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

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We have examined the ability of epidermal growth factor (EGF)-stimulated ERK activation to regulate Grb2-associated binder-1 (Gab1)/phosphatidylinositol 3-kinase (PI3K) interactions. Inhibiting ERK activation with the MEK inhibitor U0126 increased the HGF-stimulated association of Gab1 with either full-length glutathione S-transferase-p85 or the p85 C-terminal Src homology 2 (SH2) domain, a result reproduced by co-immunoprecipitation of the native proteins from intact cells. This increased association of Gab1 and the PI3K correlates with an increase in PI3K activity and greater phosphorylation of Akt. This result is in direct contrast to what we have previously reported following HGF stimulation where MEK inhibition decreased the HGF-stimulated association of Gab1 and p85. In support of this divergent effect of ERK on Gab1/PI3K association following HGF and EGF stimulation, U0126 decreased the HGF-stimulated association of p85 and the Gab1 c-Met binding domain but did not alter the EGF-stimulated association of p85 and the c-Met binding domain. An examination of the mechanism of this effect revealed that the treatment of cells with EGF + U0126 increased the tyrosine phosphorylation of Gab1 as well as its association with another SH2-containing protein, SHP2. Furthermore, overexpression of a catalytically inactive form of SHP2 or pretreatment with pervanadate markedly increased EGF-stimulated Gab1 tyrosine phosphorylation. These experiments demonstrate that EGF and HGF-mediated ERK activation result in divergent effects on Gab1/PI3K signaling. HGF-stimulated ERK activation increases the Gab1/PI3K association, whereas EGF-stimulated ERK activation results in a decrease in the tyrosine phosphorylation of Gab1 and a decreased association with the PI3K. SHP2 is shown to associate with and dephosphorylate Gab1, suggesting that EGF-stimulated ERK might act through the regulation of SHP2.

Grb2-associated binder-1 (Gab1)* has been identified in many cell types and appears to play a central role in multiple cell responses including proliferation, migration, tubulogenesis, cellular transformation, and apoptosis (1–6). Structural and functional studies suggest that Gab1 is a multisubstrate-docking protein functioning downstream of several receptor signaling pathways including the epidermal growth factor (EGF) receptor (EGFR), c-met, and the insulin receptor tyrosine kinases as well as cytokine receptors such as the gp130-associated interleukin-6 receptor and T and B cell antigen receptors (7–10). Similar to the Drosophila daughter of sevenless protein, DOS, and the insulin receptor substrate proteins 1 and 2 family, Gab1 consists of a PH domain at its N terminus, several proline-rich motifs in the C-terminus, and multiple tyrosine phosphorylation sites. But Gab1 is unique in that it contains a c-met binding domain (MBD) that includes the 13 amino acid c-met binding sequence, which mediates direct association with c-met (1, 11). The MBD also mediates indirect association with the activated EGFR via its proline-rich association with the SH3 domain of Grb2 (2, 11, 12). The PH domain has been found to be important for Gab1 localization and epithelial cellular morphogenesis in Madin-Darby canine kidney cells (5), whereas neoplastic transformation in SHE cells has been found to correlate with the loss of this domain (3).

Following ligand binding, Gab1 associates with activated c-met or EGFR and is phosphorylated on specific tyrosine residues, in turn recruiting a series of SH2 domain-containing proteins that initiate intracellular signaling cascades. One of the most important signaling proteins found to associate with Gab1 in response to various stimuli is the phosphoinositide 3-kinase (PI3K). Studies have shown that the activation of the PI3K is involved in a wide range of cellular responses including cell proliferation, differentiation, and prevention of apoptosis (13–15). We have previously demonstrated that the PI3K is required for HGF-mediated kidney epithelial cell migration and in vitro tubulogenesis (16). The importance of Gab1 for this response has been demonstrated in work by Maroun et al. (5) who found that the loss of association of Gab1 and the PI3K due to mutation of the PI3K binding sites in Gab1 results in a decrease of c-met-mediated tubulogenesis in Madin-Darby canine kidney cells. An association of the PI3K with Gab1 was also reported to be required for HGF-mediated cell survival and DNA repair (17).

Previously, it was believed that regulation of the PI3K association with Gab1 was mediated solely by receptor-dependent tyrosine phosphorylation of Gab1 on the PI3K SH2 binding motifs 447YVP451 and 472YVP476 in the MBD domain, and/or 585YVP at the carboxyl terminus. However, we have demonstrated that in addition to tyrosine phosphorylation, intracellular signal-regulated kinase; MEK, MAPK/extracellular signal-regulated kinase kinase; SHP2-WT, wild-type SHP2; SHP-CS, catalytically inactive SHP2; HGF, hepatocyte growth factor.
Gab1 is also phosphorylated on serine and threonine in response to ERK2 activation by HGF (12). In vitro phosphorylation studies revealed that ERK2 primarily phosphorylates Gab1 in the MBD domain, and an examination of the Gab1 sequence for potential ERK binding and phosphorylation sites revealed that the 472YVPM476 motif immediately following the 472YVPM476 PI3K binding site is a high probability ERK1/2 phosphorylation site within the MBD domain of Gab1. In a more recent study, we found that the phosphorylation of both Tyr472 and Thr476 resulted in a higher affinity of a YVPMTP-containing peptide for the PI3K than did the phosphorylation of Tyr472 alone. Thus, ERK1/2-mediated phosphorylation of this site can initiate a novel regulation of the Gab1/PI3K interaction. This was confirmed by demonstrating that HGF-stimulated association of the PI3K with Gab1 was partially dependent on ERK activation (7).

Our recent determination that HGF-stimulated epithelial cell morphogenesis requires ERK1/2 activation provides a potential physiologic role for ERK-regulated PI3K activation (18). Interestingly, in this same study, we found that EGF, but not HGF, activates ERK5 in addition to ERK1/2. The expression of a kinase-dead form of ERK5 in epithelial cells prevented EGF-stimulated morphogenesis, demonstrating that EGF and HGF use different MAPK signaling pathways for their morphogenic responses. Because the branching morphogenesis observed following EGF and HGF stimulation is phenotypically distinct, we decided to investigate the effects of EGF-stimulated ERK activation on the association of Gab1 with the PI3K. In contrast to the positive regulation of the Gab1/PI3K interaction that we found following HGF-stimulated ERK activation, EGF-stimulated ERK activation down-regulates the interaction of Gab1 and the PI3K. The investigation of the mechanism of this effect revealed that EGF-stimulated tyrosine phosphorylation of Gab1 was diminished in the setting of ERK activation, thereby decreasing the association of SH2-docking proteins.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Reagents**—Immortalized mIMCD-3 epithelial cells (19) or HEK cells were maintained using standard culture techniques in Dulbecco’s modified Eagle’s medium/F-12 containing 10% fetal bovine serum. Experiments were performed when the cells were 80–90% confluent. All reagents were obtained from Sigma unless otherwise noted.

**EGF 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 EGF (20 ng/ml, Sigma), HGF (40 ng/ml, Sigma), or vehicle control for 10 min. To inhibit ERK activation, cells were pretreated with 10 μM U0126 (Promega) for 20 min prior to EGF stimulation (7). U0126 has been shown to inhibit MEK1, MEK 2 (20), and MEK5 (18) at concentrations less than 50 μM, but it does not inhibit MEK3, MEK4, MEK6, MEK7, protein kinase C, protein kinase A, PDK1, or other tested serine/threonine kinases (20). U0126 was used rather than PD98059, because we have found that EGF-mediated ERK5 activation, which is critical for EGF-mediated mIMCD-3 cell morphogenesis, is fully inhibited by U0126 but only partially inhibited by PD98059 at concentrations ≥100 μM (18). Following EGF stimulation, cells were lysed in 800 μl of ice-cold radiolabel release lysis buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 0.5 mM DTT, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 2 μg/ml pepstatin A, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, and 1 mM Na3VO4.

**GST Fusion Protein Expression and Pull-down Assay**—The GST-p85 full-length N-terminal and C-terminal fusion proteins were expressed as described previously (7, 12). The bacteria were lysed with sodium deoxycholate, and the supernatants were collected and incubated with a 50 μg/ml GST-Sepharose 4B (Amersham Biosciences). The beads were washed and resuspended in 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 the empty pGEX-4T vector. Total GST fusion protein amounts were estimated visually using Coomassie Blue-stained SDS-PAGE with albumin standards. 1 mg of total protein from the appropriate mIMCD-3 whole cell lysates was then incubated with 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 resolubilization with SDS-PAGE. Proteins were electrophoretically transferred onto Immobilon-P transfer membranes (Millipore) using Trans-Blot SD semi-dry transfer cell (Bio-Rad) for 90 min at 120 volts. 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, blots were incubated with the appropriate primary antibody (Gab1 or p85, Upstate Biotechnology). After rinsing with TBS-T, 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 ECL system (Amersham Biosciences).

**Co-immunoprecipitation and Western Blotting**—800 μg of mIMCD-3 cell lysates were preclotted for 1 h at 4°C with protein A-Sepharose CL 4B (1:1 slurry in phosphate-buffered saline (Amersham Biosciences) and centrifuged at 5000 rpm for 2 min at 4°C. Supernatants were incubated with anti-Gab1 overnight followed by the 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 ice-cold lysis buffer prior to the resolution through SDS-PAGE. Western blot was performed using the methods mentioned above or following the protocol of the primary antibody manufacturer. For the resolution of the MBD domain of Gab1, lysates from mIMCD-3 cells transiently transfected with FLAG-MBD or vector control were used. Transfections were performed with LipofectAMINE 2000 (Invitrogen) as described previously (7, 12). Lysates were immunoprecipitated with anti-FLAG antibody (Sigma) as noted above.

For the expression of SHP2, HEK cells were transiently transfected with either the empty control vector, pIRES-OGF, or the vector encoding Myc-tagged wild-type SHP2 (SHP2-WT) or catalytically inactive SHP2 (SHP2-CS). These constructs, which independently express green fluorescent protein to allow rapid determination of transfection efficiency, were a kind gift of Dr. Anton Bennett. Cells were transfected using calcium phosphate as previously described (12).

**Gab1-Associated PI3K Activity Assay**—Immunoprecipitation of Gab1 from EGF and EGF-stimulated mIMCD-3 cells was performed as above. Immunoprecipitates were collected by centrifugation and washed twice with phosphate-buffered saline containing 1% Nonidet P-40 and 100 μM Na3VO4, twice with 100 mM Tris, 500 mM LiCl3, 100 μM Na3VO4, pH 7.5, and twice with 10 mM Tris, 100 mM NaCl, 1 mM EDTA, and 100 μM Na3VO4, pH 7.5. The pellets were then resuspended in 50 μl of the final wash buffer containing 12 mM MgCl2 and 20 μg of bovine serum albumin (BSA). To start the lipid kinase reaction, 10 μl of 40 μM ATP containing 30 μCi of [32P]ATP was added to each pellet and was incubated to allow incubation at room temperature for 10 min. The reaction was stopped by the addition of 20 μl of 8 M HCl and the lipids extracted using 160 μl of CHCl3/MeOH (1:1). The phases were resolved by thin-layer chromatography in MeOH/H2O/CHCl3/H2O-NH4OH (60:47:11:3.2) and visualized using the Storm PhosphorImager. Quantitation was performed on triplicate samples using ImageQuant software.

**Statistical Analysis**—All experiments were repeated on at least three separate occasions. Quantification of ECL immunoblots was performed using the NIH Image program. The values for co-immunoprecipitation experiments were normalized to the amount of immunoprecipitated protein and expressed as the mean ± S.E. The results were analyzed using the Student’s t test. A value of p < 0.05 was considered significant.

**RESULTS AND DISCUSSION**

**EGF-dependent ERK Activation Inhibits Gab1/PI3K Association**—Our observation that HGF and EGF appear to use different ERK signaling pathways for epithelial cell morphogenesis (18) led us to examine the effect of ERK activation on EGF-stimulated Gab1/PI3K association. In these experiments, we tested the ability of a bacterially expressed GST fusion protein containing the full-length p85 subunit of the PI3K to associate with Gab1 from EGF-stimulated mIMCD-3 cell lysates. Similar to previous results in Madin-Darby canine kidney cells (5), EGF stimulation was found to induce the interaction of Gab1 and p85 in vitro (Fig. 1A, upper panel). To examine the role of ERK in regulating this association, cells
EGF-dependent ERK Activation Inhibits Gab1/PI3K Association

FIG. 1. The EGF-stimulated interaction of Gab1 with p85 is up-regulated by inhibition of ERK activation. A. Bacterially expressed full-length GST-p85 (gst-p85 pulldown, upper panel), N-terminal SH2 domain of p85 (gst-NSH2 pulldown, middle panel) and C-terminal SH2 domain of p85 (gst-CSH2 pulldown, lower panel) were used for pull-down experiments of control and EGF-stimulated mIMCD-3 cell lysates ± pretreatment with U0126 (10 μM). IB:α-Gab1, immunoblotting of Gab1. B, co-immunoprecipitation of Gab1 (IB:α-Gab1) and p85 (IB:α-p85) from mIMCD-3 cells ± EGF ± U0126. IP:α-Gab1, immunoprecipitation of Gab1. C, quantitation of the co-immunoprecipitation of Gab1 and p85, as shown in B, normalized to 1 for EGF stimulation alone (*, p < 0.01, n = 3).

were pretreated with 10 μM U0126, a concentration of the MEK inhibitor that prevents both ERK1/2 and ERK5 activation (18). Inhibition of ERK activation resulted in a marked increase in the EGF-stimulated association of p85 with Gab1. To determine whether this effect was specific for the p85 SH2 domain interactions with Gab1, we used GST fusion proteins encoding the N-terminal SH2 and C-terminal SH2 domains of p85. These pull-down experiments revealed that the EGF-stimulated interaction of Gab1 with the N-terminal SH2 domain of p85 appears to be ERK activation-independent (Fig. 1A, middle panel), whereas the association of the C-terminal SH2 domain of p85 with Gab1 increased with the inhibition of ERK activation (Fig. 1A, lower panel), mimicking the results obtained with full-length p85.

The effects of EGF-mediated ERK activation on endogenous Gab1/PI3K interactions were then examined by co-immunoprecipitation from mIMCD-3 cells. Compared with control cells, the interaction of Gab1 and the PI3K was increased following the EGF stimulation (Fig. 1B). Consistent with the in vitro pull-down results, this interaction was further increased in the setting of inhibition of ERK activation. The quantitation of the results from three experiments revealed that stimulation with EGF resulted in a 9-fold increase in Gab1/PI3K association, whereas concomitant inhibition of ERK activation led to an additional 40% increase in the Gab1/PI3K association (Fig. 1C). This result was confirmed in EGF-stimulated HEK cells (data not shown) and demonstrates that EGF-mediated ERK activation down-regulates the EGF-stimulated Gab1/PI3K association.

**ERK Regulation of EGF-stimulated Gab1/PI3K Association Occurs Outside of the MBD Domain**—In contrast to the results now presented for EGF, we have previously found that HGF-mediated ERK1/2 activation causes an increase in the HGF-stimulated Gab1/PI3K association. The association of the PI3K with Gab1 occurs primarily at three consensus YXXM association sites in Gab1 (5, 11). One of these sites, 472YVPMT478, is included in the Gab1 MBD domain and also encodes the ERK1/2 consensus phosphorylation sequence PX(S/T)P. We have shown that the MBD region is the primary target of ERK2 phosphorylation of Gab1 (12) and that dual phosphorylation of the 472YVPMT478 motif on Tyr472 and Thr476 results in a higher affinity binding site for p85 than does phosphorylation on Tyr472 alone (7). Because inhibition of ERK activation decreases the Gab1/PI3K association as well as downstream Akt activation by ~50% (Fig. 2A, left panel) (7), it is likely that HGF-stimulated ERK2 phosphorylation at the 472YVPMT478 site results in a physiologically relevant increase in HGF-stimulated PI3K association.

The current experiments demonstrate that preventing EGF-stimulated ERK activation results in an increase in the association of Gab1 with p85, suggesting that HGF and EGF-stimulated ERK activation can mediate divergent downstream events (Fig. 2A, right panel). To determine whether the effects of EGF on ERK-regulated Gab1/PI3K association are also mediated by the MBD of Gab1, we examined the association of the MBD with p85 following HGF or EGF stimulation. The MBD of Gab1 can associate with and be phosphorylated by either the c-met receptor (directly through the c-met binding sequence and indirectly through association with Grb2) or the EGFR (indirectly through association with Grb2).

Immunoprecipitation of the epitope-tagged MBD domain reveals that both HGF and EGF can induce the association of the Gab1 MBD with the p85 subunit of the PI3K (Fig. 2B, lanes 3 and 5). In agreement with our previous finding that HGF-stimulated ERK1/2 activation results in the creation of a higher affinity p85 binding site, U0126 treatment caused a decrease in the HGF-stimulated MBD-p85 association that was indistinguishable from that seen with full-length Gab1 (Fig. 2B, compare A, lanes 3 and 4, with B, lanes 3 and 4). However, the inhibition of ERK activation did not influence EGF-induced p85 binding to the Gab1 MBD (Fig. 2B, lanes 5 and 6). Interestingly, the HGF-stimulated association of p85 with the Gab1 MBD was significantly stronger than that observed for EGF stimulation, possibly because the dual binding interaction between the MBD and c-met results in a more efficient tyrosine phosphorylation of the p85 binding motif. Taken together, these results are most consistent with a model in which HGF-mediated ERK2 activation increases the Gab1/PI3K association through direct phosphorylation of the p85 binding site in the MBD, whereas EGF-mediated ERK activation decreases the Gab1/PI3K association through a mechanism outside of the MBD.

**ERK Activation Decreases EGF-mediated Gab1 Tyrosine Phosphorylation**—Because p85 association is dependent on the phosphorylation of the YXXM SH2 binding sites in Gab1, the
tyrosine phosphorylation state of Gab1 in both resting and EGF-stimulated mIMCD-3 cells was examined. Immunoprecipitation with anti-phosphotyrosine followed by blotting with anti-Gab1 revealed the expected increase in tyrosine phosphorylation of Gab1 following EGF stimulation with a further increase in the setting of concomitant inhibition of ERK activation (Fig. 3A).

Immunoprecipitation of Gab1 followed by anti-phosphotyrosine immunoblotting confirmed these results (Fig. 3B) with quantitation demonstrating that inhibition of ERK activation resulted in a 62% increase in the EGF-stimulated tyrosine phosphorylation of Gab1 (Fig. 3C). In contrast, we have previously demonstrated that HGF-stimulated ERK1/2 activation does not alter c-met-mediated Gab1 tyrosine phosphorylation (7).
To investigate whether the increased Gab1 tyrosine phosphorylation, which occurs following EGF stimulation, and concomitant ERK inhibition increases the association of Gab1 with other SH2 domain-containing signaling proteins, we examined the association of Gab1 with SHP2. SHP2 is an SH2 domain-containing tyrosine phosphatase that associates with Gab1 in a phosphorylation-dependent manner at tyrosines 627 and 659 in the C terminus of Gab1 (8, 21, 22). In both anti-Gab1 and anti-SHP2 immunoprecipitates, we were able to detect the association of Gab1 with SHP2 following the stimulation of mIMCD-3 cells with EGF (Fig. 3D). After inhibition of ERK activation with U0126, there was a substantial increase in the EGF-stimulated co-immunoprecipitation of Gab1 and SHP2, similar to that seen with Gab1/PI3K association. These results demonstrate that ERK activation in the absence of ERK activation results in a higher level of tyrosine phosphorylation of Gab1 and subsequently a greater recruitment of SH2 domain-containing proteins such as the PI3K and SHP2, and they suggest that EGF-stimulated ERK activation normally serves to down-regulate Gab1 signaling.

ERK Inhibition Does Not Alter EGFR Phosphorylation or EGFR-Grb2-Gab1 Complex Formation—Two potential mechanisms, whereby EGF-stimulated ERK activation could decrease the tyrosine phosphorylation state of Gab1, are to either decrease the EGFR-dependent tyrosine phosphorylation of Gab1 or to increase the activity of a Gab1-associated phosphatase. A decrease in the EGFR-dependent tyrosine phosphorylation of Gab1 could occur because of the inhibition of the EGFR tyrosine kinase activity or a decrease in the association of Gab1 with the EGFR. As a marker for EGFR tyrosine kinase activity, we examined the tyrosine phosphorylation state of the EGFR after stimulation with EGF. Anti-phosphotyrosine immunoblots of anti-EGFR immunoprecipitates revealed an indistinguishable level of EGFR phosphorylation in cells stimulated with EGF in the presence or absence of ERK inhibition with U0126 (Fig. 4A).

The association of Gab1 with the EGFR occurs indirectly via an SH3-mediated association of Gab1 and Grb2 and a phosphotyrosine-dependent SH2-mediated association of Grb2 with the EGFR. Because ERK2 has been shown to directly phosphorylate Gab1 in the MBD region that encodes one of the prolinerich Grb2 SH3 binding sites (11), we examined the effects of EGF-stimulated ERK activation on Gab1/Grb2 and Grb2-EGFR complex formation. The SH3-mediated association of Gab1 and Grb2 was found to be constitutive and was not altered by EGF stimulation, either in the presence or absence ERK inhibition (Fig. 4B). The SH2-mediated association of Grb2 with the EGFR was increased following EGF stimulation with no alteration detected in the setting of ERK inhibition (Fig. 4C). These results demonstrate no detectable effect of ERK activation on Gab1/Grb2 or association of the receptor with Gab1 and thus do not support the model that the EGF-stimulated ERK-dependent decrease in Gab1 tyrosine phosphorylation is the result of regulation of the EGFR-mediated tyrosine phosphorylation of Gab1.

EGF Stimulates SHP2-dependent Dephosphorylation of Gab1—An alternative explanation for the ERK-mediated decrease in EGF-stimulated tyrosine phosphorylation of Gab1 is that ERK serves to either recruit and/or activate a tyrosine phosphatase to Gab1. To examine this possibility, we determined the tyrosine phosphorylation state of Gab1 in the presence of the cell-permeable phosphatase inhibitor pervanadate. The addition of pervanadate at 50 μM to serum-starved mIMCD-3 cells resulted in a 4-fold increase in basal Gab1 tyrosine phosphorylation (Fig. 5A, quantitated in B). Stimulation with EGF increased the level of tyrosine phosphorylation 9-fold over base line with an additional 2.2-fold increase in phosphorylation in the setting of pervanadate treatment. These data demonstrate that one or more phosphatases regulate the tyrosine phosphorylation state of Gab1 both in quiescent and EGF-stimulated cells.

A logical candidate for an EGF-dependent ERK-stimulated Gab1 tyrosine phosphatase is SHP2. Recently, Gab1 has been identified as a potential SHP2 substrate in the EGFR (8) and cytokine receptor signaling pathways (10). In the work by Cun nick et al. (8), SHP2 was found to be capable of dephosphorylating peptides containing either phosphorylated Tyr589 (a PI3K binding site) or Tyr659 (the SHP2 binding sites in Gab1), although the ability of SHP2 to dephosphorylate native Gab1 following EGF stimulation was not determined. To further examine the role of SHP2 in Gab1 dephosphorylation, we used the transient expression of SHP2-CS in HEK cells. In unstimulated cells, the expression of SHP2-CS resulted in a minimal increase in the tyrosine phosphorylation of Gab1 as compared with cells expressing wild-type SHP2 or the empty vector (Fig. 5C, lane 2 versus lanes 1 and 3). However, following EGF stimulation, SHP2-CS-expressing cells exhibited approximately a 2-fold increase in tyrosine phosphorylation compared with controls, a change similar to that seen following pervanadate treatment (Fig. 5A) or U0126 treatment (Fig. 3B). Of note, we have thus far been unable to detect an increase in SHP2 phosphatase activity in Gab1 immunoprecipitates from EGF-stimulated cells using the artificial substrate paranitrophenyl phosphate, preventing us from directly determining the role of ERK activation in the regulation of SHP2. Based on the results shown in Fig. 5A, we believe that this failure to detect in-
creased SHP2 activity is because of either the relative insensitivity of the assay or the instability of the Gab1/SHP2 association.

Our present data are most consistent with a model in which EGF stimulation results in tyrosine phosphorylation of Gab1 followed by recruitment of the PI3K and SHP2. SHP2 then acts to dephosphorylate Gab1 and down-regulate its activation, an effect that is enhanced by simultaneous ERK activation. ERK could be acting to either increase the association of Gab1 with SHP2 or to stimulate its phosphatase activity. Our observation that inhibition of ERK activation results in an increase in the association of Gab1 with SHP2 (Fig. 3D) would seem to rule out the former possibility. However, it is conceivable that an initial ERK-mediated increase in SHP2 association results in the dephosphorylation of multiple Gab1 tyrosine residues including Tyr627 and/or Tyr659 followed by the loss of the SHP2-Gab1 association. Interestingly, the association of SHP2 with Gab1 through Tyr627 and Tyr659 has been found to be required for EGF-induced ERK2 activation (8, 22), suggesting that the ERK-mediated decrease in tyrosine phosphorylation of Gab1 might then serve as a negative feedback regulator of further ERK2 activation.

**ERK Regulates EGF-stimulated PI3K Activity and Akt Activation**

The binding of the p85 subunit of the PI3K to phosphotyrosine residues typically results in the activation of the catalytic 110kD subunit of the enzyme and the production of phosphoinositide 3,4,5 triphosphate (PI-3,4,5-P3) at the cell membrane. To determine whether the increase in the PI3K-
Gb1 association detected following the inhibition of ERK activation plays a significant role in the activation state of the PI3K. PI3K activity was assayed in anti-Gab1 immunoprecipitates from EGF-stimulated cells. The stimulation of mIMCD-3 cells with EGF increased the activity of the PI3K by ~1.5-fold (Fig. 6A). The pretreatment of EGF-stimulated cells with U0126 resulted in an additional 2-fold increase in Gab1-associated PI3K activity.

The production of PI-3,4,5-P3 at the membrane serves as a binding site for PH domain containing proteins such as Akt. The recruitment of Akt to the cell membrane results in its phosphorylation and activation by protein kinase B, leading to enhanced cell survival and proliferation (23). To determine whether ERK regulates the activation of this signaling pathway downstream of the PI3K, we examined the activation of Akt using a phosphorylation-specific antibody. Akt was basally phosphorylated to a low level in control cells with a 1-fold increase in Akt activation following EGF stimulation (Fig. 6B, quantitated in C). Although U0126 pretreatment had no effect on basal Akt phosphorylation, EGF-stimulated cells demonstrated a further 50% increase in Akt phosphorylation in the setting of U0126-mediated ERK inhibition. These results demonstrate that EGF-stimulated ERK activation plays a significant role in down-regulating the ability of Gab1 to recruit and activate the PI3K and its downstream effectors.

Although both HGF and EGF are capable of activating Gab1 and inducing epithelial morphogenesis, the phenotypic effects of these growth factors are distinct. HGF stimulation of mIMCD-3 cells results in cell processes with multiple branches, whereas the EGFR ligands EGF and transforming growth factor-α stimulate longer processes with fewer branch points (24). The ability of Gab1 to induce these morphogenic effects is dependent on its localization to the membrane, which is mediated by the binding of the Gab1 PH domain to PI-3,4,5-P3 (5). The association of the PI3K with Gab1, resulting in local PI-3,4,5-P3 generation, thus serves to perpetuate Gab1 signaling. Therefore, the careful regulation of this interaction may serve to dictate whether the phenotypic outcome of receptor stimulation is process branching or process elongation.

Our results suggest that growth factor-selective MAPK activation is capable of providing a second level of regulation of the interaction of the PI3K and Gab1 in addition to receptor phosphorylation of the p85 binding sites. HGF-activated ERK2 appears to directly phosphorylate Gab1 near the p85 binding site, resulting in enhanced PI3K binding and activation. In contrast, EGF-stimulated ERK activation, possibly ERK5, causes a decrease in the tyrosine phosphorylation state of Gab1 and results in diminished PI3K signaling. 

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