Met adapter protein Grb2, nor did we find a change in the total tyrosine phosphorylation state of Gab1 following HGF stimulation in the presence or absence of MEK inhibition. Thus it does not appear that treatment with U0126 prevents HGF-mediated c-Met activation or c-Met-dependent tyrosine phosphorylation of Gab1. Of note, because Gab1 is known to be potentially phosphorylated on at least 8 tyrosine residues (37), a selective loss of tyrosine phosphorylation of one of the residues critical for p85 association might not be detected by this method.

To determine whether phosphorylation of threonine 476 (the predicted ERK phosphorylation site in Gab1) alters the affinity of tyrosine 472 (the most important residue for p85 binding to Gab1 (33)) for binding the SH2 domain of p85, three peptides based on the 14 amino acids encompassing this site were synthesized. As expected, the non-phosphorylated peptide failed to interact with p85, whereas pY472 pulled-down p85 at concentrations as low as 1 μM. However, in both simple pull-down experiments and competition experiments using the native p85 binding site in the PDGFR, the dual-phosphorylated peptide (pYT) displayed a higher affinity for p85 than did pY. Thus, dual phosphorylation on both tyrosine and threonine at the (pYT) displayed a higher affinity for p85 than did pY. Thus, dual phosphorylation on both tyrosine and threonine at the predicted ERK phosphorylation site in Gab1 results in a higher affinity for p85 binding than does tyrosine phosphorylation alone. The antibody used in these experiments can recognize both pS85α and β, and the association of these isoforms with Gab1 is not well distinguished using the gel separation techniques presented. Therefore the association of either pS85α, pS85β, or both may be up-regulated in this setting. Conversely, the dual phosphorylation of Gab1 could result in a decreased affinity of Gab1 for an independent PI3K regulatory isoform such as p55.

To test whether this proposed dual phosphorylation of Gab1 actually results in an alteration in PI3K downstream signaling, we examined the effects of inhibition of ERK activation on Akt phosphorylation. Akt is a PH domain containing serine/threonine kinase that is recruited to the membrane by the PI3K product PIP_3, where it is phosphorylated and activated by the membrane-associated kinase PDK1, resulting in enhanced cell survival (28). Using an antibody specific for the activated state of Akt (anti-pT^308), we found that activated Akt was detectable within 2 min following HGF stimulation. In the absence of U0126 treatment, the phosphorylation state of Akt increased further at 5 and 10 min, consistent with a further increase in PIP_3 production at the membrane. In the setting of ERK inhibition, the initial increase in phospho-Akt was not altered, but the subsequent further increase at 5 and 10 min was entirely abolished.

Taken together, these data are most consistent with the model that c-Met activation results in Gab1 recruitment to the receptor followed by phosphorylation of Gab1 at multiple tyrosine residues, including Y472VPM. This results in association of PI3K with Gab1 and local production of PIP_3. In concert, HGF induces ERK activation with the subsequent association of Gab1 and ERK resulting in phosphorylation of Gab1 at Thr476 in the PI3K docking site. This dually phosphorylated motif then can act as a higher affinity site for the p85 subunit of the PI3K, leading to a further recruitment and/or stabilization of PI3K at the membrane and subsequently greater activation of PIP_3-dependent signaling pathways. The increase in Akt phosphorylation observed in the absence of ERK inhibition suggests yet another pathway whereby ERK activation may help promote cell survival and prevent apoptosis.

In conclusion, the HGF-mediated interaction of Gab1 and PI3K is dependent on tyrosine phosphorylation by the c-Met receptor, but is further enhanced by ERK-mediated phosphorylation of Gab1, possibly at Thr476. Threonine phosphorylation at this site results in a higher affinity of the PI3K binding site Y472VPM for p85, whereas inhibition of ERK activation decreased the p85 association with Gab1 and decreased the activation of the downstream kinase Akt. Because activation of the PI3K is critical for epithelial cell migration and morphogenesis, this result may in part explain our prior observations that HGF-mediated epithelial cell migration and tubule formation are dependent on MAPK activation (22).

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