Activated ERK2 Interacts with and Phosphorylates the Docking Protein GAB1*

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Grb2-associated binder 1 (GAB1) is a docking protein found to associate with the activated c-MET receptor via the MET-binding domain (MBD) and appears to be critical for the tubulogenic actions of this receptor. Pull-down experiments with bacterially expressed MBD and full-length GAB1 revealed the presence of c-MET as well as phosphorylated ERK2 (pERK2). By using purified pERK2 and non-pERK2, we found that GAB1 associates exclusively with the phosphorylated form of the enzyme and that this association does not require mediation by a third protein. When epitope-tagged GAB1 was co-transfected with constitutively active MEK1 into A293 cells, co-immunoprecipitation of GAB1 and pERK2 was observed, demonstrating that this interaction can occur in intact cells. In vitro, both the MBD and full-length GAB1 were found to be substrates for activated ERK2. In intact cells, epitope-tagged GAB1 was found to be basically phosphorylated on serine with an increase following co-transfection with constitutively active MEK1 and the appearance of novel phosphorylation sites detected in intact cells. In vitro, both the MBD and full-length GAB1 were found to be substrates for activated ERK2. In intact cells, epitope-tagged GAB1 was found to be basically phosphorylated on serine with an increase following co-transfection with constitutively active MEK1 and the appearance of novel phosphorylation sites detected in intact cells.

In cultured epithelial cells, the tyrosine kinase receptor c-MET and its ligand hepatocyte growth factor (HGF)1 are capable of inducing mitogenesis, motogenesis (scattering/chemotaxis), and morphogenesis (formation of branching tubules) (1). In the kidney, these distinct actions may comprise the components of more complex events such as ureteric bud branching and repair of renal tubules after injury. Whereas mitogenesis and morphogenesis have been described following activation of many tyrosine kinase receptors, epithelial tubulogenesis appears to be relatively specific for ligand activation of members of the MET receptor family and the epidermal growth factor (EGF) family (2). Recently a docking protein, GAB1 (Grb-2-associated binding protein 1), was found to associate with the activated c-MET receptor via the Met-binding domain (MBD) and to be capable of inducing the tubulogenic phenotype (3–5).

GAB1 was cloned by Holgado-Madruga et al. (3) as a protein that associated with Grb-2 in an expression library assay. The amino acid sequence of GAB1 suggested that it was a docking protein. However, its MBD was comparable to the interaction sites for full-length GAB1 with an amino-terminal pleckstrin homology (PH) domain and a proline-rich carboxyl terminus, as well as multiple potential SH2 binding sites for the class 1 phosphoinositide 3-kinase, Grb2, phospholipase Cγ, and SHPTP2. Using a yeast-two-hybrid screen with the carboxyl terminus of c-MET as the bait, Weidner et al. (4) found that GAB1 could interact directly with the MET receptor at the Tyr1349–Tyr1356 multifunctional docking site of c-MET. An 82-amino acid stretch near the carboxyl terminus of GAB1 was necessary and sufficient for the binding interaction of GAB1 and c-Met and was named the Met-binding domain (MBD). The association between GAB1 and c-MET appears to involve a direct interaction between the MBD and Tyr1349 of c-MET (4), and Grb2-mediated interaction between the proline-rich region of GAB1 and Tyr1356 of c-MET (5).

The finding that the MBD of GAB1 associates directly with c-MET in a tyrosine-dependent fashion suggests that this domain might associate with other tyrosine-phosphorylated proteins and thus act in a novel fashion to mediate epithelial signaling events. To examine this possibility, we incubated GST-MBD and GST-GAB1 fusion proteins with lysates from HGF- and EGF-activated renal tubular epithelial cells and examined the co-precipitated proteins. We found that GST-MBD and GST-GAB1 associate with both the tyrosine-phosphorylated c-MET receptor and with phosphorylated ERK2 (p42 MAPK) and more weakly with ERK1 (p44 MAPK).

EXPERIMENTAL PROCEDURES

GST Fusion Protein Expression—The 2,084-nucleotide sequence of full-length GAB1, a 284-nucleotide fragment of GAB1 encoding the 82-amino acid MBD and 13 flanking amino acids, and the 287 nucleotides encoding the amino-terminal PH domain were amplified from a human fetal kidney cDNA library via the polymerase chain reaction and cloned into the pCR2.1 vector (Invitrogen) (Fig. 1). Single clones were fully sequenced and subcloned into pGEX-4T vectors (Amersham Pharmacia Biotech) for bacterial expression. A construct encoding the 416 amino acids at the NH2-terminal of GAB1 (NH2-GAB1) excluding the MBD domain was generated by BglII digest of full-length GAB1 cDNA. A construct encoding the MBD homologous region of GAB2 (named MBD2) was amplified from full-length GAB2 (kindly provided by Hailiu Gu) and subcloned into pGEX-4T. The MBD2 domain shares the XXXM phosphoinositide 3-kinase-binding site found in MBD1, but the two PXXP motifs are missing from this protein (see Gu et al. (7) for alignment). Expression of the GST fusion proteins was induced in BL21-competent bacteria with isopropyl-β-D-thiogalactoside. The bacteria were pelleted and resuspended in 10 mM Trizma base, 100 mM NaCl, 1 mM EDTA, 1 mM sodium orthovanadate, 1 µM leupeptin, 4 µg/ml 4-(2-aminoethyl)benzenesulfonyl fluoride, 5 mM dithiothreitol, and 0.3 mg/ml lysozyme, pH 7.5. The mix was incubated with 20 mg of...
deoxycholate (sodium salt), followed by DNase I and MgCl2. 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 until use. Control experiments were performed with GST-Sepharose beads generated by expression of GST alone using the empty pGEX-4T vector. Total GST fusion protein amounts were estimated visually using Coomassie Blue-stained SDS-PAGE with albumin standards.

**GAB1 Association with ERK2**

**GAB1 Co-precipitation Experiments**—Subconfluent mIMCD-3 cells were serum-starved for 24 h prior to stimulation with either HGF (40 ng/ml, Sigma) or EGF (20 ng/ml, Upstate Biotechnology Inc.) for 10 min at 37 °C. The cells were then washed twice with ice-cold phosphate-buffered saline, scraped in ice-cold 0.5% Nonidet P-40 lysis buffer (137 mM NaCl, 20 mM Trizma base, 1 mM MgCl2, 1 mM CaCl2, 0.5% Triton X-100, 0.5% iodoacetamide, 25 µg/ml leupeptin, 0.75 µg/ml antipain, and 25 µg/ml antipain) and vortexed vigorously. Non-solubilized debris was removed by microcentrifugation at 13,000 × g for 10 min at 4 °C, and the supernatant was collected. (In the MEK-1 inhibitor experiment, the cells were preincubated with PD98059 (2'-amino-3'-methoxyflavone) (Calbiochem) at a concentration of 50 µM for 20 min prior to stimulation.) One mg of protein from the whole cell lysates was then incubated with the GST fusion protein of interest bound to glutathione-Sepharose 4B (Amersham Pharmacia Biotech) for 30 min on a rotator at 4 °C. The glutathione beads were washed 3 times with ice-cold lysis buffer followed by resolation via SDS-polyacrylamide gel electrophoresis. The proteins were electrophoretically transferred into Immobilon-P membranes (Millipore), immunoblotted with the appropriate antibody, and proteins detected using ECL (Amersham Pharmacia Biotech). For experiments using purified ERK2, bacterially expressed inactive (non-phosphorylated) and active (phosphorylated) ERK2 was purchased from New England Biolabs. 125 µmol of the GST-GAB1, GST-MBD, or GST-MBD-2 beads were then incubated with 0.25 µmol of purified ERK2 in 0.5% Nonidet P-40 lysis buffer in the absence of cell lysates for 30 min at 4 °C, followed by washing three times in lysis buffer and separation by SDS-PAGE. For competition experiments, either 50 µg of inactive ERK2 was preincubated with PD98059 (2'-amino-3'-methoxyflavone) for 10 min at 4 °C, and 0.25 µmol of purified GST-MBD fusion proteins were separated by SDS-PAGE and detected via autoradiography. For phosphopeptide mapping of GST-MBD, the GST-MBD-2 beads were washed three times with ice-cold lysis buffer followed by resolution via SDS-PAGE followed by autoradiography.

**In Vivo Association Experiments**—The nucleotide sequences of full-length GAB1 were cloned into the pFLAG-CMV2 expression vector with the FLAG tag at the amino terminus of the fusion protein. A293 human embryonic kidney (HEK) cells were then transfected with 2.5 µg of pFLAG-GAB1 ±2.5 µg of dominant negative MEK1 or MEK1-AS-E (constitutively active MEK1, plasmids a generous gift of Dr. Roger Davis (9)) using standard calcium phosphate transfection.

Immunoprecipitation was performed as described previously (10). Briefly, 24 h after transfection, the cells were washed once with ice-cold phosphate-buffered saline, scraped in 500 µl of ice-cold 1% Nonidet P-40 lysis buffer (157 mM NaCl, 20 mM Trizma base, 1 mM MgCl2, 1 mM CaCl2, 10 mM NaF, 1 mM sodium orthovanadate, 10% glycerol, 1% Igepal CA-630, 1 mM phenylmethylsulfonfyl fluoride, 0.5 µg/ml leupeptin, 0.75 µg/ml pepstatin A, and 0.05 µM antipain), and vortexed vigorously. Non-solubilized debris was removed by microcentrifugation at 13,000 × g for 10 min at 4 °C, and the supernatant was collected.

**Kinase Assays**—In vitro MAPK phosphorylation was performed by the addition of 0.5 µmol of purified active ERK2 (or the equivalent amount of inactive ERK2) to 18 µmol of the appropriate fusion proteins in 1× kinase buffer (10 µM ATP, 5 mM MnCl2, 5 mM MgCl2, and 20 mM HEPES), 10 µCi of [γ-32P]ATP (NEN Life Science Products) for 30 min at 30 °C. The beads were then washed three times, and the fusion proteins were separated by SDS-PAGE and detected via autoradiography.

In vitro phosphorylation of GAB1 was performed by calcium phosphate co-transfection of A293 HEK cells with amino-terminal FLAG-tagged GAB1 ± the appropriate MEK construct as above. Twenty-four hours following transfection, the media were changed to phosphate-free media containing 0.6 mg/ml 32Porthophosphate for 4 h. The cells were then lysed in 1% Nonidet P-40 lysis buffer and immunoprecipitated for 3 h with the M5 anti-FLAG antibody, washed three times, and separated by SDS-PAGE and detected via autoradiography.

For phosphopeptide mapping of GAB1, the band corresponding to either **in vitro** or **in vivo** 32P-labeled GAB1 was excised and resuspended in 50 mM NaHCO3, 0.1% SDS, and 0.5% β-mercaptoethanol overnight. 200 µg of bovine serum albumin was added as a carrier, and the phosphopeptide precipitated overnight with trichloroacetic acid and then pelleted for 10 min at 13,000 × g. The pellet was washed once with 95% ethanol, dried down, and resuspended in 6 µl H2O for 1 h at 110 °C. The resulting amino acids were dried down again, resuspended in 5% acetic acid, 0.5% pyridine at pH 3.0, separated by thin layer electrophoresis, and visualized by autoradiography. For phosphate mapping, the **in vitro** 32P-labeled GAB1 was excised from the blot and coated with 0.5% PV-P-300 in 100 mM acetic acid at 37 °C for 30 min, washed extensively with water, and digested with 10 µg of l-tosylamido-2-phenylglycinnitrophenyl ketone-treated trypsin in 50 mM ammonium bicarbonate overnight at 37 °C. The eluted peptides were dried down again and resuspended in 1.9 buffer (2% formic acid, 8% acetic acid). Samples were spotted on cellulose plates and resolved by thin layer electrophoresis at pH 1.9 in the first dimension and by thin layer chromatography in phosphochromatography buffer (37.5% n-butyl alcohol, 25% pyridine, 7.5% acetic acid) in the second dimension. Peptides were visualized by autoradiography of cellulose plates.

**RESULTS**

**GAB1 Associates with both c-MET and ERK2 in a Ligand-dependent Manner via the MBD—**Cell lysates from HGF-activated mIMCD-3 cells were incubated with GST-MBD fusion protein for 30 min. An anti-phosphotyrosine blot of the co-precipitated proteins revealed three dominant bands, 145, —60, and —42 kDa (Fig. 2A, middle panel). Immunoblotting with an antibody to the β-subunit of c-MET confirmed that the 145-Da protein was the activated MET receptor (Fig. 2A, left panel). An antibody to the phosphorylated form of ERK1 and ERK2 demonstrated that the 42-kDa band was activated ERK2 (p42) and that phosphorylated ERK1 (p44) was also weakly present in the GST-MBD precipitate (Fig. 2A, right panel). Neither of these proteins were present in the unstimulated cell lysates. Similarly, incubation of full-length GST-GAB1 fusion protein with HGF-stimulated lysates resulted in co-precipitation of c-MET as well as ERK2 and ERK1 (Fig. 2B). These results confirmed the observation of Weidner and coworkers (4) that the MBD of GAB1 associates with the c-MET receptor in a phosphorylation-dependent manner and additionally demonstrated that GAB1 binds to activated ERK2 via the MBD following HGF stimulation.

As the GAB1 protein previously has been shown to associate indirectly with the EGFR (via Grb2) following EGF stimulation (3), we compared GST-MBD precipitation of HGF- and EGF-stimulated cells (Fig. 3A). Anti-phosphotyrosine blotting again revealed three primary bands in the HGF-stimulated cell lysates, with identical bands observed at the 60- and 42-kDa positions in EGF-stimulated cells (Fig. 3A, right panel). Thus,
GST-MBD associates with phosphorylated ERK2 following cell stimulation with either HGF or EGF. Prolonged exposure revealed a faint phosphotyrosine band at the appropriate size for the EGFR. The weakness of the EGFR band may be consistent with the observation that GAB1 does not directly associate with this receptor.

Following stimulation with HGF, ERK2 is maximally phosphorylated within 5–10 min with a slow decrease in activation over the next 30 min (Fig. 3B, left panel). Examination of GST-MBD pull-down from lysates reveals that the MBD-ERK interaction parallels this temporal pattern, arguing that ERK remains available for interaction with GAB1 throughout its activation (Fig. 3B, right panel). IP, immunoprecipitated; IB, immunoblot.

The GAB1-MBD Interaction Is Specific for the GAB1 MBD—To determine if ERK2 associates with GAB1 as part of a complex with the c-MET receptor, we examined anti-c-MET immunoprecipitates for the presence of phosphorylated ERK2. As before, GST-MBD co-precipitated the c-MET receptor following HGF stimulation, as well as co-precipitation of active ERK2 (Fig. 4A, 1st and 2nd lanes), whereas immunoprecipitates of c-MET failed to bring down phosphorylated ERK2 under either basal or stimulated conditions (Fig. 4A, 3rd and 4th lanes).

To map further the site within the GAB1 protein responsible for mediating the interaction with ERK2, GST fusion proteins representing full-length GAB1, the MBD, the GAB1 PH domain, and the amino terminus of GAB1 truncated just prior to the MBD (NH2-GAB1) were compared for their ability to co-precipitate ERK2. In an attempt to determine relative binding capacity, the quantity of each GST fusion protein was estimated on SDS-PAGE via comparison to an albumin standard, and 18 pmol of each fusion protein was used for co-precipitation. The PH domain of GAB1 and the NH2 terminus of GAB1 failed to associate with ERK2 in HGF-stimulated cell lysates, whereas the binding capacity of the MBD and full-length GAB1 were judged essentially equal (Fig. 4B). Thus it appears that GAB1 associates with ERK2 exclusively via the Met-binding domain.

Recently GAB2 has been cloned and found to have significant homology with GAB1, including the carboxyl terminus of the MBD domain (7). We therefore tested the ability of the GAB2 MBD (MBD2) to associate with activated ERK2. The region of GAB2 corresponding to the GAB1 MBD was polymerase chain reaction-amplified and cloned into pGEX as before. Both GST-MBD2 and GST-MBD1 (the MBD domain of GAB1) interacted with Grb2 in a constitutive manner in mIMCD-3 cell lysates (Fig. 4C, upper panel); however, GST-MBD2 failed to associate with phosphorylated ERK2 in HGF-stimulated cells (Fig. 4C, lower panel).

The GAB1-MBD Interaction Requires MEK Phosphorylation of ERK2—The most likely interpretation of the above results is that the MET-binding domain of GAB1 associates directly with ERK2 and ERK1 in a phosphospecific manner, as has been demonstrated for c-MET binding. However, the antibody to phospho-MAPK does not detect non-phosphorylated ERK2 or ERK1, making it possible that ERK2 is constitutively phosphorylated in these cells.

Fig. 2. GAB1 associates with both c-MET and ERK via the MBD. Bacterially expressed MBD and full-length GAB1 were used for co-precipitation experiments of mIMCD3 cell lysates. A, an anti-phosphotyrosine blot of GST-MBD precipitates from HGF-stimulated cells reveals the presence of phosphoproteins at ~145, 60, and 42 kDa (middle panel). Immunoblotting with anti-MET (left panel) and anti-phospho-MAPK (right panel) confirms that the p145 and p42 proteins are the β-subunit of c-MET and pERK2, respectively. Phosphorylated ERK1 is also weakly detected. B, precipitation of HGF-stimulated cell lysates using full-length GST-GAB1 again reveals the presence of both c-MET (left panel) and phospho-ERK1/2 (right panel). IP, immunoprecipitated; IB, immunoblot.

Fig. 3. The MBD-ERK interaction occurs with both HGF and EGF. A, stimulation of mIMCD-3 cells with either HGF or EGF results in co-precipitation of phosphorylated ERK with GST-MBD. B, the time course of ERK activation following stimulation of mIMCD-3 cells by HGF is shown (left panel). Pull-down of these lysates with GST-MBD reveals maximal interaction with pERK2 after 5 min activation by HGF and a gradual decline with more prolonged stimulation. IP, immunoprecipitated; IB, immunoblot.
associated with GAB1 and is only recognized by the phosphospecific antibody following receptor activation and MAPK phosphorylation. To distinguish between these possibilities, we examined immunoblots of GST-MBD-precipitated cell lysates using an antibody that detects ERK1 and -2 regardless of their phosphorylation state (ERK1/2, Fig. 5A). With prolonged exposure, both the phospho-MAPK and the ERK1/2 antibodies detected only small amounts of ERK2 associated with GST-MBD in lysates from quiescent mIMCD-3 cells, with a dramatic increase following cell stimulation with HGF, consistent with the interaction between GAB1 and ERK2 requiring ERK2 phosphorylation. To determine if the ERK phosphorylation required for MBD-ERK interaction was via the classical MAPK pathway, the MEK1 inhibitor, PD98059, was added to intact mIMCD-3 cells prior to ligand activation. At 50 μM, PD98059 inhibited HGF-induced phosphorylation of ERK2 by 110% (the small degree of basal ERK2 phosphorylation seen in these cells was inhibited as well) and EGF-induced ERK2 phosphorylation by 92% (data not shown).

Following stimulation with either HGF or EGF, the MBD-ERK2 association was substantially reduced in the setting of MEK1 inhibition (Fig. 5B), demonstrating that the association of ERK2 and the MBD is dependent on MEK1 phosphorylation of ERK2.

GAB1 Interacts Directly with Phosphorylated ERK2 and Co-precipitates from Intact Cells—The GAB1 MBD contains a proline-rich region capable of interacting with SH3 domain-containing proteins such as Grb2 (see Fig. 4C) (3, 4, 6). We therefore considered the possibility that the interaction between GAB1 and phosphorylated ERK2 was mediated by a third protein containing an SH3 domain and a more conventional phosphoprotein binding domain. Commercially purchased purified phospho- and non-pERK2 were utilized to investigate this possibility. Both GST-MBD1 and GST-GAB1 co-precipitated purified pERK2, whereas GST-MBD2 again failed to associate with pERK2 (Fig. 6A). Blotting with the antibody that detects both phospho- and non-phospho-ERK2 confirmed that purified non-phosphorylated ERK2 fails to associate with GAB1 (data not shown).

To determine if ERK2 association with the MBD involves direct association of the MBD with phosphorylated residues on ERK2, we examined the ability of phenylphosphate or of a synthetic peptide mimicking the dual phosphorylation site of ERK2 to diminish the MBD-ERK2 association (11–13). The isolated GST-MBD was preincubated for 30 min with either the phosphorylated or non-phosphorylated 11-amino acid ERK2 competitive peptide at a 1000-fold molar excess compared with the GST-MBD. In a parallel experiment, GST-MBD was preincubated with 50 mM phenylphosphate. The mixtures were then used for co-precipitation of purified pERK2 as described before. Neither the phosphorylated competitive peptide nor phenylphosphate diminished the affinity of GST-MBD for active ERK2 (Fig. 6B).

The ability of GAB1 to interact with pERK2 in intact cells was examined by transfecting human embryonic kidney cells (HEK cells) with epitope-tagged GAB1 and constitutively active MEK. Whole cell lysates of transfected cells revealed the presence of phospho-ERK1 and -2 and GAB1 in the transfectants (Fig. 6C, 1st and 2nd lanes). Immunoprecipitation of GAB1 with an anti-FLAG antibody revealed the presence of phosphorylated ERK1 and -2 in the GAB-transfected cells (4th lane) but not the vector control (3rd lane).

GAB1 Is a Substrate for ERK Phosphorylation—Examination of the sequence of GAB1 revealed several consensus ERK phosphorylation sites (14), including 452PNSP, 474PMTP,
co-immunoprecipitate FLAG-GAB1 and pERK was compared in lysates and either pFLAG-GST-MBD beads is shown in the affinity of MBD for pERK2. The total amount of pERK2 added to the constitutively active MEK resulted in the novel phosphorylation of two peptide fragments (fragments 2 and 3; Fig. 8, middle panel) and a marked increase in the phosphorylation of fragment 1. In vitro phosphorylation of GST-GAB1 with purified pERK2 again resulted in phosphorylation of peptides 1–3 (Fig. 8, right panel), suggesting that the in vivo phosphorylation of these fragments of GAB1 was directly mediated by pERK. The in vivo basally phosphorylated fragments 4 and 6 were not detected in the in vitro experiment, arguing that these sites are phosphorylated by a different serine/threonine kinase. In addition, novel fragments were phosphorylated in the in vitro experiment consistent with the ability of the purified kinase to phosphorylate GAB1 at non-physiologic sites.

**GAB1 Association with ERK2**

The GAB1-ERK interaction is direct and occurs in intact cells. A, both GST-MBD1 and GST-GAB1 co-precipitate purified, bacterially expressed pERK2 in the absence of cell lysates, whereas GST–MBD2 fails to associate with pERK2. B, preincubation of GST-MBD for 30 min with an excess of a non-phosphorylated (1st lane) or phosphorylated (2nd lane) 11-amino acid peptide mimicking the ERK phosphorylation site fails to diminish the affinity of GST-MBD1 for purified pERK2 when compared with GST-MBD1 treated with vehicle control (4th lane). Similarly, phenylphosphate (3rd lane) does not alter the affinity of MBD for pERK2. The total amount of pERK2 added to the GST-MBD beads is shown in the 5th lane. C, the ability of anti-FLAG to co-immunoprecipitate FLAG-GAB1 and pERK was compared in lysates from A293 cells co-transfected with constitutively active MEK (caMEK) and either pFLAG-GAB1 or the empty pFLAG vector. Whole cell lysates confirm the presence of phosphorylated ERK1 and -2 in the constitutively active MEK-transfected cells (bottom panel, 1st and 2nd lanes) with the appearance of FLAG-GAB in the cells co-transfected with pFLAG-GAB1 (2nd lane). Immunoprecipitation (IP) of FLAG-GAB1 with an anti-FLAG antibody reveals the co-immunoprecipitation of both pERK1 and -2 (4th lane), whereas these proteins were absent in immunoprecipitates of control cells co-transfected with the empty pFLAG vector (3rd lane). IB, immunoblot.

Phosphopeptide mapping of GAB1 immunoprecipitated from cells co-transfected with GAB1 and dominant negative MEK revealed three major basal phosphorylation sites (fragments 4–6; Fig. 8, left panel) with weak phosphorylation of a fourth site (fragment 1). Co-transfection of GAB1 with constitutively active MEK resulted in the novel phosphorylation of two peptide fragments (fragments 2 and 3; Fig. 8, middle panel) and a marked increase in the phosphorylation of fragment 1. In vitro phosphorylation of GST-GAB1 with purified pERK2 again resulted in phosphorylation of peptides 1–3 (Fig. 8, right panel), suggesting that the in vivo phosphorylation of these fragments of GAB1 was directly mediated by pERK. The in vivo basally phosphorylated fragments 4 and 6 were not detected in the in vitro experiment, arguing that these sites are phosphorylated by a different serine/threonine kinase. In addition, novel fragments were phosphorylated in the in vitro experiment consistent with the ability of the purified kinase to phosphorylate GAB1 at non-physiologic sites.

**DISCUSSION**

In the present study, we confirm that the GAB1 MET-binding domain associates with the β-subunit of the c-MET receptor in a phosphorylation-dependent fashion. Furthermore, we demonstrate the novel observation that this domain is capable of mediating the association of GAB1 with both ERK2 and ERK1 following activation of the MAPK cascade. In addition, an unidentified tyrosine-phosphorylated protein of approximately 60 kDa appears to associate constitutively with the MBD of GAB1 in epithelial cell lysates. Antibodies to Shc and Src have failed to recognize this protein.

The site on GAB1 that interacts with ERK is defined in these studies as the MBD. Bacterially expressed constructs encompassing the PH domain or the entire amino terminus of GAB1 up to the MBD domain were capable of high affinity association with phosphatidylinositol 3,4,5-trisphosphate but failed to associate with phosphorylated ERK2. Equimolar amounts of the MBD and full-length GAB1 co-precipitate pERK2 to the same degree, suggesting that the association is mediated entirely by the MBD.

Based on the observation that the MBD of GAB1 has a proline-rich sequence capable of mediating interactions with SH3-containing proteins, we examined the possibility that the GAB1-ERK2 interaction was mediated by a third protein such as Grb2. Purified bacterially expressed pERK2 was found to associate with GST-GAB1 in the absence of cell lysates, arguing against interaction via an unidentified cytosolic protein. Arguing specifically against Grb2 as a mediator of the GAB1-ERK interaction is our finding that the homologous MBD region of the recently cloned GAB1 family member, GAB2 (7), had an equal affinity for Grb2 but failed to associate with pERK2. This experiment demonstrates that the GAB1-ERK2 interaction is not mediated via Grb2 and is apparently unique for the MBD of GAB1. Thus it appears that GAB1 and pERK directly interact and do not require an intermediary protein.

Despite the above observations, the exact mechanism of the interaction between the MBD and ERK2 remains somewhat unclear. We initially predicted that the interaction between GAB1 and pERK2 was mediated by direct binding to either the phosphothreonine or phosphotyrosine residues in the ERK activation domain. This was tested by determining whether a phosphopeptide sequence mimicking this site could compete for ERK association, an approach that has been successfully used for both SH2 and PTB interactions (15–18). Although GAB1

**References**

[1] L. Rameh, personal communication.
only binds to ERK1 and -2 following their phosphorylation by MEK, the inability of the phosphorylated peptide or phenylphosphate to compete for binding argues that the MBD does not directly bind to these phosphorylated residues. In further support of this is our observation that binding of phosphorylated ERK2 to GAB1 does not significantly inhibit ERK kinase activity (data not shown), arguing that the active site is not continuously occupied. A reasonable explanation would be that phosphorylation of ERK results in a conformational change in the protein that allows binding to GAB1 or that GAB1 only recognizes pERK in the homodimerized form (19). In agreement with this possibility is our observation that GAB1 failed to bind phosphorylated ERK2 in a far Western assay (data not shown), suggesting that the binding epitope was not recognized in the denatured protein.

Transient co-expression of epitope-tagged GAB1 and constitutively active MEK in HEK cells resulted in co-immunoprecipitation of pERK1 and -2 with GAB1, demonstrating that this interaction can occur in vivo. Of note, immunoprecipitates of native GAB1 from HGF-stimulated mIMCD-3 cells failed to co-immunoprecipitate pERK2 (data not shown). When the amount of GAB1 in the immunoprecipitates was compared with that in whole cell lysates, we found that only 10–20% of total GAB1 was immunoprecipitated with the commercially available antibody, raising the possibility that our failure to detect pERK is due to the technical limitations of this approach. Alternatively, it is possible that the in vivo interaction is that of an enzyme and substrate and is therefore transient in nature and only detectable when the proteins are overexpressed. In support of this hypothesis, we have found that GAB1 serves as an excellent substrate for active ERK2 both in vitro and in vivo, and pERK rapidly disassociates from GAB1 in

**Fig. 7. GAB1 is a phosphorylation substrate for ERK.** A, autoradiography of an in vitro incubation of equimolar amounts of GST-PH, GST-MBD, GST-NH2 GAB1, or full-length GST-GAB1 with purified pERK2 in the presence of [32P]ATP. The MBD and full-length GAB1 are heavily phosphorylated, whereas the PH domain of GAB1 is not detectably phosphorylated, and the NH2 GAB1 is only weakly phosphorylated. B, A293 cells were co-transfected with pFLAG-GAB1 and either dominant negative (dn) MEK or constitutively active (ca) MEK followed by [32P]orthophosphate labeling and anti-FLAG immunoprecipitation (IP). GAB1 from cells co-transfected with GAB1 + constitutively active MEK demonstrates a decrease in gel mobility (left panel, 3rd lane versus 1st and 2nd lanes) and a modest increase in total phosphorylation (left panel, autoradiogram). Elution of the phosphorylated GAB1 protein followed by acid digestion and phosphoamino acid analysis reveals that GAB1 is basally phosphorylated almost exclusively on serine, with an increase in phosphorylation following co-transfection with constitutively active MEK (right panel, 3rd lane, shows the gel mobility of phosphoamino acid standards). IB, immunoblot.

**Fig. 8. in vivo and in vitro phosphopeptide mapping of tryptic fragments of GAB1.** Phosphopeptide mapping of GAB1 immunoprecipitated from cells co-transfected with FLAG-GAB1 and dominant negative MEK revealed three major basal phosphorylation sites (fragments 4–6, left panel) with weak phosphorylation of a fourth site (fragment 1). Co-expression of GAB1 with constitutively active MEK resulted in the novel phosphorylation of 2 peptide fragments (fragments 2 and 3, middle panel) and a marked increase in the phosphorylation of fragment 1. In vitro phosphorylation of GST-GAB1 with purified pERK2 (right panel) again resulted in phosphorylation of peptides 1–3.
solution.

Phosphopeptide mapping of GAB1 expressed in intact cells reveals that the basal serine phosphorylation occurs primarily on three tryptic fragments (Fig. 7B, fragments 4–6). The phosphorylation of these sites was not diminished following co-transfection with dominant negative MEK nor were these sites phosphorylated in vitro with purified ERK (Fig. 8), arguing that GAB1 is basally phosphorylated at these sites by a different serine/threonine kinase. Following co-transfection with constitutively active MEK, GAB1 is phosphorylated on three novel tryptic fragments (Fig. 8). Phosphorylation of the same fragments was found following in vitro phosphorylation of GAB1 by purified pERK2, confirming that these sites are directly phosphorylated by activated ERK2. Of note, ERK2 also phosphorylated several fragments in vitro which were not detected in the in vivo assay, making it likely that these sites are non-physiologic.

The potential role that the GAB1-ERK interaction may play in epithelial cell signaling is intriguing. GAB1 has been shown to associate with the membrane via its PH domain in a physiologic fashion (5) and could therefore act to recruit a small fraction of active ERK to a membrane location. For example, recruitment of activated ERK to a membrane-bound EGFR-GAB1 complex following EGF addition might explain the observation that threonine 669 in the EGFR is a major site of ERK phosphorylation (20, 21). Alternatively, the ability of ERK to phosphorylate GAB1 suggests that ERK might regulate GAB1 interactions with other signaling molecules such as the phosphoinositide 3-kinase (as has been demonstrated for insulin receptor substrate 1 (22)) or phospholipase C. Finally, the fact that the same GAB1 domain binds to both the c-MET receptor and ERK1/2 via the MET-binding domain. The interaction with ERK is direct, requires phosphorylation of ERK by its upstream kinase MEK, and results in the novel serine phosphorylation of GAB1 by ERK. This interaction results in GAB1 phosphorylation by ERK and may therefore serve to regulate GAB1 signaling in epithelial cells.

In conclusion, the docking protein GAB1 binds to both the c-MET receptor and ERK1/2 via the MET-binding domain. The interaction with ERK is direct, requires phosphorylation of ERK by its upstream kinase MEK, and results in the novel serine phosphorylation of GAB1 by ERK. This interaction results in GAB1 phosphorylation by ERK and may therefore serve to regulate GAB1 signaling in epithelial cells.

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