A Conserved Inositol Phospholipid Binding Site within the Pleckstrin Homology Domain of the Gab1 Docking Protein Is Required for Epithelial Morphogenesis*

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The receptor for hepatocyte growth factor (HGF)1, scatter factor, Met, belongs to the family of receptor tyrosine kinases including Sea and Ron (1, 2). Met is expressed on epithelial and endothelial cells as well as on muscle precursor cells and neuronal cells and mediates the numerous biological responses attributed to the mesenchymally derived HGF (3–6). Increasing evidence demonstrates an important in vivo role for Met and HGF during the development of liver and placenta, as well as the development and innervation of skeletal muscle, the ductal growth of mouse mammary explants, and directing the growth of axonal cones (7–11). In culture, HGF is a potent mitogen for primary hepatocytes and renal tubule cells and stimulates epithelial cell dissociation, motility, and invasion (5, 12–14). Importantly, HGF triggers an intrinsic morphogenic program in epithelial cells grown in collagen matrices (15, 16). However, until recently the molecular basis underlying the morphogenic response of epithelial cells has remained unclear.

To characterize signaling pathways downstream from the Met receptor involved in epithelial morphogenesis, we and others have used receptor chimeras to demonstrate that the Met receptor cytoplasmic domain is sufficient for the biological responses attributed to HGF and that the Met tyrosine kinase activity is required for these responses (4–6, 17). From structure/function studies, two tyrosine residues within the carboxyl terminus of Met (Tyr1349 and Tyr1356), which are highly conserved between the other members of the Met receptor tyrosine kinase gene family, Sea and Ron, are crucial for cell scatter and branching morphogenesis in Madin-Darby canine kidney (MDCK) epithelial cells (2, 4, 5, 17). Tyrosine 1356 forms a multisubstrate binding site that couples the Met receptor directly with the Grb2 and Shc adapter proteins and is required for recruitment or phosphorylation of the p85 subunit of phosphatidylinositol 3-kinase (PI3K), phospholipase Cγ1 (PLCγ1), the phosphatase SHP2, the c-Cbl proto-oncogene, and the Gab1 (Grb2-associated binder-1) multisubstrate binding protein (17–24).

From a search for Met substrates that are implicated in epithelial morphogenesis, we have recently identified Gab1 as a protein that becomes highly phosphorylated following HGF stimulation of epithelial cells that undergo a morphogenic program (23, 24). Gab1 was initially identified in a library screen as a Grb2-binding protein that is phosphorylated downstream from multiple receptor tyrosine kinases including epidermal growth factor, insulin, and TrkA receptors and, more recently, members of the cytokine receptor family, interleukin-3, interleukin-6, interferon-α, and -γ receptors, and the erythropoietin receptor (25–28). Gab1 is a member of the IRS-1 family of multisubstrate binding proteins, including IRS-1, IRS-2, p62 dok, and daughter of sevenless (29–33), and defines a new subfamily that includes a related protein, Gab2 (34). The greatest homology observed with the other IRS-1 family members lies within the N terminus of Gab1, which contains a pleckstrin homology (PH) domain, suggesting a conserved role for the PH
PIP₃ Binding in Gab1 Cellular Localization and Function

domain within these proteins (25, 35). However, unlike IRS-1, Gab1 lacks a PTB domain and instead has been shown in vivo to be recruited to the Met receptor both indirectly, through the Grb2 adapter protein, and directly, involving the multisubstrate binding site tyrosines, Tyr₁²⁴⁹ and Tyr₁³⁰⁶ in the Met C terminus (20, 23, 36, 37). Gab1 contains multiple tyrosine residues, and following HGF stimulation of epithelial cells, it couples the Met receptor with the p85 subunit of PI3K and associated PI3K activity, SHP2, and PLCγ₁ (24).

MDCK cells expressing Met receptor mutants with decreased ability to recruit Gab1 fail to form branching tubules upon Met activation (21, 23). Overexpression of Gab1 rescues the tubulogenesis defect of these mutants (24). To investigate the mechanism through which Gab1 mediates this function, we have undertaken a structure/function analysis of Gab1 and demonstrated that the Gab1 PH domain is essential for the ability of Gab1 to promote branching morphogenesis (24). In addition, we have established that the Gab1 PH domain is required to target Gab1 to the proximity of the cellular membrane, at sites of cell-cell contact in epithelial cells, and that this localization is also dependent on the activity of PI3K (24).

Increasing evidence supports a role for PH domains in the regulated targeting of proteins to cell membranes through their interactions with inositol phospholipids (reviewed in Refs. 38–40). Amino acid residues implicated in phospholipid binding are highly conserved among PH domains, including that of Gab1, suggesting that the Gab1 PH domain also interacts with membrane phospholipids (41, 42). This possibility is further supported by the recent findings that the Gab1 PH domain can bind to PIP₃ in vitro and in a modified yeast two-hybrid system (41). To establish whether phospholipid binding by the Gab1 PH domain is a prerequisite for its function, we have mutated conserved amino acids in its β2 strand (Trp²⁶ and Arg²⁹) implicated in phospholipid binding from studies of Bruton’s tyrosine kinase (BTK) and other PH domain-containing proteins (41–45). We show here that a similar mutation at residues Trp⁹⁹/Arg⁹⁰ in the PH domain of Gab1 generates Gab1 proteins with reduced ability to bind phosphotyrosinol 3,4,5-trisphosphate (PIP₃) in vitro, reduced cellular localization to sites of cell-cell contact, and decreased ability to rescue Met-dependent morphogenesis.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and DNA Transfections—**MDCK cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS). The generation of MDCK cell lines expressing CSF-Met receptor and its mutant N1358H, by retroviral infection, has been previously described (6, 21). For the generation of stable cell lines expressing wild type Gab1 and its mutant N1358H, by retroviral infection, has been previously described (6, 21). For the generation of stable cell lines expressing wild type Gab1 and its mutant N1358H, by retroviral infection, has been previously described (6, 21). For the generation of stable cell lines expressing wild type Gab1 and its mutant N1358H, by retroviral infection, has been previously described (6, 21).

**Antibodies—**Antibodies raised in rabbit against a C-terminal peptide of human Met were used (47). Anti-phosphotyrosine (4G10) was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-HA (HA.11) was purchased from Babco (Richmond, CA). Anti-SHP2 was kindly provided by Dr. G-S. Feng.

**Site-directed Mutagenesis—**Site-directed mutagenesis within the Gab1 PH domain was performed using the Chameleon™ double-stranded site-directed mutagenesis kit ( Stratagene), according to the manufacturer’s instructions. Trp²⁶/Arg²⁹ residues were converted to either Cys or Ala residues. The mutagenesis primers were the following: for the C/C mutant, 5′-TCGCCCCCGGAGAAGGCTAAGGCTTAAAGCTGCGTGGTTACAGTCTGGTTAGTGTGC TGTCACCTGCGTAAGGTGGATACG-3′ and 5′-TCGCCCGGAGAAGGCTAAGGCTTAAAGCTGCGTGGTTACAGTCTGGTTAGTGTGC TGTCACCTGCGTAAGGTGGATACG-3′ for the A/A mutant, 5′-TCGCCCGGAGAAGGCTAAGGCTTAAAGCTGCGTGGTTACAGTCTGGTTAGTGTGC TGTCACCTGCGTAAGGTGGATACG-3′. The underlined sequences are mutated.

**Phospholipid Binding Assay—**GST fusion proteins were generated by cloning wild type Gab1 and A/A and C/C Gab1 mutants as a BamHI-EcoRI fragment into pGEX-5X-2 (Amersham Pharmacia Biotech) and expressed in E. coli BL21 strain. GST fusion proteins were purified on glutathione-Sepharose according to the manufacturer’s instructions. GST fusion proteins (10 pmol) were bound to 30 μl of glutathione-Sepharose beads (Amersham Pharmacia Biotech) in 30 ml HEPES, pH 7, 100 mM NaCl, 1 mM EDTA, 0.025% Nonidet P-40 (HNE). Labeled phosphotyrosines were sonicated in the same buffer containing 20 μg/ml Ser(P). Unlabeled competitor lipids (dipalmityl) phosphatidylinositol 3,4-bisphosphate or phosphatidylinositol 3,4,5-trisphosphate; Matreya, Inc.) were sonicated at 100 μm with Ser(P) and 25 μM phosphatidylinositol 3,4-bisphosphate and diluted with Ser(P)/HNE, and constant amounts of labeled 32P-P-PIP, (800,000 cpm) were added. Beads with bound fusion proteins were incubated with 40 μl of labeled phospholipid with or without competitor for 1 h at 22 °C, and the beads were washed twice with 1 ml of HNE, 0.1% Nonidet P-40. Beads were subjected to extraction with 60 μl of CHCl₃/CH₃OH (1:1) and added directly to scintillation fluid for quantitation. Data presented were corrected for nonspecific binding by subtracting the counts bound to GST.

**Immunofluorescence—**MDCK cells overexpressing wild type Gab1 or the Gab1 mutants were plated on glass coverslips (Beller Co. Inc.) in a 24-well dish (Nunc) for the indicated times in DMEM containing 10% FBS. Cells were fixed in 2% paraformaldehyde in PBS for 30 min at room temperature, washed twice in PBS, and incubated for 10 min in PBS containing 50 mM ammonium chloride. Following one additional wash in PBS, cells were treated with PBS containing 0.1% Triton X-100 and 5% FBS (buffer A) for 10 min at room temperature. Anti-HA was diluted (1:300) in buffer A, and after three washes in the same buffer, C59-conjugated anti-mouse (1:2000) was added for 10 min, followed by three washes in buffer A. The glass coverslips were mounted on slides in Immunofluore medium (ICN) and visualized using a Nikon Labophot-2 epifluorescence microscope. Photographs were taken using Eastman Kodak Co. TMZ3200 film.

**HGF Stimulation of MDCK Cell Lines Expressing Wild Type and Mutant Gab1—**Cells were added to 1 × 10⁴/100-mm dish. Twenty-four hours later, cells were washed once with DMEM and then starved overnight in 10 ml of DMEM containing 0.02% FBS. HGF (kindly provided by Dr. G. F. Vande Woude) was added at 100 units/ml in 2 ml for the indicated times. Cells were immediately lysed in 1 ml of lysis buffer (50 mM Hepes, pH 7.4, 150 mM NaCl, 10% glycerol, 0.1% Triton X-100, 1 mM phenylmethysulfonyl fluoride, 1 μg/ml each leupeptin and aprotinin, 1 mM NaVO₄).

**Immunoprecipitations and Western Blotting—**MDCK cell lysates (2 mg of total protein), or 293T cell lysates (50 μg) were incubated with the indicated antibodies for 1 h at 4 °C with gentle rotation. Twenty micro- liters of a 50% slurry of either protein A- or protein G-Sepharose was added for an additional 1 h to collect immune complexes. Following three washes in lysis buffer, proteins were resolved by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membranes were blocked for 1 h with 3% bovine serum albumin in TBST (10 mM Tris-HCl, pH 7.4, 2.5 mM EDTA, 150 mM NaCl, 0.1% Tween 20) and then with primary antibody (1:1000) for an additional hour. Following five washes in TBST, proteins were probed with secondary anti-mouse antibody (Jackson ImmunoResearch Laboratories, Inc.) or protein A (Life Technologies, Inc.) conjugated to horseradish peroxidase. The proteins were visualized with an ECL detection system (Amersham Pharmacia Biotech).

**Collagen Assays—**The ability of MDCK cells to form branching tubules was assayed as described previously (17). Briefly, 5 × 10⁶ cells were resuspended in 500 μl of collagen solution (Vitrogen 100 from Celsrix) prepared following the manufacturer’s instructions and layered onto the collagen solution in a 24-well plate. Cells were maintained in Liebowitz medium containing 5% FBS and allowed to form cysts for 5–7 days. For stimulations, HGF (5 units/ml) or recombinant human CSF-1 (50 ng/ml; kindly provided by Genetics Institute, Boston, MA) was added to the Liebowitz medium containing 5% FBS. Tubules were apparent by light microscopy 5–10 days after the addition of stimuli. The medium was changed every 4 days, and photographs were taken using a Nikon Diaphot 200 microscope.
were taken at day 14 on Kodak TMY400 films at a magnification of 10×
For the quantitation of the morphogenetic response, 60 colonies in each of three independent cultures (wells) were scored for their ability to form branching tubules. The results were plotted as the average percentages derived from the number of cysts able to undergo tubulogenesis in cells expressing the Gab1 PH domain mutant proteins compared with cells expressing wild type Gab1.

RESULTS

Mutation of Conserved Residues in the Gab1 PH Domain Decreases the in Vitro Binding of Gab1 to Inositol 3,4,5-Trisphosphate—We have previously demonstrated that Gab1 functions as a multisubstrate docking protein that mediates branching morphogenesis downstream from the Met receptor (23, 24). From structure/function analysis, we have established that the Gab1 PH domain is essential for the ability of this protein to promote morphogenesis and is both necessary and sufficient for the localization of Gab1 to sites of cell-cell contact (24). While cellular localization of Gab1 is also dependent on the activity of PI3K (24), the exact mechanism through which Gab1 localizes to sites of cell-cell contact has not been determined.

While PH domains are found in proteins with a broad range of activities, the three-dimensional structure of this motif is common to all; seven β-strands form two anti-parallel β-sheets that end with an α-helix (reviewed in Refs. 38 and 39). Insight into the role of this domain comes from the identification and characterization of inositol phospholipids as specific ligands for several PH domains (39, 42, 43, 45, 48–52). Thus, such protein-lipid interactions provide a mechanism through which PH domain-containing proteins can be recruited to membranes where they are required to function. In vitro binding studies of a number of isolated PH domains and use of a modified yeast two-hybrid system have allowed the grouping of PH domains based on their lipid binding specificity (41, 42, 53). From the crystal structure (51, 54) and the alignment of several PH domains within the various groups, a model involving a consensus amino acid sequence was generated, which includes a WRK/KXR motif in the lipid binding domain (41, 42). Alignment of the Gab1 PH domain with other PH domains known to bind PI3K products indicates that the highly conserved Arg\(^{28}\) residue in the BTK PH domain, within the β2 strand, is also found in the PH domain of Gab1 (Arg\(^{29}\)) (Fig. 1A). A less conserved residue, Trp\(^{26}\), is found in several but not all PH domains (Fig. 1A). To investigate the role of the Gab1 PH domain in epithelial morphogenesis and in Gab1 cellular localization, we have undertaken site-directed mutagenesis within the putative phospholipid binding site of the Gab1 PH domain and first studied the effect of such mutations on the ability of Gab1 to bind PI\(^3\). The conserved Trp\(^{26}/\text{Arg}^{29}\) residues were replaced either by alanine residues (A/A), rendering the charge similar to PI3K products. As shown in Fig. 2, mutation of the Trp/Arg residues into either alanine or cysteine residues results in a 50% reduction in the ability of these mutated proteins to bind PI\(^3\) as compared with GST-wild type Gab1. Furthermore, we compared the ability of unlabeled PI\(^3\) and PI3K to compete with \(^{32}\)P-PI3 binding and show that unlabeled PI\(^3\) (100 μM) efficiently displaces \(^{32}\)P-PI3, whereas a similarly high concentration (100 μM) of unlabeled PI2 does not. These results not only indicate that the loss of the conserved residues in the Gab1 PH domains correlates with a reduction in phospholipid binding but also that the remaining

FIG. 1. Alignment of the PH domain of Gab1 with PH domains from other proteins known to bind PI3K products. A, PH domains from Gab1 (amino acids 7–35), GenBank\(^\text{TM}\) accession number HSU43885 (25)), daughter of sevenless (amino acids 7–35, GenBank\(^\text{TM}\) accession number X9744 (31)), BTK (amino acids 5–33, GenBank\(^\text{TM}\) accession number 129788 (76)), and AKT (amino acids 9–31, GenBank\(^\text{TM}\) accession number X65687 (77)) were aligned using the Mac Vector ClustalW formatted alignment program. The β1 and β2 strands are delineated by the arrows below the sequences. Conserved residues are highlighted in gray boxes. The Trp\(^{26}\) and Arg\(^{29}\) residues are marked by arrows above the sequences. B, schematic representation of Gab1 proteins used in this study. Shown is a wild type Gab1 protein in comparison with the A/A and the C/C mutant Gab1 proteins as well as the Gab1 mutant lacking the entire PH domain (ΔPH). The mutated residues are indicated by an asterisk above the corresponding residues in the wild type molecule.

FIG. 2. Reduced binding of A/A and C/C mutant Gab1 proteins to PI\(^3\). PI3K binding assays were performed as described under "Experimental Procedures." GST fusion proteins were immobilized on glutathione-Sepharose beads and incubated with \(^{32}\)P-PI3, in the presence or absence of competitor phospholipids. Following washing, the bound phospholipids were extracted, and radioactivity was quantitated by liquid scintillation. The data were corrected for nonspecific binding by subtracting the counts bound to GST alone, and are the means ± S.D. of duplicate determinations. Open bars, no competitor; thin stripes, 100 μM PI2; thick stripes, 10 μM PI3; black bars, 100 μM PI2.

PH domain-lipid interaction is preferentially directed to PI\(^3\).

The Conserved Phospholipid Binding Site within the Gab1 PH Domain Is Required for the Ability of Gab1 to Localize to Sites of Cell-Cell Contact—PH domains have been shown to be essential for the localization of multiple proteins to the plasma membrane. For example, deletion of the PH domain from PLC\(\gamma1\) or specific mutations that alter phospholipid binding within this domain resulted in the relocalization of this protein to the cytoplasm (55, 56). We have shown that the PH domain of Gab1 is necessary for the localization of Gab1 to the proximity of the cell membrane at sites of cell-cell contact in epithelial cells and that the PH domain of Gab1 alone is sufficient

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for membrane recruitment of Gab1 (24). Moreover, Gab1 localization at the plasma membrane is dependent on PI3K activity (24), suggesting that PI3K-generated phospholipids were directly or indirectly implicated in the recruitment of Gab1 to the plasma membrane.

It is widely accepted that the molecular basis through which PH domains are targeted to membranes involves binding to membrane phospholipids. The PH domain of Gab1 binds PIP₃² (Fig. 2, and Ref. 41); therefore, to test the possibility that PIP₃ targets Gab1 to the plasma membrane, we have generated stable cell lines expressing HA epitope-tagged A/A or C/C mutant Gab1 proteins with reduced PIP₃ binding. The level of expression of the mutant proteins is analogous to that observed in cells expressing wild type Gab1, indicating that mutation of the phospholipid binding site within the Gab1 PH domain has no detectable effect on protein stability (Fig. 3A). To investigate the cellular localization of the mutant Gab1 proteins, MDCK cell lines expressing each mutant were grown on glass coverslips and subjected to indirect immunofluorescence using anti-HA followed by CY3 conjugated anti-mouse. Photographs were taken at a magnification of ×60.

The Conserved Trp²⁶/Arg²⁹ Residues Are Not Required for Phosphorylation of Gab1 or Its Association with the Met Receptor—Association of the Met receptor tyrosine kinase with Gab1 in coimmunoprecipitation assays is dependent on Tyr¹₃₅₆ of the Met C terminus or on the presence of a Grb2 binding site downstream from Tyr¹₃₅₆ (23, 24, 36, 37). To determine whether the conserved Trp²⁶/Arg²⁹ residues in the Gab1 PH domain are required for the interaction Gab1 with the Met receptor, transient transfection assays in 293T cells were performed. The ability of wild type Gab1 and mutants thereof to coimmunoprecipitate with either wild type Met receptor or the Grb2 binding mutant (N1358H) was evaluated. Wild type Met coimmunoprecipitated with the A/A and C/C Gab1 mutant proteins as efficiently as with wild type Gab1. This is revealed using anti-Met for the immunoprecipitations followed by Western blotting using anti-HA or the converse (Fig. 4). The N1358H Met receptor mutant, as described previously, associates less efficiently with Gab1 when compared with wild type Met. However, both A/A and C/C Gab1 mutants are comparable with wild type Gab1 in their efficiency to coimmunoprecipitate with N1358H Met (Fig. 4). Stripping of the blots and reprobing with anti-HA shows that similar levels of Gab1 proteins are expressed in the different experimental groups (Fig. 4).

We have previously demonstrated in MDCK epithelial cells that Gab1 becomes highly phosphorylated following Met receptor activation (23, 24). As a consequence, Gab1 associates with several signaling proteins including the phosphatase SHP2 and the p85 subunit of PI3K (24). Further, we have demonstrated that the kinetics of Gab1 phosphorylation is distinct following activation of the Met receptor or the epidermal growth factor receptor. In the former, HGF induces sustained Gab1 phosphorylation that lasts at least 60 min before returning to basal levels (24). Importantly, stimulation of Gab1-over-expressing MDCK cells with epidermal growth factor does not induce branching morphogenesis. In these cells, stimulation with epidermal growth factor results in transient phosphorylation of Gab1, which returns to base line within 15 min of stimulation (24), suggesting that the kinetics of Gab1 phosphorylation is implicated in Gab1 biological activities.

To investigate whether phospholipid binding of the Gab1 PH domain is implicated in Gab1 phosphorylation downstream from the Met receptor, stable MDCK epithelial cell lines expressing either HA epitope-tagged Gab1 A/A or C/C mutant proteins were stimulated with HGF. An increase in the level of phosphorylation of A/A and C/C Gab1 mutant proteins is observed within 15 min of stimulation, which is maintained for 60 min (Fig. 5). This increase is comparable both in intensity and duration with that observed in cells expressing wild type Gab1. Further, mutation of the Trp²⁶/Arg²⁹ conserved residues into either alanine or cysteine residues does not detectably alter the ability of these proteins to coimmunoprecipitate with SHP2 (Fig. 5) or p85 (not shown). Thus, mutations in the β strand of the PH domain that result in reduced phospholipid binding by Gab1 do not interfere with Met-dependent Gab1 phosphorylation or the binding of Gab1 to signaling proteins. These results
are consistent with our previous findings that deletion of the entire PH domain had no effect on Gab1 recruitment to the Met receptor, Gab1 phosphorylation, or its ability to bind cellular signaling proteins.

An Intact Gab1 PH Domain Is Required for Efficient Rescue of Branching Tubulogenesis Downstream from the Met Receptor—Structure/function studies using chimeric CSF-Met receptor mutants have revealed that receptor mutants impaired in their association with Gab1 are unable to induce branching tubules (17, 21). Thus, cell lines expressing a CSF-Met receptor mutant that is specifically unable to bind Grb2 (N1358H) and shows reduced Gab1 recruitment fail to form branching tubules in response to CSF-1 (23, 24). Importantly, overexpression of Gab1 in these lines rescues the ability of CSF-Met to promote branching tubules in response to CSF-1. This response requires the Gab1 PH domain, since mutant Gab1 proteins lacking the entire PH domain fail to rescue (24). To establish if the Gab1 PH domain point mutants A/A or C/C, which retain the PH domain yet show reduced phospholipid binding and reduced localization at sites of cell-cell contact, are able to rescue branching tubulogenesis downstream from the N1358H CSF-Met receptor mutant, five independent cell lines overexpressing each mutant Gab1 protein were generated by transfection. The ability of these to form branching tubules following activation of CSF-Met N1358H mutant receptor with CSF-1 was assayed and compared with the ability of each cell line to form tubules following activation of the endogenous Met receptor by HGF. The level of expression of Gab1 in two representative cell lines is shown (Fig. 6A); the tubulogenic response is shown for one clone, and the quantitation of this response is shown for two clones (Fig. 6, B and C).

As previously demonstrated, cells expressing the N1358H CSF-Met Grb2 mutant do not form branching tubules in response to CSF, and overexpression of a wild type Gab1 protein in these lines rescues the tubulogenic response, whereas cells expressing a ΔPH Gab1 mutant are unable to rescue (Fig. 6, B and C). Cells expressing the A/A and the C/C Gab1 mutants form cysts, as do parental cells when grown in a collagen matrix; however, they show reduced ability to form branching tubules when the chimeric CSF-Met N1358H protein is stimulated with CSF-1. Comparison of cell lines expressing the Gab1 PH domain A/A and C/C mutants with cell lines expressing wild type Gab1 reveals that 50% of preformed cysts from the A/A mutant-expressing cell lines undergo branching tubulogenesis (Fig. 6, B and C). Interestingly, the response of cells expressing the C/C mutant Gab1 protein is more severely altered. Only two of the five cell lines tested are able to form branching tubules, and quantitation of this response reveals that only 18 and 0.8% of cysts in these two cell lines are able to form branching tubules when compared with cells expressing wild type Gab1 (Fig. 6, B and C). Furthermore, the expression of the A/A and C/C Gab1 mutants per se does not interfere with the intrinsic ability of these cells to form branching tubules in response to HGF (Fig. 6, B and C). Thus, the reduced binding of A/A and C/C Gab1 mutants to PIP3 correlates with reduced ability of these proteins to rescue branching morphogenesis downstream from the CSF-Met N1358H receptor.

DISCUSSION

Hepatocyte growth factor and the Met receptor tyrosine kinase regulate dispersal of epithelial cell sheets in culture and promote the inherent morphogenic program of epithelia when grown in matrix cultures (5, 15, 16). We have recently demonstrated that the multisubstrate docking protein Gab1 is required for epithelial morphogenesis downstream from the Met receptor (24). Met receptor mutants that are unable to associate with Gab1 are impaired in their ability to promote branching tubulogenesis in MDCK cells, and overexpression of Gab1 rescues the Met-dependent tubulogenic response (21, 24). Structure/function analysis had revealed that the Gab1 PH domain is required for the rescue of tubulogenesis and for localization of Gab1 to the proximity of the cell membrane at sites of cell-cell contact (24). However, the mechanism through which the Gab1 PH domain provides its function in branching morphogenesis and cellular localization had remained unclear. In this paper, we show that conserved residues within the β2 strand of the PH domain, Trp26 and Arg29, are required for efficient binding of Gab1 to the PI3K product PIP3. Moreover, the ability of Gab1 to bind PIP3 is required for the localization of Gab1 at sites of cell-cell contact and rescue of Met-mediated branching tubulogenesis.

A large body of literature implicates PH domains in the targeting of proteins to cellular membranes, through interaction with inositol phospholipids (39, 45, 48, 49, 51, 52, 55–60). Recently, PH domains have been subdivided into four groups based on their inositol phospholipid binding specificity (41, 42, 53). PH domains in group I are those with the highest affinity for the PI3K product PIP3. Included in this group are PH domains from the protein tyrosine kinase BTK, as well as the IRS-1 family member daughter of sevenless and Grp-1. Group II members bind either PIP2 or PIP3, with a lower affinity of binding and include PLC-δ1. Group III contains AKT/PI3K, which binds preferentially to phosphatidylinositol 3,4-bisphosphate, although binding to PIP3 has also been reported (45, 48, 61, 62). PH domains from group IV bind weakly to inositol phospholipids as, for example, the PH domain from dynamin (41, 42, 53). Importantly, such studies have allowed the identification of a consensus sequence in PH domains that bind to

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**Fig. 5. The A/A and C/C mutant Gab1 proteins are phosphorylated following Met activation.** MDCK cells expressing wild type Gab1 (clone 9, WT-9), A/A Gab1 (clone 2, A/A-2), and C/C Gab1 (clone 1, C/C-1) were serum-starved for 24 h prior to stimulation with 100 units/ml HGF for the indicated times. Gab1 proteins were immunoprecipitated with anti-HA and subjected to Western blotting with anti-PY (left panel). Membranes were stripped and reprobed with anti-SHP2 (middle panel). 50 μg of proteins from lysates of the different experimental groups were resolved on a 10% SDS-polyacrylamide gel, transferred to nitrocellulose, and blotted with anti-HA to ensure equivalent levels of proteins in all groups (right panel).
undergone branching tubulogenesis in the cells expressing mutant
The responses are plotted as the average percentage of cysts that have
mutants was performed as described under "Experimental Procedures."
N1358H CSF-Met receptor with either wild type Gab1 or the Gab1
protein, are shown. Gab1 proteins with mutations of the conserved
conserved among proteins that bind products of PI3K (Fig. 1)
throughout the molecule to inositol phospholipid binding. Additional lipid
sites were required (24). Hence, we conclude that the generation of
was dependent the presence of the Gab1 PH domain and on
was required (24). Hence, hence, these
studies together with the interaction of Gab1 with PIP3 both in vitro2 (Fig. 2)
and in a modified yeast two-hybrid system (41)
provide strong evidence that PIP3 is a physiological ligand for
the Gab1 PH domain.

While the integrity of the PH domain is critical for efficient
PIP3, W(R/K)X(R/K) (Ref. 41, Fig. 1). However, while the phospho-
lipid binding specificity has been determined for many pro-
teins, the biological relevance for such lipid-protein inter-
actions has been characterized predominantly for PH domain-
containing proteins with enzymatic activities, BTK, AKT,
PLCg1, and PLCb1, and not for members of the family of
multisubstrate proteins like Gab1 (48, 63–69). This study is the
first to define the biological relevance of lipid-protein inter-
actions for the Gab family of multisubstrate proteins.

FIG. 6. Trp26/Arg29 residues are required for efficient rescue of
branching tubulogenesis downstream from the N1358H CSFMet mutant receptor. A, lysates from MDCK cells expressing the
CSF-Met N1358H mutant receptor and either wild type Gab1 or Gab1
mutants, as indicated in the figure, were subjected to immunoprecipita-
tion with anti-HA followed by Western blotting using the same anti-
body. Two representative lines, each expressing A/A or C/C mutant
Gab1 proteins, are shown. B, quantitation of the tubulogenic response
following stimulation with HGF and CSF in cell lines expressing
N1358H CSF-Met receptor with either wild type Gab1 or the Gab1
mutants was performed as described under “Experimental Procedures.”
The responses are plotted as the average percentage of cysts that have
undergone branching tubulogenesis in the cells expressing mutant
Gab1 compared with wild type Gab1-expressing cells. The values were
derived from three independent experiments. C, MDCK cell lines ex-
pressing the CSF-Met N1358H mutant receptor together either with wild type
Gab1 (WT-3), the A/A Gab1 mutant (A/A-6), the C/C Gab1 mutant
(C/C-7), or the ΔPH Gab1 mutant (ΔPH-3) were grown in collagen for
5 days, during which they formed cysts. Recombinant human CSF-1 (50
ng/ml) or HGF (5 units/ml) was added, and 14 days later branching
structures were visualized at a magnification of × 10. Photographs were
taken with Kodak TMY 400 film.
rescue of tubulogenesis by Gab1, we show that this is not related to alterations in the ability of the mutant Gab1 proteins to be recruited to the Met receptor or to their tyrosine phosphorylation following stimulation of cell lines with HGF (Figs. 4 and 5). Moreover, following stimulation of cells with HGF, the WR mutant Gab1 proteins associate with SHP2 and the p85 subunit of PI3K to similar levels as wild type Gab1, indicating that the association of Gab1 with known substrates is not altered. However, we do observe that the electrophoretic mobility of phosphorylated WR mutant Gab1 proteins is slightly faster than that of wild type Gab1 proteins (Fig. 4), although no detectable changes in the electrophoretic mobility of the unphosphorylated mutant proteins are observed (Figs. 3A and 6A). Thus, these mutations may result in decreased and/or altered phosphorylation of Gab1 (Fig. 4). These results are consistent with our previous data showing that the Gab1 PH domain is dispensable for Gab1 phosphorylation and association with Met and suggest a distinct role for the Gab1 PH domain in signaling downstream from the Met receptor. These data support two mechanisms for recruitment of Gab1 to the membrane. One involves the interaction of the Gab1 PH domain with PIP3 and is required for localization of Gab1 at cell-cell junctions in epithelial cells. However, Gab1 lacking its entire PH domain or mutated in the WR motif in its PH domain can still be recruited to the Met receptor at the cell membrane (Ref. 24, Fig. 4).

In conclusion, Gab1 functions as a multisubstrate docking protein that is required for epithelial morphogenesis downstream from the Met receptor (24). Structure/function analyses have revealed that the Gab1 PH domain is required for the localization of Gab1 at sites of cell-cell contact and its biological activities. Here, we have presented molecular and biochemical evidence that PIP3 is a physiological ligand for the Gab1 PH domain required for subcellular localization of Gab1 to sites of cell-cell contact and biological activities of Gab1. We propose that the recruitment of Gab1 and its associated signaling proteins to membrane domains rich in PIP3 and/or its stabilization at those sites is crucial for Gab1 to amplify/compartmentalize signals generated downstream from the Met receptor. Recently, both amplification of PI3K and loss of function of a 3′ lipid phosphatase, PTEF/MMAC1, have been shown in multiple human tumors (72–75), demonstrating that the regulation of PI3K at the cellular membrane may be critical for the control of multiple biological processes including tumorigenesis. Thus, understanding the molecular mechanisms through which Gab1 subcellular localization and function are regulated during epithelial morphogenesis will provide insight into how the epithelial organization may be altered during the progression of cancers.

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