The Gab1 docking protein forms a platform for the assembly of a multiprotein signaling complex downstream from receptor tyrosine kinases. In general, recruitment of Gab1 occurs indirectly, via the adaptor protein Grb2. In addition, Gab1 interacts with the Met/hepatocyte growth factor receptor in a Grb2-independent manner. This interaction requires a Met binding domain (MBD) in Gab1 and is essential for Met-mediated epithelial morphogenesis. The Gab1 MBD has been proposed to act as a phosphotyrosine binding domain that binds Tyr-1349 in the Met receptor. We show that a 16-amino acid motif within the Gab1 MBD is sufficient for interaction with the Met receptor, suggesting that it is unlikely that the Gab1 MBD forms a structured domain. Alternatively, the structural integrity of the Met receptor residues upstream of Tyr-1349 located in the C-terminal lobe of the kinase domain, are required for Grb2-independent interaction with the Gab1 MBD. Moreover, the substitution of Tyr-1349 with an acidic residue allows for the recruitment of the Gab1 MBD and for phosphorylation of Gab1. We propose that Gab1 and the Met receptor interact in a novel manner, such that the activated kinase domain of Met and the negative charge of phosphotyrosine 1349 engage the Gab1 MBD as an extended peptide ligand.

The Gab family of docking proteins consists of Gab1, Gab2, and Gab3 in mammals, Daughter of Sevenless in Drosophila, and Suppressor of Clear-1 in Caenorhabditis elegans. Gab proteins are non-enzymatic and are characterized by a conserved N-terminal pleckstrin homology domain that, where investigated, binds phosphatidylinositol 3,4,5-triphosphate and mediates the phosphatidylinositol 3,4,5-triphosphate-dependent membrane association of Gab proteins (1–3). In addition, Gab family proteins contain multiple conserved tyrosine residues within consensus binding sites for SH2 domain-containing proteins, as well as proline-rich regions that interact with SH3 domain-containing proteins. Gab proteins play a crucial role in transmitting signals from a variety of stimuli, including growth factors, cytokines, and T and B cell antigens, to downstream effectors involved in normal growth, differentiation, and development programs (reviewed in Ref. 4).

Gab1 (Grb2-associated binder 1) was the first mammalian gab gene cloned and was originally identified as a Grb2-binding protein in an interaction screen using a cDNA library prepared from glioblastoma (5). Gab1 is widely expressed and is phosphorylated downstream from numerous receptor tyrosine kinases, cytokine receptors, G protein-coupled receptors, and antigen receptors. In vivo, Gab1 is critical for embryonic development, because gab1-deficient mice die in utero displaying defects in the heart, placenta, and skin, as well as reduced liver size (6, 7). In vitro, Gab1 promotes cell survival, neurite outgrowth, and DNA synthesis in neuronal cells downstream from the TrkA receptor (8, 9) and regulates an invasive epithelial morphogenic program downstream from the HGF/Met receptor tyrosine kinase (1, 10, 11).

In epithelial cells, Gab1 is the major substrate for the Met receptor tyrosine kinase (12), and, upon tyrosine phosphorylation, Gab1 provides binding sites for proteins involved in signal transduction, including the tyrosine phosphatase, SHP-2, the p85 subunit of PI3K, phospholipase Cγ, as well as the Crk adaptor protein (10, 11, 13–15). The association of Gab1 with several of these proteins, as well as an intact Gab1 pleckstrin homology domain, is required for the ability of Gab1 to promote the morphogenic program of Madin-Darby canine kidney epithelial cells downstream from the Met receptor (1, 10, 11, 16). In contrast, the Gab2 protein fails to promote a morphogenic response, indicating that Gab1 and Gab2 are functionally distinct downstream from the Met receptor (15, 17).

Gab1 and Gab2 contain highly conserved, but atypical binding sites for the C-terminal SH3 domain of the adaptor protein Grb2, and are recruited indirectly via a Grb2-dependent interaction to the epidermal growth factor receptor, the fibroblast growth factor receptor-1, and the interleukin-3 receptor beta common chain (16) (2, 18–21). Whereas the recruitment of Gab2 to the Met receptor is strictly Grb2-dependent (29, 54), the interaction between Gab1 and the Met receptor is distinct, in that Gab1 is recruited by both a Grb2-dependent and Grb2-independent mechanism (12, 22). The Grb2-dependent recruitment requires tyrosine 1356 in the Met receptor C terminus, which forms a Grb2 SH2 domain binding site (12), and intact Grb2 SH3 domain binding sites on Gab1 (18). The Grb2-independent recruitment of Gab1 requires an 83-amino acid, proline-rich region of Gab1, termed the Met binding domain (MBD). This interaction was initially identified in a yeast-two hybrid interaction assay and requires the kinase activity of the Met receptor and phosphorylation of tyrosine 1349 in the Met
Direct Binding of Gab1 and Met

EXPERIMENTAL PROCEDURES

Plasmids and Mutagenesis—The generation of GST-MBD, GST-MBDlGrb2, and MBdGrb2-nte was previously described (18). GST-MBD16 and GST-MBD16 R499A were generated by insertion of double-stranded oligonucleotides into BamHI/EcoRI sites of pGEX 2TK. MBD16-F, GATCTTTTGGAATTCAGGTACTCTCCTGCTCATATGGCCTTGAAGATTG; MBD16-R, AATTCACCTTCTAAAAAGGCCATATGAGCAGGAGAGGTCATGGCATCTCAGAAAG. Alanine-scanning mutations in Tpr Met-PMX were generated via the QuikChange mutagenesis protocol (Stratagene). Met C-terminus truncation mutants were generated by insertion of stop codons at amino acids 1348 and 1353, respectively. Tpr Met A and B mutants were p85 and Shc binding variants, respectively, and are described in a previous study (23). All mutants were sequenced prior to use.

Cell Culture, DNA Transfections, and Total Cell Extracts—293T cells were seeded at 1 × 10^6/100-mm Petri dish and transfected 24 h later by the cationic phosphate precipitation method (24) with 2 µg of DNA, whereas 293 cells were transfected with 10 µg of DNA by the SuperFect method (Qiagen). Cells were serum-starved in 1% fetal bovine serum (FBS) for 24 h and harvested in either 0.5% Triton X-100 lysis buffer for co-immunoprecipitation assays (0.5% Triton X-100, 50 mM HEPES, pH 8.0, 150 mM NaCl, 10% glycerol, 2 mM EDTA, 1.5 mM MgCl₂) or in RIPA buffer for GST-pull-down assays (0.5% SDS, 50 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate). Inhibitors (10 µg of aprotinin/ml, 10 µg of leupeptin/ml, 1 mM phosphomethylsulfonyl fluoride, 1 mM sodium fluoride, and 1 mM sodium vanadate) were included in each lysis buffer. Following a 10-min incubation on ice, the lysates were centrifuged at 13,000 rpm for 10 min at 4°C. GST Fusion Proteins, in Vitro Association Assays, Immunoprecipitations, and Western Blotting—Fusion proteins were produced in the DH5α or BL21 gold Escherichia coli strain, by induction with isopropyl-1-thio-β-D-galactopyranoside. GST fusion proteins (0.5–1 µg) were immobilized on glutathione-Sepharose beads for 30 min at room temperature and washed three times prior to a 1-h incubation with the indicated cell lysates (lysed in RIPA buffer). For some experiments, the following peptides were added at the indicated concentrations: Gab1 MBD16 (FGMQVPPPAMFGFRSS) or Gab1 MBD16 R499A (FGMQVPPPAHMGFRASS). The peptides were synthesized at the Sheldon Bio-technology Center (Montreal, Quebec, Canada). Immunoprecipitation and Western blotting were performed as previously described (10). Lysates prepared from 293T cells transiently expressing Tpr Met mutants (300 µg) were immunoprecipitated with anti-Met antibody, loaded on an 8% polyacrylamide gel, transferred to membrane, and blocked overnight in 10% milk in TBST containing 1 mM sodium vanadate. GST-Grb2 and GST-MBD were eluted from glutathione-Sepharose beads with reduced glutathione, and the concentration was determined by Bradford assay. Purified protein (20 µg) was incubated with 2 µl of glutathione-conjugated horseradish peroxidase (G-6400, Sigma) for 30 min at room temperature. Washed membranes were then incubated with this reaction mixture in 10 ml of TBST for 30 min, prior to washing and ECL. Membranes were stripped and Western-blotted with anti-Met 144 antibody. The protocol is described in a previous study (25).

Antibodies and Reagents—Antibodies against the extreme C terminus (144 Met) and the extracellular domain (DL-21, Upstate Biotechnology Inc., Lake Placid, NY) of human Met protein were used. RC20H was purchased from Transduction Laboratories, anti-GST and anti-Grb2 were from Santa Cruz Biotechnology (Santa Cruz, CA), anti-pY1349Met was from Cell Signaling (Mississauga, Ontario, Canada), and anti-Gab1 was from Upstate Biotechnology Inc.

Three-dimensional Modeling of Met—The catalytic domain of Met was modeled using Swiss Model Comparative Protein Modeling Server and was visualized with Swiss-Pdb viewer (27–29). The Met kinase domain was modeled on available structures of the insulin and IGF-1 receptors (Protein Data Bank ID codes, 1K3A, 1HRK, 1HR3, and 1GAGA).

RESULTS

A Peptide Motif within the Gab1 MBD Is Sufficient to Interact with the Met Receptor—The mechanism of interaction between the Gab1 MBD and the Met receptor, as defined from yeast two-hybrid analyses, is still undefined. This is complicated by the fact that one of the Grb2 SH3 domain binding sites is located within the MBD, which contributes to the indirect recruitment of Gab1 to Met. To define the requirements for an interaction that is independent of Grb2, we have utilized a GST fusion protein derived from the MBD region of Gab1 that is lacking the Grb2 binding site (MBDlGrb2) (18). Previous studies had identified 13 amino acids within the MBD as essential for Grb2-independent recruitment of Gab1 (15, 17). To investigate the requirements for Met receptor binding, we first established whether a 16-amino acid peptide containing these 13 amino acids (MBD16, FMGVPPPAMFGFRSS (Fig. 1A)) would act as a competitor in association studies in vitro. Addition of increasing levels of this peptide (10–250 µM) was able to compete the association of GST-MBDlGrb2 from Met in an in vitro association assay (Fig. 1B). This was specific for the interaction of Met with the Gab1 MBDlGrb2, because the peptide failed to compete the association of a GST-Grb2 fusion protein with Met (Fig. 1B) or the association of a WT Gab1MBD fusion protein with Grb2 (Fig. 1C). Our previous studies had shown that Arg-499 within the Gab1MBD was required for the interaction of Met with GB (25). Arg-499 was expressed to Ala (Gab1MBD16 R499A) failed to compete the association of the Gab1MBDlGrb2 with Met (Fig. 1D).

We had previously demonstrated that insertion of these 16 amino acids into Gab2 was sufficient to confer the capacity for Grb2-independent Met binding on Gab2 (17). However, it was unclear if this short sequence was sufficient to interact with Met independently in a direct manner, or if surrounding amino acids in Gab2, which share homology with the Gab1 MBD, were also required. To investigate this, we created a fusion protein containing only these 16 amino acids fused to GST (GST-MBD16). When used in an in vitro association assay, the GST-MBD16 fusion protein was sufficient to associate with Met, albeit at a lower efficiency than the full-length MBDlGrb2. Moreover, consistent with our previous studies, the conversion of Arg-499 to Ala abolished the ability of the GST-MBD16 fusion protein to bind to Met (Fig. 1E). No association of Met was detected with GST alone, indicating that binding was specific to the 16 amino acids in the Met binding domain, referred to as the Met binding motif (MBM).

Structural Integrity of Met Is Required for Interaction with the Gab1 MBD—The observation that a 16-amino acid peptide...
A peptide motif within the Gab1 MBD is sufficient to interact with the Met receptor. A, schematic representation of Gab1, highlighting binding sites within the MBD, including those for the SH2 domains of the p85 subunit of PI3K, and the C-terminal SH3 domain of Grb2. Amino acids 486–501 within the MBD are indicated, with critical residues 487–499 underlined, and Arg-499 is represented in boldface. B, competition of Met from GST-MBDGrb2 and GST-Grb2 fusion proteins using the Gab1 MBD16 peptide (FGMQVPPPAHMGFRRSS). The indicated concentrations of peptides were incubated for 1 h with 100 μg of lysate prepared from 293T cells transiently expressing Tpr Met, prior to addition of GST fusion proteins (1 μg) previously bound to glutathione-Sepharose beads. Bound Met was detected by Western blot with the Met 144 antibody. C, competition of Met or Grb2 from GST-MBD using the Gab1 MBD16 peptide. The indicated concentrations of peptides were incubated for 1 h with 100 μg of lysate prepared from 293T cells transiently expressing Tpr Met, prior to addition of GST fusion proteins (1 μg) previously bound to glutathione-Sepharose beads. Bound Met was detected by Western blot with the Met 144 antibody. D, competition of Met or Grb2 from GST-MBD using the Gab1 MBD16 peptide with arginine 499 substituted for an alanine (MBD16-R499A, FGMQVPPPAHMGFRRSS). The indicated concentrations of peptides were incubated for 1 h with 100 μg of lysate prepared from 293T cells transiently expressing Tpr Met, prior to addition of GST-MBD fusion protein or GST alone (1 μg) previously bound to glutathione-Sepharose beads. Bound Met or Grb2 were detected by Western blot with the Met 144 antibody. E, competition of Met or Grb2 from GST-MBD using the Gab1 MBD16 peptide with arginine 499 substituted for an alanine (MBD16-R499A, FGMQVPPPAHMGFRRSS), as in B, E, proteins from lysates prepared from 293T cells transiently expressing Tpr Met were incubated with the indicated GST fusion proteins previously bound to glutathione-Sepharose beads for 1 h. Bound Met was detected by Western blot with the Met 144 antibody.

derived from Gab1 can bind to Met is inconsistent with the Gab1 MBD forming a structured domain in a manner similar to a PTB or SH2 domain. In general, an interaction between two proteins requires at least one of the interacting regions to form a structured domain. To assess whether the structural integrity of the Met receptor, rather than the Gab1 MBD, is required for this interaction, we performed in vitro association assays with denatured Met protein. Proteins from lysates of 293T cells transiently transfected with Met expression plasmids were immunoprecipitated with Met antibody, boiled in 1% SDS to promote protein unfolding, and boiled proteins were then added to the in vitro association assay. As a control, the same assay was performed using the same lysates that were not previously boiled. Importantly, GST-Grb2 and GST-Grb2 SH2 domain fusion proteins bound equally to denatured or non-denatured Met proteins (Fig. 2A). This was not surprising, because the interaction of Grb2 with Met requires only a short phosphotyrosine motif corresponding to Tyr-1356 (YYVN) in the Met C terminus and the structured SH2 domain of Grb2 (30, 31). In contrast, although the GST-MBDGrb2 fusion protein associated with non-denatured Met protein, it failed to bind Met from the boiled lysate containing denatured protein (Fig. 2A). This indicates that the interaction of the Gab1 MBD with the Met receptor requires the structural integrity of Met. This is further supported by the inability of the MBD to bind a denatured Met protein following SDS-PAGE and transfer to nitrocellulose membrane in a Far Western blot assay. In contrast, a GST-Grb2 fusion protein efficiently binds Met in a Far Western assay (Fig. 2B).

Delineation of the Gab1 MBD Binding Site on Met—To define the requirements in Met for interaction with the Gab1 MBD, we undertook a structure-function approach using previously characterized mutants of Met. The substitution of tyrosine 1349 with phenylalanine severely reduced the association between GST-MBDGrb2 and Met in an in vitro association assay (Fig. 3A) or by co-immunoprecipitation when both proteins are transiently overexpressed in 293T cells (Fig. 3B). In contrast, the substitution of tyrosine 1336 with phenylalanine had little effect on MBDGrb2 binding by either assay, whereas, as expected, a mutant with both Tyr-1349 and Tyr-1356 substituted with phenylalanine residues or a kinase inactive mutant (K1110A) were both unable to bind to a MBDGrb2 fusion protein (Fig. 3, A and B). This identifies an important role for phosphotyrosine 1349 in the Grb2-independent interaction with the Gab1 MBD and supports previous data obtained using a yeast two-hybrid interaction assay (22).

To determine whether amino acids upstream or downstream from Tyr-1349 are required for Grb2-independent recruitment of the Gab1 MBD, we used existing mutants of the Met oncprotein where amino acids 1353–1362 of Met were replaced
with amino acids distinct from those in the Met receptor but still capable of recruiting signaling proteins. Mutant A contained amino acids derived from the platelet-derived growth factor receptor, and mutant B contained amino acids derived from the TrkA receptor, with binding sites for the p85 subunit of PI3K and the Shc adapter protein, respectively (23). When subjected to an in vitro association assay, both the A and B variants were capable of associating with the MBD-Grb2 fusion protein. As expected, substitution of Tyr-1349 to Phe in the Met receptor kinase domain and C terminus. Residues substituted with alanine are indicated. B, GST-MBDΔGrb2 fusion protein bound to glutathione-Sephrose beads was incubated in RIPA lysis buffer with 350 μg of lysate prepared from 293T cells transiently expressing Tpr Met WT or alanine-scanning mutants. Bound Met was detected by Western blot with the 144 Met antibody. Total cell lysates (20 μg) were also loaded to ensure equal expression of the Tpr Met WT or alanine-scanning mutants. Bound Met was detected by Western blot with the 144 Met antibody. Total cell lysates (20 μg) were also loaded to ensure equal expression of the Tpr Met mutants. C, GST-MBDΔGrb2 fusion protein bound to glutathione-Sepharose beads was incubated in RIPA lysis buffer with 350 μg of lysate prepared from 293T cells transiently expressing Tpr Met WT or alanine-scanning mutants were incubated with either GST-MBDΔGrb2 or GST-Grb2 fusion proteins bound to glutathione-Sepharose beads. Bound Tpr Met was detected by Western blot with the 144 Met antibody. Total cell lysates (20 μg) were also loaded to ensure equal expression of the Tpr Met mutants. D, GST-MBDΔGrb2 fusion protein bound to glutathione-Sepharose beads was incubated in 0.5% Triton lysis buffer with 1.5 mg of lysate prepared from 293T cells transiently expressing Tpr Met WT or alanine-scanning mutants were incubated with either GST-MBDΔGrb2 or GST-Grb2 fusion proteins bound to glutathione-Sepharose beads. Bound Tpr Met was detected by Western blot with the 144 Met antibody. Total cell lysates (20 μg) were also loaded with anti-pY1349Met, pTyr (RC20H), or 144 Met, as indicated. Bound Tpr Met was detected by Western blot with the 144 Met antibody. Total cell lysates (20 μg) were also loaded to ensure equal expression of the Tpr Met mutants. The amino acid sequence of mutant A (p85 binding mutant) and B (Shc binding mutant) in comparison to WT Tpr Met are indicated (23). D, GST-MBDΔGrb2 fusion protein bound to glutathione-Sepharose beads was incubated in 0.5% Triton lysis buffer with 1.5 mg of lysate prepared from 293T cells transiently expressing the WT Tpr Met receptor and the mutants indicated. Bound Met was detected by Western blot with the DL-21 Met antibody, directed against the extracellular domain of Met. Total cell lysates (20 μg) were also loaded to ensure equal expression of the Met receptor mutants. E, schematic representation of the Met receptor C terminus and the C-terminal truncation mutants used in D.

Fig. 3. Delineation of the Gab1-MBD binding site on Met. A, GST-MBDΔGrb2 fusion protein bound to glutathione-Sepharose beads was incubated with 350 μg of lysate prepared from 293T cells transiently expressing Tpr Met mutants in RIPA lysis buffer. Bound Met was detected by Western blot with the 144 Met antibody. Total cell lysates (20 μg) were also loaded to ensure equal expression of the Tpr Met mutants. B, 293T cells were transiently co-transfected with expression vectors for Tpr Met WT or mutant and myc-tagged MBDΔGrb2. Lysates (800 μg) were immunoprecipitated with either anti-Met 144 or anti-myc, run on a polyacrylamide gel, and Western blotted with anti-Met 144 or anti-myc. Total cell lysates (20 μg) were also loaded to ensure equal expression of the indicated proteins. C, GST-MBDΔGrb2 fusion protein bound to glutathione-Sepharose beads was incubated in RIPA lysis buffer with 350 μg of lysate prepared from 293T cells transiently expressing Tpr Met WT or mutant and myc-tagged MBDΔGrb2. Lysates (800 μg) were immunoprecipitated with either anti-Met 144 or anti-myc, run on a polyacrylamide gel, and Western blotted with anti-Met 144 or anti-myc. Total cell lysates (20 μg) were also loaded to ensure equal expression of the Tpr Met mutants.

Fig. 4. Residues upstream of Tyr-1349 are required for association with the Gab1 MBD. A, schematic representation of the Met receptor kinase domain and C terminus. Residues substituted with alanine are indicated. B, GST-MBDΔGrb2 fusion protein bound to glutathione-Sepharose beads was incubated in RIPA lysis buffer with 350 μg of lysate prepared from 293T cells transiently expressing Tpr Met WT or alanine-scanning mutants. Bound Tpr Met was detected by Western blot with the 144 Met antibody. Total cell lysates (20 μg) were also loaded to ensure equal expression of the Tpr Met mutants. C, lysates prepared from 293T cells transiently expressing Tpr Met WT or alanine-scanning mutants were incubated with either GST-MBDΔGrb2 or GST-Grb2 fusion proteins bound to glutathione-Sepharose beads. Bound Tpr Met was detected by Western blot with the 144 Met antibody. Total cell lysates (20 μg) were also loaded with anti-pY1349Met, pTyr (RC20H), or 144 Met, as indicated.
fusion protein (Fig. 4B), even though all mutant proteins were expressed at similar levels. Importantly, mutant Met proteins were tyrosine-phosphorylated and capable of associating with GST-Grb2 to similar levels as WT Met (Fig. 4C). Hence, the reduction in their association with GST-MBDΔGrb2 was not due to a change in the overall tyrosine phosphorylation of the mutants. We conclude that these mutations specifically alter the ability of Met proteins to associate with Gab1 MBDΔGrb2, without affecting the ability of Met to associate with other interacting partners such as Grb2. This also implies that the structure of the Met receptor kinase domain is not compromised, such that it could no longer phosphorylate tyrosine residues in the C-terminus. Indeed, as shown using a phosphospecific Tyr-1349 antibody, all mutant proteins are tyrosine-phosphorylated on Tyr-1349 (Fig. 4C), indicating that the diminished association of GST-MBDΔGrb2 is not due to the inability of Met mutant proteins to phosphorylate Tyr-1349. These data identify that residues upstream of Tyr-1349 are required for binding to the Gab1 MBD.

A Negative Charge at Residue 1349 Is Sufficient for MBD Binding—Phosphorylation of Tyr-1349 is required for binding to the MBDΔGrb2 (Fig. 3, A and B). However, the role of this phosphorylation is unclear, because the association of the Gab1 MBDΔGrb2 requires structural integrity in Met and hence does not bind Met in a similar manner to the association of PTB or SH2 domains with linear phosphopeptide motifs (32–35). Because Tyr-1349 is localized two amino acids downstream from the last helix of the Met kinase domain, and Met structural integrity was required for association with the MBDΔGrb2, we reasoned that phosphorylation of Tyr-1349 may play a role in modulating the structure of Met, allowing association with the Gab1MBDΔGrb2 protein. To investigate this, we created mutant proteins with tyrosine to glutamic acid substitutions of Tyr-1349 and Tyr-1356, to determine whether the MBDΔGrb2 requires the actual phosphotyrosine entity for binding to Met or just requires the negative charge of the phosphotyrosine. Similar substitutions have been used previously to mimic the effects of a negatively charged phosphotyrosine on conformational changes, without allowing the binding of SH2 domain-containing proteins (36). Whereas a Y1349F mutant protein was unable to associate with the MBDΔGrb2, the substitution of Tyr-1349 with glutamic acid (Y1349E) rescued the ability of the Y1349F mutant Met protein to bind to MBDΔGrb2, although at a reduced level to the WT protein. Moreover, association of MBDΔGrb2 was observed, even when both tyrosines 1349 and 1356 were substituted for glutamic acid residues, Y1349E/Y1356E (Fig. 5A). This was specific for the MBDΔGrb2, because mutants Y1349E/Y1356E or Y1356E were unable to bind GST-Grb2 (Fig. 5B). Consistent with this, following transient transfection assays, a Y1349E/Y1356E mutant induced tyrosine phosphorylation of the endogenous Gab1 protein in 293 cells, whereas in cells expressing the Y1349F/Y1356F mutant Gab1 was only basally phosphorylated (Fig. 5B). This ability of the Y1349E/Y1356E mutant to bind and phosphorylate Gab1 in the absence of phosphotyrosines 1349/1356 also correlated with the ability of this mutant to transform fibroblasts (Fig. 5C). Whereas the Y1349E/Y1356E mutant of Tpr-Met fails to phosphorylate Gab1 and is unable to transform fibroblasts (Fig. 5C) (37–40), the Y1349E/Y1356E mutant transformed fibroblasts to a low level (6 foci/µg of DNA). These results indicate that negative charge at Tyr-1349 is essential for binding of MBDΔGrb2.

Residues Critical for MBD Binding Lie within the Met Receptor Kinase Domain—From sequence alignments, residues identified through alanine-scanning mutagenesis as being critical for MBD binding (Phe-1341, Phe-1344, and Ile-1345, Fig. 6A) are located within the kinase domain of the Met receptor (41). Kinase domains are extremely well conserved among serine/threonine and tyrosine kinases and consist of two subdomains. The N-terminal lobe is composed of a five-stranded β-sheet (β1–5) and one α-helix (helix αC), whereas the larger C-terminal lobe contains at least two β strands (β7/β8) and 7–8 α helices (αD, αE, αF, αP, and αl/αJ) (42). ATP is coordinated primarily by the N-terminal lobe, whereas substrate peptide binding and catalysis are performed by residues in the C-terminal lobe (reviewed in Ref. 43). Although the structure of the Met receptor kinase domain has not been solved, the three-dimensional structure of the Met kinase domain modeled on the insulin and IGF-1 receptors predicts that these residues lie within the C-terminal lobe (Fig. 6A). Notably, Phe-1341 is located in the loop between α-helix I and α-helix J, whereas Phe-1344 and Ile-1345 compose α-helix J (Fig. 6B), indicating that the binding of the GST-MBDΔGrb2 to Met requires residues within the kinase domain. The remainder of the residues critical for Gab1 MBD binding (Gly-1346, His-1348, and pY1349) did not appear in the model because they are located C-terminal to the kinase domain. Phe-1341, Phe-1344, and Ile-1345 are predicted to lie in close contact (less than 5 Å) to residues in the α-helix E (Lys-1179, Ile-1182, Leu-1186, and Gln-1187), as well as Lys-1215 located in the loop between β-strand 7 and β-strand 8 (Fig. 6B). Because we have shown that the MBD cannot bind to a denatured Met protein (Fig. 2A), the location of these residues in the kinase domain supports our observation that the structural integrity of the kinase domain itself may be required.

**DISCUSSION**

Gab1 is tyrosine-phosphorylated and participates in signal transduction downstream from a broad range of growth factors.
and cytokines. A fundamental role for Gab1 in Met/HGF receptor-specific signaling is supported by ablation of the gab1 gene in mice. gab1-/- embryos display reduced liver size and placental defects and are characterized by strongly reduced and delayed migration of myogenic precursor cells into the limbs (6, 7), a phenotype similar to mice harboring mutations in met or hgf genes (44, 45). In addition, our laboratory and others have previously demonstrated that Gab1 is required for the initiation of a Met receptor-mediated invasive morphogenic program in epithelial cells (31). The morphogenic capacity of Gab1 requires the ability of Gab1 to interact with the Met receptor in both a Grb2-dependent and Grb2-independent manner (17). The Grb2-independent interaction requires the integrity of 13 amino acids within a proline-rich region in Gab1 termed the Met binding domain (MBD) (15, 17). The Gab1 MBD has been proposed to be a phosphotyrosine binding (PTB)-like domain (22), although it has no known homology with other phosphotyrosine binding modules. Our data support the interpretation that the Gab1 MBD interacts with the Met receptor in a novel and previously unsuspected manner. Instead of the classic interaction of a phosphotyrosine binding domain with a phosphotyrosine-containing motif, we show that the structural integrity of the kinase domain of Met and a negative charge at tyrosine 1349 are required to engage the Gab1MBD as a peptide ligand.

A Peptide Motif within the Gab1 MBD Is Sufficient to Interact with the Met Receptor—A GST fusion protein containing only 16 amino acids derived from the Gab1 MBD (GST-MBD16), referred to as the Met binding motif (MBM), was sufficient to interact with Met. Consistent with this, only residues found within the MBD16 peptide (amino acids 487–499) were identified by random PCR mutagenesis as critical for Met binding (15). However, interaction of the Gab1 MBD16 was reduced when compared with the full-length GST-Gab1MBD fusion protein indicating that the surrounding residues in the Gab1MBD likely contribute to a higher affinity binding (Fig. 1E). Residues outside of a minimal domain-binding motif can contribute to affinity (46). For example, a full-length Nef protein can bind to SH3 domains 300 times more efficiently than a peptide corresponding to known SH3 domain-interacting PX domain in Nef (47). In support of this, in a yeast two-hybrid binding assay, amino acids 450–532 corresponding to the Gab1 MBD were found to be necessary and sufficient for binding of Gab1 to Met, whereas shorter constructs retained only minimal binding to Met (22). Together this supports a role for residues in Gab1 outside of the Met binding motif for efficient binding to Met.

Consistent with the Met binding motif in Gab1 being sufficient to bind Met, using multiple structure prediction methods, including PHD (48–50) and PSIPRED (51, 52), the Gab1 MBD is predicted not to form any secondary structure and, from this data, is unlikely to function as a domain (data not shown). This is likely due in part to the proline-rich nature of the MBD; 25% of the residues are prolines in comparison to the expected 6%. The amino acid proline is established as a potent breaker of both α-helical and β-sheet structures in globular proteins (53–56). Moreover, in addition to the Met binding motif, the MBD contains binding sites for other proteins, including the p85 SH2 domain (two binding sites), the Grb2 SH3 domain, and Erk1/2 (15, 18, 57, 58). These sites are non-overlapping, suggesting that the MBD is relatively extended and accessible. We propose then that the MBD is actually a loosely structured region with a central Met binding motif and surrounding residues that provide additional contacts with the Met receptor.

The Integrity of the Met Kinase Domain Is Required for Gab1 Interaction—In general, an interaction between two proteins requires at least one of the interacting regions to form a structured domain. The conclusion that the MBD functions as a
peptide ligand led us to speculate that the Met receptor may contain a structured domain that interacts with the MBM peptide. In support of this, we have shown through denaturation studies that the structural integrity of Met is required for its interaction with the MBD (Fig. 2, A and B). This is in contrast to the interaction of Met with Grb2, which requires only a short TVNV phosphotyrosine-containing motif involving Tyr-1349 in the C terminus of Met, and the structured SH2 domain of Grb2 (Fig. 2, A and B). In addition, we have shown that the interaction between the MBD and Met requires residues upstream of Tyr-1349, as well as the presence of a phosphotyrosine or a negatively charged residue at 1349 (Figs. 5–5). Several of these residues (Phe-1341, Phe-1344, and Ile-1345) lie within the end of the C-terminal lobe of the kinase domain. Together these data indicate that the interaction of the Gab1MBD with Met requires the structural integrity of the Met receptor kinase domain.

Although the Met catalytic domain has not been crystallized, the modeling of the Met receptor kinase domain on structures of the insulin and IGF-1 kinase domains indicated that residues required for MBD binding are located in the loop between α-helix I and α-helix J (Phe-1341) and comprise α-helix J (Phe-1344 and Ile-1345) (Fig. 6). All kinase domains whose structures have been solved thus far have been done so in the absence of their C-terminal regions, with the exception of the Tie2 receptor (59). Therefore, it is unclear how the 47-amino acid C terminus of Met, including Gly-1346, His-1348, and pY1349, critical for MBD binding, fit into the model. The crystal structure of Tie2 suggests that the C-terminal tail blocks access to the substrate binding site of the kinase domain and must undergo a conformational change upon activation of the receptor to expose both the substrate binding site and tyrosines in the C terminus required for binding of signaling proteins (59). The Met C terminus may adopt a similar conformation, because a peptide of the Met receptor multifunctional docking site (amino acids 1345–1363) can bind to the Met kinase domain in an undefined manner, and inhibit the kinase activity of the receptor (60).

We propose that residues identified through alanine-scan-ning mutagenesis in the C-terminal lobe of the kinase domain function to create a binding surface for the MBM peptide and that phosphorylation of Tyr-1349 located at the junction between the kinase domain and the C terminus is required for a conformational change in Met that exposes a binding surface for Gab1 (Fig. 7, model). Consistent with this, the substitution of Tyr-1349 for a charged glutamic acid is sufficient for Gab1 binding and phosphorylation, whereas a similar substitution at 1356 abrogates the association of the Grb2 SH2 domain (Fig. 5B). However, we cannot rule out the possibility that mutation of these residues may affect the binding of the proline-rich MBM to another location in the Met kinase domain.

The peptide binding surfaces of domains that bind to proline-rich ligands, including SH3, WW, GYF, and EVH1, tend to contain elongated patches of aromatic and hydrophilic residues that create a contiguous binding surface. Residues Phe-1341, Phe-1344, and Ile-1345 on Met are aromatic and/or hydrophilic and are predicted to lie in close contact with side chains of residues in α-helix E (Lys-1179, Ile-1182, Gly-1183, Leu-1186, and Gln-1187), as well as Lys-1215 located in the loop between β-7 and β-8 (Fig. 6B). A potential binding surface for the MBM ligand is present in the region comprising helices E, I, and J (Fig. 6B). However, the presence of two basic residues (Lys-1179 in α-helix E and Lys-1215 in the loop between β-7 and β-8) could be a deterrent to MBM binding. Activation of the kinase and phosphorylation of tyrosines with the activation loop, as well as phosphorylation of Tyr-1349, could lead to a conforma-

The peptide binding surface of SH3 domains generally also contains at least one negatively charged acidic residue that interacts with a positively charged basic residue located within the proline-rich ligand. The MBM contains a basic residue, Arg-499, that is critical for interaction with Met (15, 17) (Fig. 1, D and E). However, the MBD binding region on Met contains only one acidic residue, Glu-1347, that was not essential (Fig. 4B). Alternatively, because phosphorylation of Tyr-1349 or the creation of a negative charge is critical (Fig. 2, A and B), the positively charged Arg-499 in the MBM may interact with the negatively charged phosphate group on Tyr-1349, in a manner reminiscent of, but opposite to, that of SH2 domains and phosphotyrosine ligands. Hence, in a similar manner to an SH3 domain, the kinase domain of Met may contain a contiguous binding surface of aromatic and hydrophilic residues, in addition to a negatively charged amino acid (phosphotyrosine 1349), that engage the prolines and the critical arginine, respectively, of the MBM.

Although the Grb2-independent interaction is specific for Gab1 and Met, specificity in binding to particular receptors has also been shown for other docking/adapter proteins. For example, the Grb7/Grb10/Grb14 family of adapter proteins, in addition to their SH2 domains, contain novel receptor-specific interaction domains (BPS) that allow them to interact differentially with receptors (61, 62). Moreover, a domain in IRS-2, the kinase regulatory loop-binding (KRLB) domain, interacts specifically with the insulin receptor but not the highly related IGF-1 receptor (63). Intriguingly, MBM, BPS, and KRLB all bind to kinase domains, and all three interactions require receptor kinase activity (61, 64). It is not clear yet whether the BPS or the KRLB are indeed domains or, like the MBD, would also function as extended peptide motifs. The presence of domains or motifs in docking proteins that interact differentially with a subset of receptor tyrosine kinases may thus be a common mechanism through which docking proteins can modulate distinct biological responses downstream from receptor tyrosine kinases. Solving the three-dimensional structure of Met, in conjunction with Gab1, will permit a clearer understanding of this novel interaction, and could provide an approach to interfere specifically with the invasive response triggered by Gab1 downstream from the Met receptor.

Acknowledgments—We are grateful to Drs. Frank Sicheri and Kalle Gehring as well as members of the Park laboratory for helpful discussions.

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J. Biol. Chem. 2003, 278:30083-30090.
doi: 10.1074/jbc.M302675200 originally published online May 22, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M302675200

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