Distinct Recruitment and Function of Gab1 and Gab2 in Met Receptor-mediated Epithelial Morphogenesis

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The Gab family of docking proteins (Gab1 and Gab2) are phosphorylated in response to various cytokines and growth factors. Gab1 acts to diversify the signal downstream from the Met receptor tyrosine kinase through the recruitment of multiple signaling proteins, and is essential for epithelial morphogenesis. To determine whether Gab1 and Gab2 are functionally redundant, we have examined the role of Gab2 in epithelial cells. Both Gab1 and Gab2 are expressed in epithelial cells and localize to cell-cell junctions. However, whereas overexpression of Gab1 promotes a morphogenic response, the overexpression of Gab2 fails to induce this response. We show that Gab2 recruitment to the Met receptor is dependent on the Grb2 adapter protein. In contrast, Gab1 recruitment to Met is both Grb2 dependent and Grb2 independent. The latter requires a novel amino acid sequence present in the Met-binding domain of Gab1 but not Gab2. Mutation of these residues in Gab1 impairs both association with the Met receptor and the ability of Gab1 to promote a morphogenic response, whereas their insertion into Gab2 increases Gab2 association with Met, but does not confer on Gab2 the ability to promote epithelial morphogenesis. We propose that the Grb2-independent recruitment of Gab proteins to Met is necessary but not sufficient to promote epithelial morphogenesis.

INTRODUCTION

Hepatocyte growth factor (HGF) is a mesenchymally derived factor with pleiotropic activities. In vivo, HGF stimulates cell proliferation and survival, as well as cell dispersal, motility, and a morphogenic program in a wide range of cellular targets, including epithelial, endothelial, and hematopoietic cells, and neurons (reviewed in Gherardi and Stoker, 1991; Michalopoulos, 1995; Zarnegar and Michalopoulos, 1995). These effects are fundamental for the diverse biological functions of HGF observed in vivo, including embryogenesis, organogenesis, angiogenesis, tissue regeneration, axonal outgrowth, wound healing, and invasion by tumor cells (reviewed in Rosen *et al.*, 1994; Jeffers *et al.*, 1996; Birchmeier and Gherardi, 1998).

The biological responses to HGF are mediated by its cell surface receptor, the Met tyrosine kinase. To characterize signaling pathways downstream of the Met receptor involved in epithelial morphogenesis, we have used chimeric colony-stimulating factor (CSF)-Met receptors, where the

extracellular domain of Met was replaced with that of the CSF-1 receptor. These approaches have demonstrated that the Met receptor cytoplasmic domain is sufficient for the biological responses attributed to HGF and that Met tyrosine kinase activity is required for these responses (Komada and Kitamura, 1993; Weidner *et al.*, 1993; Zhu *et al.*, 1994b). Two tyrosines in the C terminus (1349 and 1356) of Met are crucial for the induction of branching morphogenesis in epithelial cells (Zhu *et al.*, 1994a; Fournier *et al.*, 1996; Weidner *et al.*, 1996). Tyrosine 1356 forms a multisubstrate binding site, coupling the Met receptor directly with the Grb2 and Shc adapter proteins, and indirectly with the Gab1 docking protein and the Cbl ubiquitin ligase (Ponzetto *et al.*, 1994; Fixman *et al.*, 1996, 1997; Fournier *et al.*, 1996; Bardelli *et al.*, 1997; Nguyen *et al.*, 1997), whereas tyrosine 1349 contributes to the direct recruitment of the Gab1 docking protein to Met (Weidner *et al.*, 1996). Met receptor mutants specifically lacking the ability to recruit the Grb2 adaptor protein (N1358H) fail to elicit a morphogenic response (Fournier *et al.*, 1996). This Met receptor mutant has a decreased association with the docking protein Gab1, and overexpression of Gab1 in these cells rescues the morphogenesis defect, thereby identifying Gab1 as an essential mediator of Met receptor-induced epithelial morphogenesis (Maroun *et al.*, 1999a).

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Gab1 (Grb2-associated binder 1) was originally identified as a Grb2 binding protein from a glioblastoma tumor-derived cDNA library (Holgado-Madruga *et al.*, 1996). The Gab family of docking proteins, including Gab1, Gab2, Gab3, Daughter of Sevenless, and Suppressor of Clear-1, belong to a group of docking proteins, including insulin receptor substrates 1–4, downstream of kinases 1–5, and fibroblast growth factor receptor substrate 2 (FRS2) (Herbst *et al.*, 1996; Holgado-Madruga *et al.*, 1996; Raabe *et al.*, 1996; Carpino *et al.*, 1997; Kouhara *et al.*, 1997; Yamanashi and Baltimore, 1997; Yenush and White, 1997; Gu *et al.*, 1998; Jones and Dumont, 1998; Lemay *et al.*, 2000; Grimm *et al.*, 2001; Schutzman *et al.*, 2001; Wolf *et al.*, 2002). These proteins lack enzymatic activities, but after activation of receptor tyrosine kinases, cytokine receptors, and G protein-coupled receptors, they become phosphorylated on tyrosine residues, providing binding sites for multiple proteins involved in signal transduction. In this manner, they act to potentiate and diversify the signals downstream from receptors by virtue of their ability to assemble multiprotein complexes.

Gab1 is the major phosphorylated protein downstream of the Met receptor in epithelial cells (Nguyen *et al.*, 1997). After stimulation with HGF, Gab1 couples with the p85 subunit of phosphatidylinositol 3-kinase (PI3-kinase), and the majority of Met-dependent PI3 kinase activity is associated with Gab1 (Maroun *et al.*, 1999a). In response to HGF, Gab1 also associates with phospholipase C (PLC)- γ 1, the tyrosine phosphatase SHP-2, the adapter protein Crk (Garcia-Guzman *et al.*, 1999; Maroun *et al.*, 1999a; Gual *et al.*, 2000; Lamorte *et al.*, 2000; Sakkab *et al.*, 2000) and acts to recruit these signaling proteins to the Met receptor (Maroun *et al.*, 1999a). The Gab1-dependent recruitment of SHP-2 is required for sustained mitogen-activated protein kinase (MAPK) activity and epithelial morphogenesis downstream from the Met receptor (Maroun *et al.*, 2000). Gab1 contains an amino-terminal pleckstrin homology (PH) domain that binds PIP3 in a PI3-kinase-dependent manner (Isakoff *et al.*, 1998; Maroun *et al.*, 1999b; Rodrigues *et al.*, 2000). This association is required for localization of Gab1 at cell-cell junctions in epithelial cells and for Gab1-dependent morphogenic responses (Maroun *et al.*, 1999b). Gab1 also contains two binding sites for the C-terminal Src homology 3 (SH3) domain of Grb2 (Lock *et al.*, 2000; Schaeper *et al.*, 2000; Lewitzky *et al.*, 2001).

Gab1 is recruited to the Met receptor by both indirect and direct mechanisms. Gab1 associates constitutively with the C-terminal SH3 domain of the adapter protein Grb2, allowing for the recruitment of Gab1 via the interaction of the SH2 domain of Grb2 with Y1356 of the Met receptor (Bardelli *et al.*, 1997; Fixman *et al.*, 1997; Nguyen *et al.*, 1997). However, deletion of the Grb2 SH3 domain binding sites in Gab1, uncoupling Gab1 from Grb2, does not inhibit the ability of Gab1 to rescue morphogenesis (Lock *et al.*, 2000). This implies that Gab1 can also be recruited to Met in a Grb2-independent manner and provides physiological support for yeast two-hybrid studies and in vitro association assays, indicating that Gab1 can associate directly with the Met receptor through tyrosines 1349 and 1356 (Weidner *et al.*, 1996; Nguyen *et al.*, 1997). The Grb2-independent interaction requires the proline-rich Met binding domain (MBD) of Gab1, which may represent a novel type of phosphotyrosine binding domain (Weidner *et al.*, 1996).

A Gab1-related protein, Gab2, was identified recently (Gu *et al.*, 1998; Nishida *et al.*, 1999; Zhao *et al.*, 1999). Gab1 and Gab2 have highly homologous PH domains; contain tyrosine residues within a consensus for recruitment of p85, SHP-2, and Crk (Gu *et al.*, 1998; Pratt *et al.*, 2000; Crouin *et al.*, 2001); and share conserved Grb2 SH3 domain binding sites (Lock *et al.*, 2000; Schaeper *et al.*, 2000). They are both phosphorylated upon epidermal growth factor (EGF), interleukin-6, interleukin-3, thrombopoietin, and erythropoietin stimulation, as well as T-cell receptor engagement (Holgado-Madruga *et al.*, 1996; Gu *et al.*, 1998; Nishida *et al.*, 1999; Wickrema *et al.*, 1999; Zhao *et al.*, 1999; Kong *et al.*, 2000; Bouscary *et al.*, 2001), and have overlapping but distinct patterns of expression (Gu *et al.*, 1998; Nishida *et al.*, 1999). Studies of knockout mice suggest that both Gab1 and Gab2 have distinct functions during development; Gab1 is embryonic lethal and Gab2 is deficient in the allergic response (Itoh *et al.*, 2000; Sachs *et al.*, 2000; Gu *et al.*, 2001). However, the contributions of Gab1 and Gab2 to biological functions downstream from the same receptor have not been evaluated.

We show that both Gab1 and Gab2 are expressed in Madin-Darby canine kidney (MDCK) epithelial cells, show a similar localization to cell-cell junctions, and associate with similar signaling proteins after stimulation with HGF. In spite of this, Gab2, in contrast to Gab1, is unable to rescue the morphogenic program of epithelial cells expressing Met receptor mutants. We have identified amino acids in Gab1 that are absent in Gab2 and are responsible for Grb2-independent association of Gab1 with the Met receptor. We show that these residues are essential for epithelial morphogenesis elicited by Gab1, and when introduced into Gab2 they confer Grb2-independent recruitment of Gab2 to the Met receptor, but are insufficient to allow the rescue of the morphogenic program by Gab2. We propose that the Grb2-independent recruitment of Gab proteins to the Met receptor is necessary but not sufficient to promote epithelial morphogenesis.

MATERIALS AND METHODS

Plasmids and Mutagenesis

Wild-type (WT) Gab1-pCDNA 1.1-HA, Gab1 Δ Grb2, GST-MBD (pGEX 2TK), and GST-MBD Δ Grb2 were described previously (Lock *et al.*, 2000). All mutants were generated with the QuikChange mutagenesis kit (Stratagene, La Jolla, CA) unless otherwise noted, and sequenced before use. All primers are written 5' to 3'. Gab2 Δ Grb2-pEBB-HA was generated by making the mutations Δ 348–355/P500A/R504A in Gab2-bluescriptKS+ by using the following primers: P518A/R504A-F, GAGATCCAGCCAGCCCCTGTCAACGCAAACCTCAAGCC; P518A/R504A-R, GGCTTGAG-GT-TTGCGTTGACAGGGGCTGGCTGGATCTC; Δ 348–355-F, CTGG-AGATTCAGCGATCGCT-AGTCAGGCAGAAACATC; and Δ 348–355-R, GATGTTTCTGCCTGACTAGCGATCGCTGAATC-TCCAG. Gab2 Δ Grb2 was then inserted back into the pEBB-HA vector. GST-Gab2MBD Δ Grb2 was generated using primers P500A/R504A. The Δ 3P mutation (deletes prolines 491–493) was generated in WT Gab1, Gab1 Δ Grb2, GST-Gab1MBD, and GST-Gab1MBD Δ Grb2 by using the following primers: Δ 491–493-F, CCTTTGGAATGCAAGTCGCTCACATGG-GCTTCAGGTCC and Δ 491–493-R GGACCTGAAGCCATGTGAGCGACTTGCAATTCAGG. Alanine scanning mutagenesis on WT Gab1 pCDNA1.1 was performed with the Chameleon double-stranded mutagenesis kit (Stratagene) by using the following primers: G487A, GGACCTTTGACTTTTCAAGCTTTG-

CAATGCGAGTCC-CTCCTCC; R489A, GGACCTTTGACTTTTCAAGCTTTTGAATGGCAGTCCCTCCTG-CTC; P491A, CTTTCTCTCTTGGCATGCGAGTCGCTCCTCTGCTCATATATGG; P492A, CTTCTTTTGAATGCGGGTACCTGCTC-CTGCTCATATGGGCTTC; and P493A, CTTCTTTG-GAATGCGGGTACCTCCTGCTGCTCATATGGGCTTC. The MBD2/MBD1 (484-end) chimera (Gab2 aa 390–475, Gab1 484–532) was generated by adding in restriction sites with cohesive ends: *Xba*I site into Gab2MBD at nucleotide 1501 (aa 476 Asp to Ser, aa 477 Pro to Arg) and an *Nhe*I site into Gab1MBD at nucleotide 1458 (aa 484 Ser to Ala). Gab2MBD*Xba*I-pGEX 4T-1 was digested with *Xba*I and *Pst*I, and this fragment was replaced with the *Nhe*I/*Pst*I-digested fragment of Gab1MBD*Nhe*I to destroy the cut site and recreate the original Gab1 amino acid sequence (Ser, Ser). The MBD2/1/2–13aa insert chimera (Gab1 insert of aa 484–496, replaces aa 476–478 of Gab2) was generated by creation of an *Nde*I site in Gab2 at nucleotide 1531 (aa 477 Pro to His, aa 478 Leu to Met). An *Nde*I site is found in Gab1 at nucleotide 1491, and MBD2/MBD1 pGEX 4T-1 was digested with *Nde*I and *Pst*I and the fragment was replaced the *Nde*I/*Pst*I-digested fragment from Gab2MBD*Nde*I. MBD2/MBD1 (495 to end), which is Gab2 aa 390–484, Gab1 495–532, was created by insertion of the *Nde*I/*Pst*I digested fragment of MBD1 into *Nde*I/*Pst*I-digested MBD2*Nde*I pGEX 4T-1 vector. MBD2/1/2* (Gab1 insert of aa 484–502, replaces aa 476–484 of Gab2) was generated with MBD2/1/2(484–496) as template, by using the following primers: 484–502-F, CTCCTGCTCATATGGGCTTCCGATCGAGTCCACTTCCTATTACACAGAGGC; and 484–502-R, GCCTCTGTGAATAGGAAGTGGACTCGATCGGAAGCCCATATGAGCAGGAG. MBD2/1/2*-Δ3P was generated from MBD2/1/2* with the following primers: 2/1/2Δ491-F, CTTCTTTTGAATGCGATCGTCCATGCGGCTTCCGATCGAGTCC; and 2/1/2Δ491-R, GGACTCGATCGGAAGCCCATGTGAGCGACTCGCATTCCAAAGGAAG. MBD2/12*-ΔGrb2 was generated with the P500A/R504A primers. The full-length Gab2/1/2(484–502), termed Gab2/1/2*, construct was created in bluecriptKS+ as described above for the MBD2/1/2* construct, and then placed back into the pEBB-HA vector.

Cell Culture, DNA Transfections, and Whole Cell Extracts

For transient transfections, 293T cells were seeded at 1×10^6 /100-mm Petri dish and transfected 24 h later by the calcium phosphate precipitation method (Wigler *et al.*, 1979) with 2 μ g of DNA. COS-1 cells were seeded at 8×10^5 /100-mm Petri dish and transfected 24 h later with 6 μ g of plasmid DNA by a standard DEAE-dextran precipitation method as described previously (Rodrigues *et al.*, 1991). For cotransfections of Met receptor with Gab DNAs, 3 μ g of Met receptor DNA and 1 μ g of Gab DNA were used. COS-1 and 293T cells were serum starved in 0.1% fetal bovine serum (FBS) for 24 h and harvested in 0.5% Triton X-100 lysis buffer (0.5% Triton X-100, 50 mM HEPES, pH 8.0, 150 mM NaCl, 10% glycerol, 2 mM EGTA, 1.5 mM $MgCl_2$, 10 μ g aprotinin/ml, 10 μ g leupeptin/ml, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium fluoride, and 1 mM sodium vanadate). After a 10-min incubation on ice, the lysates were centrifuged at 13,000 rpm for 10 min. The generation of MDCK cell lines expressing wild-type CSF-1 Met receptor and mutants thereof by retroviral infection has been described previously (Zhu *et al.*, 1994a; Fournier *et al.*, 1996). Stable cell lines expressing WT HA-Gab1, HA-Gab1ΔGrb2, HA-Gab1Δ3P, HA-Gab2, HA-Gab2ΔGrb2, and HA-Gab2/1/2* were generated as described previously (Maroun *et al.*, 1999a; Lock *et al.*, 2000).

Stimulation of MDCK Cells

MDCK cells were seeded at 10^6 /100-mm dish. Twenty-four hours later, they were washed twice with DMEM and then starved for 24 h in 10 ml of DMEM containing 0.02% FBS. HGF was added at 100

U/ml in 2 ml for the indicated time. The cells were immediately lysed on ice in 1 ml of 0.5% Triton X-100 lysis buffer.

Glutathione S-Transferase (GST) Fusion Proteins, In Vitro Association Assays, Immunoprecipitations, and Western Blotting

Fusion proteins were produced in the DH5, or BL 21 gold *Escherichia coli* strain, by induction with isopropyl-1-thio- β -D-galactopyranoside. GST fusion proteins (0.5–1 μ g) immobilized on glutathione-Sepharose beads were incubated with either 300 μ g of lysate from 293T cells transiently expressing the Met receptor, or 350 μ g of lysate from MDCK cells expressing Gab1 or Gab2, stimulated or not with HGF for 15 min. After rocking in 0.5% Triton X-100 lysis buffer for at least 2 h at 4°C, bound proteins were washed three times with lysis buffer. Approximately 1 mg of protein was used for immunoprecipitations from 293T or MDCK cell lysates. Immunoprecipitation and Western blotting were performed as described in Maroun *et al.* (1999a).

Immunofluorescence

MDCK cells overexpressing HA-Gab1 or HA-Gab2 were plated at 1×10^4 for 3 d in DMEM containing 10% serum on glass coverslips in a 24-well dish. For stimulations, 5×10^4 cells were plated overnight in 10% serum-containing medium, washed two times, and serum starved in 0.02% serum for 6 h, and stimulated with 50 U of HGF per milliliter for 15 min. Cells were fixed in 2% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min at room temperature, and washed twice in PBS. Cells were treated for 5 min at room temperature with PBS containing 0.2% Triton X-100. Anti-hemagglutinin (HA) (1:300 in 0.2% Triton-PBS) was added to the cells for 30 min, and after three washes, CY3-conjugated goat anti-mouse IgG (1:2000; Jackson ImmunoResearch Laboratories, West Point, PA) was added for 30 min, and the cells were washed three times in 0.2% Triton-PBS and once with water. The glass coverslips were mounted onto slide with Immunofluore medium (ICN, St-Laurent, Quebec, Canada) and visualized with an Axiovert 135 incident-light fluorescence microscope (Carl Zeiss, Thornwood, NY).

Collagen Assays

MDCK cells were resuspended in a collagen matrix as described previously (Maroun *et al.*, 1999a). HGF (15 U/ml) or recombinant CSF rhCSF-1 (50 ng/ml) was added to the medium after 5 d. Quantitation of the morphogenic response was performed as described previously (Maroun *et al.*, 1999a).

Antibodies and Reagents

Antibodies against a C-terminal peptide of the human Met protein were used (Rodrigues *et al.*, 1991), as well as DL-21 (Upstate Biotechnology, Lake Placid, NY). Anti-phosphotyrosine (4G10) and anti-Gab1 were purchased from Upstate Biotechnology or RC20H from Transduction Laboratories (Lexington, KY), anti-HA (HA-11) was from BabCO (Richmond, CA), and anti-GST was from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phosphoMAPK, phosphoJNK, and c-Jun NH_2 -terminal kinase (JNK) were obtained from New England Biolabs (Nepean, Ontario Canada). Rabbit anti-SHP-2 and rabbit anti-MAPK were kindly provided by Dr. Nicole Beauchemin (McGill Cancer Centre, Montreal, PQ, Canada), and Dr. John Blenis (Harvard Medical School, Boston, MA), respectively. HGF and recombinant CSF were generously provided by Dr. George Van de Woude (Van Andel Research Institute, Grand Rapids, MI) and Genetics Institute (Boston, MA), respectively. HA-tagged Gab2, Gab2MBD-pGEX 4T-1, and anti-Gab2 sera were gifts from Dr. Ben Neel (Beth Israel-Deaconess Medical Center, Boston, MA).

Figure 1. Gab2 is phosphorylated upon HGF stimulation and is localized at cell-cell junctions in MDCK epithelial cells. (A) Endogenous Gab1 and Gab2 were immunoprecipitated from 1 mg of protein lysate prepared from serum-starved MDCK cells stimulated or not with HGF (100 U/ml) for the indicated times. Proteins were separated by SDS-PAGE, transferred to nitrocellulose membrane, and Western blotted (WB) with 4G10 anti-phosphotyrosine, stripped, and reprobed with anti-Gab2 or anti-Gab1. (B) MDCK cells (1×10^4) stably expressing either WT HA-tagged Gab1 or WT HA-tagged Gab2 were grown for 72 h on glass coverslips in DMEM containing 10% FBS. (C) MDCK cells (5×10^4) expressing either HA-Gab1 or HA-Gab2 were grown overnight on glass coverslips in DMEM containing 10% FBS, serum starved in DMEM containing 0.02% FBS for 6 h, and stimulated with HGF (50 U/ml) for 15 min. Cells were fixed in 2% paraformaldehyde and subjected to indirect immunofluorescence by using anti-HA, followed by CY3-conjugated anti-mouse antibody. Photographs were taken at a magnification of 63 $\times$.

SH2 domain containing GST fusion proteins was generously provided by Dr. Bruce Mayer (University of Connecticut Health Center, Farmington, CT) (GST-Crk II SH2); Dr. Tony Pawson (Samuel Lunenfeld Research Institute, Toronto, Ontario, Canada) (GST-PLC γ and GST-p85 SH2); and Dr. Gen-Sheng Feng (Burnham Institute, La Jolla, CA) (GST-SHP-2).

RESULTS

Gab1 and Gab2 Show a Similar Localization to Cell-Cell Junctions in MDCK Cells

To assess whether Gab family proteins mediate similar biological responses, we have examined the functional role of Gab2 in MDCK epithelial cells. Using Gab2 and Gab1 specific sera, we show that Gab2 is endogenously

expressed in MDCK cells, and that Gab2 is phosphorylated within 5 min after HGF stimulation and is sustained for at least 2 h, similar to the time course of endogenous Gab1 phosphorylation (Figure 1A). Because both Gab1 and Gab2 are expressed in MDCK cells and are phosphorylated upon HGF stimulation, this indicates a potential redundancy of function. To investigate this possibility, we generated stable cell lines expressing HA-tagged Gab2. We showed previously that Gab1 is localized to sites of cell-cell attachment in the presence of serum but localizes diffusely to the cytosol after serum starvation (Maroun *et al.*, 1999a). In a similar manner to Gab1, Gab2 is localized at sites of cell-cell contact (Figure 1B). Under serum-starved conditions, both Gab1 and Gab2 show a similar diffuse localization and are recruited to the membrane

Figure 2. Gab2 is tyrosine phosphorylated at reduced levels in response to HGF compared with Gab1, but associates with similar signaling proteins. (A) MDCK cells expressing HA-Gab1 or HA-Gab2 were stimulated or not with HGF for the indicated times. Equal amounts of protein were subjected to immunoprecipitation with anti-HA, followed by Western blotting (WB) with RC20H anti-phosphotyrosine. Blots were stripped and reprobed with anti-HA to show equal Gab protein levels in the immunoprecipitate. (B) MDCK cells expressing HA-Gab1, or high levels of HA-Gab2, were stimulated with HGF for 15 min. Gab proteins were immunoprecipitated with anti-HA, separated by SDS-PAGE, and Western blotted with anti-SHP-2 antibody. Membranes were subsequently stripped and reprobed with RC20H anti-phosphotyrosine and anti-HA. Total cell lysates (20 μ g) were used to show levels of SHP-2 protein via anti-SHP-2 Western blotting. (C) Multiple plates each of MDCK cells expressing HA-Gab1, low levels of HA-Gab2 (low Gab2), or high levels of HA-Gab2 (high Gab2) were stimulated or not with HGF for 15 min and lysates were pooled. Lysates were either incubated with the indicated SH2 domain containing GST fusion proteins, or immunoprecipitated with anti-HA antibody. Bound HA-Gab proteins were detected by Western blot with HA antibody. Tyrosine phosphorylation of the Gab proteins in the lysates was evaluated by Western blot of the HA immunoprecipitation with RC20H anti-phosphotyrosine antibody. (D) MDCK cells expressing HA-Gab1 or high levels of HA-Gab2 were stimulated with HGF for the times indicated. Proteins from 40 μ g of total cell lysates were separated by SDS-PAGE and WB with pMAPK or pJNK antibodies. Membranes were stripped and subsequently blotted with MAPK or JNK antibodies.

upon HGF stimulation (Figure 1C), indicating that Gab1 and Gab2 share a similar subcellular distribution.

Gab2 Is Tyrosine Phosphorylated at Reduced Levels Compared with Gab1, but Associates with Similar Signaling Proteins

When expressed at similar levels in stable cell lines, an HA-tagged Gab2 protein is consistently phosphorylated at lower levels than an HA-tagged Gab1 protein (Figure 2A).

To address whether Gab2 is capable of associating with similar signaling proteins as Gab1 after Met receptor activation, we used cell lines that express Gab2 to levels higher than Gab1 (highGab2; Figure 2). Under these conditions, Gab1 and Gab2 show comparable levels of phosphorylation after stimulation of cells with HGF (Figure 2B, middle panels, and C, top two panels). To investigate proteins that can associate with Gab1 and Gab2 in MDCK cells, we performed either coimmunoprecipitation assays to detect endogenous proteins, or used GST fusion proteins encoding the SH2 domains of signaling proteins known to bind to Gab1 and Gab2 (Nguyen *et al.*, 1997; Gu *et al.*, 1998; Garcia-Guzman *et*

al., 1999; Maroun *et al.*, 1999a; Gual *et al.*, 2000; Lamorte *et al.*, 2000; Pratt *et al.*, 2000; Sakkab *et al.*, 2000; Crouin *et al.*, 2001). SHP-2 was identified as a major binding protein for Gab2 (Gu *et al.*, 1998), and by coimmunoprecipitation, both Gab1 and Gab2 associate with endogenous SHP-2 protein after stimulation of cells with HGF (Figure 2B). GST fusion protein pull-down assays of cell lysates prepared from the MDCK cell lines overexpressing Gab1 or Gab2, showed that after HGF stimulation, Gab2 is able to associate with various SH2 domain-containing fusion proteins, including Crk II, p85, PLC- γ , and SHP-2 (Figure 2C). In addition, after HGF stimulation, cell lines expressing high levels of Gab2 elicited MAPK and JNK phosphorylation to similar levels as cell lines expressing Gab1 or parental cells (Figure 2D). Hence, although the level of Gab2 phosphorylation is lower than that of Gab1, when overexpressed, both Gab1 and Gab2 interact with similar signaling proteins involved in Met-mediated signaling.

Gab2 Overexpression Fails to Rescue Met-dependent Epithelial Morphogenesis

Using chimeric CSF-Met receptor mutants in structure-function analyses, we demonstrated that receptors that selectively fail to bind the Grb2 adapter protein (N1358H) fail to promote a morphogenic response in MDCK cells in response to CSF stimulation (Fournier *et al.*, 1996). The overexpression of WT Gab1 (Maroun *et al.*, 1999a), or a Gab1 protein lacking Grb2 binding sites (Lock *et al.*, 2000), rescues this morphogenesis defect. To determine whether Gab1 and Gab2 have similar functions in MDCK cells, we tested whether overexpression of Gab2 in the N1358H CSF-Met (Met Δ Grb2) cell line could rescue the morphogenesis defect upon CSF stimulation. Multiple stable cell lines that overexpress Gab2 in the CSF-Met Δ Grb2 cell line were isolated, and expression levels of representative lines are shown in Figure 3A. When tested for their ability to rescue epithelial morphogenesis, cell lines expressing low levels of Gab2 (i.e., Gab2-B3 and Gab2-H1; Figure 3A) were unable to rescue tubulogenesis upon stimulation, where cells remained as cysts, similar to the vector control (Figure 3, B and C). In cell lines expressing 5 to 7 times more Gab2 than Gab1 (i.e., Gab2-C1 and C9; Figure 3A), a few cysts (<10%) were able to form elongated tubule-like structures. These, however, had a simple structure, in contrast to the complex, branched tubules formed in cells overexpressing Gab1 (Figure 3B) (Maroun *et al.*, 1999a). The majority of the cysts derived from cell lines expressing high levels of Gab2 showed either no response or a partial response (Figure 3B). A partial response is defined as any structure that is no longer a cyst, but whose length is <5 times its width and is unbranched. These results demonstrate that even when overexpressed at higher levels than Gab1, Gab2 promotes only a minor morphogenic response. This response is insignificant compared with cells expressing Gab1, where branching tubule formation is ~80% (Figure 3C; Maroun *et al.*, 1999a). Importantly, all cell lines elicited a morphogenic response after stimulation with HGF, indicating that the signals required for this response are intact (our unpublished data). These results indicate that Gab2 and Gab1 are not functionally redundant in MDCK cells.

To evaluate the effect of Gab1 and Gab2 overexpression on signaling by the CSF-Met N1358H (Met Δ Grb2) receptor, we

compared the MAPK and JNK responses upon CSF stimulation. Although this receptor is unable to bind Grb2, it is still able to induce MAPK activation upon CSF stimulation (Figure 3D, vector), possibly due to its ability to recruit and phosphorylate the Shc adapter protein and to promote Shc-Grb2 coupling (Fournier *et al.*, 1996). In addition, a recent article suggests that Met may couple with β 4 integrin, providing an additional signaling platform through which extracellular signal-regulated kinase (ERK) signals can be elicited (Trusolino *et al.*, 2001). Notably, CSF stimulation of WT MDCK cells does not induce MAPK phosphorylation (our unpublished data), indicating that activation of MAPK in the CSF-Met Δ Grb2 cell lines occurs exclusively through signals generated from the CSF-Met Δ Grb2 receptor. Importantly, overexpression of either Gab1 or Gab2 produced similar MAPK or JNK responses upon stimulation of the CSF-Met Δ Grb2 mutant receptor (Figure 3D). These results suggest that the inability of Gab2 to rescue the morphogenic phenotype of the mutant Met Δ Grb2 receptor cell line is not due to significant alterations in MAPK or JNK activation.

Gab2 Association with Met Is Grb2 dependent

To resolve why Gab2 overexpression fails to rescue the morphogenesis defect, we established whether Gab2 is phosphorylated upon activation of the CSF-Met Δ Grb2 mutant receptor. Whereas Gab1 is readily phosphorylated after stimulation of the CSF-Met Δ Grb2 mutant, Gab2 is poorly phosphorylated, even when overexpressed (Figure 4A). However, a noticeable mobility shift is observed upon CSF stimulation. The lower level of Gab2 phosphorylation relative to Gab1 might reflect differences in the ability of the Met receptor to induce phosphorylation of Gab2, or in the mechanism of recruitment of Gab1 and Gab2 to the Met receptor. Gab1 is recruited to the Met receptor by two distinct mechanisms. One is indirect through the adapter protein Grb2 and tyrosine 1356 on the Met receptor, and the other is Grb2-independent and requires tyrosines 1349 and 1356 of the Met receptor (Weidner *et al.*, 1996; Bardelli *et al.*, 1997; Fixman *et al.*, 1997; Nguyen *et al.*, 1997; Lock *et al.*, 2000). Gab1 contains two proline-rich motifs that function as binding sites for the C-terminal Grb2 SH3 domain (Lock *et al.*, 2000; Schaeper *et al.*, 2000). These sites are conserved in Gab2, and mutation of either of these sites individually reduces the association of Gab2 with Grb2 (our unpublished data), as shown for Gab1 (Lock *et al.*, 2000), whereas mutation of both abrogates association with Grb2 as assessed by in vitro association assay (Figure 4B). To define whether Gab2 phosphorylation by the Met receptor was Grb2 dependent or independent, the phosphorylation of a Gab2 Δ Grb2 protein was assessed in stable MDCK cell lines after stimulation with HGF. Whereas WT Gab2 was tyrosine phosphorylated after stimulation of cells with HGF, a Gab2 Δ Grb2 mutant was not detectably phosphorylated (Figure 4C). In contrast, a Gab1 Δ Grb2 mutant was efficiently phosphorylated (Figure 4C). These data suggest that Gab2 lacks the ability to be recruited to the Met receptor in a Grb2-independent manner.

Grb2-independent recruitment of Gab1 to the Met receptor has been mapped within the Gab1 MBD (Weidner *et al.*, 1996; Lock *et al.*, 2000; Schaeper *et al.*, 2000). The ability of the Gab2 MBD region to associate with Met in vitro, was assessed by pull-down assays of Met receptor proteins ex-

Figure 3. Gab2 is unable to rescue a Met-mediated morphogenic program. MDCK cells expressing the CSF-Met Δ Grb2 receptor mutant (N1358H) were stably transfected with vectors encoding WT Gab1 (Maroun *et al.*, 1999a) or WT Gab2. (A) Total cell lysates of representative cell lines were separated by SDS-PAGE and Western blotted (WB) with anti-HA. (B) Cells (5×10^3) were grown in collagen for 5 d, during which time they formed cysts. RhCSF-1 or HGF was added, and 14 d later, branching tubules were visualized at a magnification of $10\times$. Representative results for one Gab2 cell line is shown. (C) Quantitation of the tubulogenic response. Results are derived from at least three independent experiments, with at least five different Gab2-expressing cell lines. None of the cysts formed tubules in the absence of stimulation. A partial response is defined as any structure that is no longer a cyst, but whose length is <5 times its width and is unbranched. Structures referred to as partial response do not develop into branched tubules over time. (D) CSF-Met Δ Grb2 (N1358H) MDCK cells expressing HA-Gab1 or high levels of HA-Gab2 were stimulated with CSF (100 ng/ml) for the times indicated. Proteins from 40 μ g of total cell lysates were separated by SDS-PAGE and WB with pMAPK or pJNK antibodies. Membranes were stripped and subsequently blotted with MAPK or JNK antibodies.

pressed by transient transfection in 293T cells. When over-expressed, the Met receptor is activated in the absence of growth factor stimulation and is tyrosine phosphorylated (Lock *et al.*, 2000). Association of the Met receptor with GST fusion proteins containing the MBD derived from Gab1 (MBD1) or Gab2 (MBD2) revealed that compared with MBD1, MBD2 binds less efficiently to the Met receptor, even though equal levels of fusion proteins were used (Figure 4D). Elimination of the Grb2 binding site in the Gab2 MBD (MBD2 Δ Grb2) abolishes this weak association, whereas the

deletion of the Grb2 binding site in the Gab1 MBD1 (MBD1 Δ Grb2) decreases but does not eliminate the association of the Gab1 MBD1 with the Met receptor (Figure 4D) (Lock *et al.*, 2000). To establish whether, as suggested by these experiments, Gab2 is recruited to the Met receptor in a Grb2-dependent manner, 293T cells were transiently co-transfected with the Met receptor and with either WT Gab1, or Gab2, or Gab1 or Gab2 mutants lacking Grb2 binding sites (Gab1 Δ Grb2 or Gab2 Δ Grb2; Figure 4E). After immunoprecipitation, WT Gab1 or a Gab1 Δ Grb2 mutant readily

Figure 4. Gab2 association with Met is Grb2 dependent. (A) CSF-MetΔGrb2 (N1358H) MDCK cells expressing HA-Gab1 or high levels of HA-Gab2 were stimulated with CSF (100 ng/ml) for 15 min. Equal amounts of protein were subjected to immunoprecipitation with anti-HA, followed by Western blotting (WB) with RC20H anti-phosphotyrosine. Blots were stripped and reprobed with anti-HA to show equal Gab protein levels in the immunoprecipitate. (B) Deletion of the proline-rich Grb2 SH3 domain-binding sites conserved between Gab1 and Gab2 abolishes the association of Gab2 with GST-Grb2 by an in vitro association assay. The Gab2ΔGrb2 mutant includes Δ348–355/P500A/R504A. (C) MDCK cells stably expressing HA-Gab1, HA-Gab1ΔGrb2, high levels of HA-Gab2, or high levels of HA-Gab2ΔGrb2 were stimulated with HGF (100 U/ml) for the times indicated. Equal amounts of protein were subjected to immunoprecipitation with anti-HA, followed by WB with RC20H anti-phosphotyrosine. Blots were stripped and reprobed with anti-HA to show equal Gab protein levels in the immunoprecipitate. (D) Gab1 GST fusion proteins MBD1 and MBD1ΔGrb2, or Gab2 GST fusion proteins MBD2 and MBD2ΔGrb2 were incubated with lysates prepared from 293T cells transiently expressing the Met receptor. Bound Met receptor protein was detected by WB analysis with anti-Met antibody. Levels of GST fusion proteins were determined by Western blotting with anti-GST. (E) 293T cells were transiently cotransfected with the Met receptor and either full-length WT HA-Gab1, HA-Gab1ΔGrb2, WT HA-Gab2, or HA-Gab2ΔGrb2. Proteins from cell lysates were immunoprecipitated (IP) with anti-Met antibody, separated by SDS-PAGE, and probed with anti-HA. Proteins from total cell lysates were separated by SDS-PAGE and probed with anti-Met and anti-HA to show expression levels.

coimmunoprecipitates with the Met receptor, whereas only low levels of WT Gab2 protein coimmunoprecipitate with Met and the Gab2ΔGrb2 protein fails to coimmunoprecipitate even though similar levels of proteins are expressed (Figure 4C). These data indicate that Gab1 and Gab2 are recruited through distinct mechanisms to the Met receptor, where Gab2 is Grb2 dependent and Gab1 recruitment is Grb2 independent.

Grb2-independent Recruitment of Gab1 Requires Amino Acids Absent in Gab2

By sequence alignment, the MBD regions of Gab1 and Gab2 are poorly conserved, but share homology within two p85 binding sites, and the Grb2 SH3 domain binding site (Figure 5A). Notably, Gab1 contains 10 amino acids that are absent in Gab2. The mutation of three proline residues within this 10-amino acid region in Gab1 (Δ3P = Δ491–493; Figure 5A) severely reduced the association of a GST-MBD1Δ3P fusion protein with the Met receptor in an in vitro association assay, compared with GST-MBD1 WT and GST-MBD1ΔGrb2 fusion proteins (Figure 5B). Similarly, coimmunoprecipitation of the Met receptor with the full-length Gab1Δ3P mutant protein is significantly reduced compared with WT Gab1 or Gab1ΔGrb2 protein (Figure 5C). Moreover, association with the Met receptor is abolished with either a GST-MBD1Δ3P/ΔGrb2 fusion protein (Figure 5B) or the full-length Gab1Δ3P/ΔGrb2 protein (Figure 5C). The

Gab1Δ3P mutant associates with a GST-Grb2 fusion protein in vitro to a similar level as WT Gab1 (Figure 5D). Thus, the reduction in association with the Met receptor is not due to an impaired ability of the Gab1Δ3P mutant to associate with Grb2, and the structure of this mutant has not been grossly altered.

To identify the amino acids critical for interaction with Met, we have individually substituted each proline residue and additional residues surrounding the prolines with alanines, and tested the ability of these mutants to coimmunoprecipitate with Met, by using the constitutively activated form of the Met receptor, Tpr-Met. The substitution of proline 491 or proline 492 with alanine disrupts the association of Gab1 with Met, and the substitution of glycine 487 showed reduced association, whereas the substitution of glutamine 489 or proline 493 had no effect (Figure 4E). Thus, the Δ3P mutant and more specifically a substitution of alanine for proline 491 or 492 abrogate the Grb2-independent recruitment of Gab1 to the Met receptor.

Insertion of Novel Amino Acid Sequence from Gab1 into Gab2 Confers Grb2-independent Binding

To define the domain required for direct recruitment of Gab1 to Met, we generated chimeric fusion proteins of Gab1 and Gab2 MBD regions (Figure 6A) and tested these for their ability to bind to Met in an in vitro association assay (Figure 6B). A chimeric protein containing the C terminus of the

Figure 5. Grb2-independent recruitment of Gab1 requires amino acids absent in Gab2. (A) Alignment of the MBD regions of Gab1 and Gab2. The conserved PI3K and Grb2 C-terminal SH3 domain-binding sites and the Δ 3P deletion mutant are indicated. (B) Deletion of prolines 491–493 (Δ 3P) significantly reduces association with Met via in vitro association assay. Gab1 MBD GST fusion proteins (MBD1, MBD1 Δ Grb2, MBD1 Δ 3P, and MBD1 Δ Grb2/ Δ 3P) or GST alone were incubated with lysates prepared from 293T cells transiently transfected with the Met receptor. Bound Met receptor protein was detected by Western blot (WB) with anti-Met. Equal levels of GST fusion proteins were used, as determined by Western blot with anti-GST. (C) 293T cells were transiently cotransfected with the Met receptor and either full-length WT HA-Gab1, HA-Gab1 Δ Grb2, HA-Gab1 Δ 3P, or HA-Gab1 Δ Grb2/ Δ 3P. Proteins from lysates were immunoprecipitated with anti-Met antibody, separated by SDS-PAGE, and WB with anti-HA. Proteins from total cell lysates were separated by SDS-PAGE and probed with anti-Met and anti-HA to show expression levels. (D) Gab1 Δ 3P mutant associates with GST-Grb2 to similar levels as WT Gab1, as shown by in vitro association assay. (E) Alanine scanning mutagenesis identifies prolines 491 and 491 as essential for association with Met. Amino acids G487, Q489, P491, P492, and P493 were substituted individually with alanine. Lysates prepared from COS cells transiently cotransfected with Met and Gab1 mutants were immunoprecipitated with anti-HA antibody and Western blotted with anti-Met.

Gab1MBD [MBD2/1(484-end); Figure 6B, lane 4], showed similar Met binding capacity to MBD1 (Figure 6B, lane 1), whereas a chimeric protein, containing the C terminus of the Gab1MBD [MBD2/1(495-end); Figure 6B, lane 5], showed a reduced ability to associate with the Met receptor, suggesting that amino acids between 484 and 495 of Gab1 were important for binding. However, the insertion of these 13 amino acids from Gab1, into the Gab2 MBD [MBD2/1/2(484–496); Figure 6B, lane 6], was insufficient to confer full binding, whereas a Gab2MBD protein with the insertion of 19 amino acids from Gab1 (MBD2/1/2*484–502) associated with the Met receptor to the same extent as a WT Gab1 MBD1 fusion protein (Figure 6B, lane 7). This is in agreement with data from Schaeper *et al.* (2000), who showed that insertion of 13 amino acids, GMQVPPPAHMGFR (aa 487–499), into Gab2 was sufficient to mediate Met association in a yeast two-hybrid assay (Schaeper *et al.*, 2000). The six additional amino acids identified herein reflect our cloning strategy to generate these mutants, and from our mutational analyses these amino acids are not essential for Grb2-independent binding.

Consistent with a requirement of prolines 491–493 for the association of the Gab1 MBD with Met, the deletion of these three prolines abolishes the Grb2-independent Met binding of the Gab2 MBD2/1/2* [MBD2/1/2* Δ 3P; Figure 6B, lane 8]. As expected, a Gab2 MBD2/1/2* Δ Grb2 fusion protein (lane 9) retained the ability to associate with Met, but showed decreased association, similar to that of the Gab1 MBD1 Δ Grb2 fusion protein (Figure 5B). The difference between the MBD2/1/2 (lane 6) and the MBD2/1/2* (lane 7) proteins is six amino acids of Gab1 (GFRSSP, aa 497–502; Figure 6C), which correspond to amino acids 479–484 (GYPSTA) in Gab2 (Figure 6C). The only significant differences between these proteins are Arg 499 and Pro 502 of Gab1 to Pro and Ala, respectively, in Gab2. To define the requirement for these residues, we generated mutants where Arg 499 and Pro 502 were substituted with Pro and Ala, respectively, in the context of the GST-MBD2/1/2* and tested for their abilities to bind to Met. The Gab2 MBD2/1/2*R499P (Figure 6B, lane 10) has a severely decreased association with Met, whereas MBD2/1/2*P502A (Figure 6B, lane 11) binds Met to the same level as MBD2/1/2* (lane 7). GST

Figure 6. Insertion of the novel amino acid sequence from Gab1 into Gab2 confers Grb2-independent binding. (A) Schematic of chimeric GST fusion proteins derived from Gab1 MBD (MBD1) and Gab2 MBD (MBD2) as used in Figure 6B. (B) In vitro association assay to determine the ability of chimeric MBD2/MBD1 fusion proteins, or GST alone, to associate with the Met receptor from transiently transfected 293T cells. Bound Met receptor protein was detected by Western blot with anti-Met antibody and levels of GST fusion proteins used were determined by Western blot with anti-GST. (C) Insertion of amino acids 484–502 from Gab1 into Gab2 (termed MBD2/1/2* or Gab2/1/2* in the full-length protein) conferred Met receptor association on Gab2, as shown in B and D. (D) The Gab2/1/2* protein can immunoprecipitate with Met to similar levels as WT Gab1. 293T cell lysates expressing Met and either HA-Gab1, HA-Gab2, or HA-Gab2/1/2* were immunoprecipitated with anti-Met antibody, separated by SDS-PAGE, and Western blotted with anti-HA to detect Gab proteins (low exposure is shown). Proteins from total cell lysates were separated by SDS-PAGE and probed with anti-HA and anti-Met to show expression levels. (E) MDCK cells expressing WT HA-Gab1, HA-Gab1Δ3P, HA-Gab2, or HA-Gab2/1/2* were stimulated or not with HGF for the indicated times. HA-tagged Gab proteins were immunoprecipitated, separated by SDS-PAGE, and WB with RC20H anti-phosphotyrosine. Blots were stripped and reprobed with anti-HA to show Gab protein levels in the immunoprecipitate.

alone (lane 12) does not bind Met. Therefore, the ability to confer Grb2-independent binding on Gab2 is mediated by the substitution of 19 amino acids from Gab1, SSF GMQVP-PPAHMGFRSSP, where the first and second prolines and the last arginine are critical, and the first glycine is preferred (Figures 5E and 6B).

In support of this, substitution of these 19 residues into Gab2 generates a Gab2 protein (Gab2/1/2*) that coimmunoprecipitates with the Met receptor to similar levels as WT Gab1 (Figure 6D). Moreover, in stable MDCK cell lines expressing the Gab2/1/2* protein, Gab2/1/2* is phosphorylated to higher levels than Gab2, although it was not as highly phosphorylated as Gab1 (Figure 6E). In accordance

with these results, in stable MDCK cell lines, the Gab1Δ3P protein is significantly less phosphorylated than WT Gab1 (Figure 6E). This indicates that not only are these amino acids from Gab1 required for Grb2-independent association with the Met receptor but also they are required for efficient phosphorylation of Gab proteins by the Met receptor.

Grb2 Independent Recruitment of Gab1 Is Essential for Epithelial Morphogenesis

To evaluate the biological significance of the Grb2-independent binding of Gab1, we have tested the ability of Gab1Δ3P and Gab2/1/2* to rescue tubulogenesis. Overexpression of

Figure 7. Met binding site is essential but not sufficient for rescue of epithelial morphogenesis. (A) MDCK cells expressing the CSF-MetΔGrb2 receptor mutant (N1358H) were stably transfected with vectors encoding HA-Gab1Δ3P or HA-Gab2/1/2*. Proteins from total cell lysates (20 μg) of representative cell lines were separated by SDS-PAGE and Western blotted with anti-HA. (B) CSF-MetΔGrb2 MDCK cells expressing HA-Gab1 or HA-Gab2/1/2* were stimulated with CSF (100 ng/ml) for 15 min. Gab proteins were immunoprecipitated with anti-HA, followed by Western blotting (WB) with RC20H anti-phosphotyrosine. Blots were stripped and reprobed with anti-HA to show equal Gab protein levels in the immunoprecipitate. (C) Cells were grown in collagen, as described in MATERIALS AND METHODS. Results are derived from at least three independent experiments, with five stable Gab1Δ3P- and Gab2/1/2*-expressing cell lines. Representative results are shown for one cell line each. (D) Quantitation of the tubulogenic response shown in C. Representative results of two Gab1Δ3P and 4 Gab2/1/2* cell lines are shown. (E) Quantitative results from multiple cell lines are combined to show average ability of Gab1, Gab2, Gab1Δ3P, and Gab2/1/2* to promote tubulogenesis. Results are derived from at least three independent experiments.

the Gab1Δ3P mutant (Figure 7A) was unable to rescue branching tubulogenesis in response to CSF-1 in five independent cell lines (Figure 7, C and D; two representative cell lines are shown), indicating that a functional Met binding motif in Gab1 is essential for epithelial morphogenesis. Importantly, when overexpressed, the Gab2/1/2* chimera fails to rescue the tubulogenic defect of the MetΔGrb2 mutant (Figure 7, C–E). In a similar manner to WT Gab2 (Figure 3B), cell lines that express low levels of Gab2/1/2* (Figure 7A) remain as cysts upon stimulation of the MetΔGrb2 receptor. Cell lines that express high levels of Gab2/1/2* (Figure 7A) are able to generate a partial response, with a higher percentage of long, unbranched tubules than Gab2-overexpressing cells (Figure 7C). However these cell lines are unable to

generate a full branching morphogenic response comparable with that of the Gab1 expressing cell lines (Figure 7A, and C–E; four representative cell lines are shown). Importantly, Gab2/1/2* protein becomes phosphorylated upon stimulation of the CSF-MetΔGrb2 receptor, although consistently to lower levels than Gab1 (Figure 7B). These results demonstrate that the direct binding of Gab1 to Met is essential but not sufficient for branching tubulogenesis, implying that Gab1 and Gab2 do not have redundant roles in MDCK cells.

DISCUSSION

Gab1-null mice are embryonic lethal (Itoh *et al.*, 2000; Sachs *et al.*, 2000), whereas those of Gab2 are viable and generally

healthy but have a defect in the allergic response (Gu *et al.*, 2001). Whether these differences are mainly attributed to distinct tissue expression during development or to a distinct function of Gab proteins has not been directly evaluated. To address this issue, we have used the MDCK epithelial cell system to study the roles of Gab1 and Gab2 in epithelial morphogenesis downstream of the Met receptor tyrosine kinase. We show that both Gab1 and Gab2 are expressed in MDCK cells (Figure 1A) and that they show a similar subcellular localization and concentrate at cell-cell junctions in colonies of epithelial cells (Figure 1B) (Maroun *et al.*, 1999a). Gab2 becomes phosphorylated upon HGF stimulation (Figure 1A) and can associate with similar signaling proteins as Gab1 (Figure 2, B and C) (Weidner *et al.*, 1996; Nguyen *et al.*, 1997; Maroun *et al.*, 1999a). However, in contrast to Gab1, Gab2 is unable to rescue the epithelial morphogenesis defect of the Met Δ Grb2 receptor mutant (Figure 3, B and C), providing evidence for the first time for a distinct role for Gab1 and Gab2 downstream from the Met receptor.

We have previously shown that Gab1 is an essential mediator of Met receptor-induced epithelial morphogenesis (Maroun *et al.*, 1999a). Overexpression of Gab1 in MDCK epithelial cells rescues the inability of a Met receptor mutant (Met Δ Grb2) to induce branching morphogenesis (Maroun *et al.*, 1999a). In contrast, the overexpression of Gab2 fails to rescue the morphogenic defect (Figure 3, B and C). Compared with Gab1, only low levels of Gab2 associate with the Met receptor, and Gab2 is phosphorylated only weakly in response to stimulation of the Met receptor (Figures 2A, and 4, D and E). The reduced Gab2 association with the Met receptor compared with Gab1 reflects different mechanisms of recruitment of these two related docking proteins. The recruitment of Gab2 to Met, and its subsequent phosphorylation, are dependent predominantly on a Grb2 binding site in Gab2 (Figure 4, C–E), whereas the recruitment of Gab1 to Met is both Grb2 dependent and Grb2 independent (Figure 5, B and C) (Weidner *et al.*, 1996; Nguyen *et al.*, 1997; Lock *et al.*, 2000; Schaeper *et al.*, 2000). The Grb2-independent association of Gab1 with Met is thought to be direct, as proposed from yeast two-hybrid studies (Weidner *et al.*, 1996; Schaeper *et al.*, 2000). However, the nature of the interaction remains undefined.

A comparison of the MBD regions of Gab1 and Gab2 revealed a 10-amino acid segment in Gab1 (aa 484–493) that is lacking in Gab2 (Figure 5A). From our structure–function analyses, and in agreement with Schaeper *et al.* (2000), these amino acids are critical for the Grb2-independent recruitment of Gab1 to the Met receptor and their insertion confers Grb2-independent recruitment of Gab2 to Met (Gab2/1/2*; Figure 6, A–D). The mutation of three proline residues within this region of Gab1 (Gab1 Δ 3P, Δ 491–493) abolishes Grb2-independent recruitment of Gab1 (Figure 5, B and C) and decreases HGF-induced tyrosine phosphorylation of Gab1 (Figure 6E). Notably, the Gab1 Δ 3P mutant fails to rescue the branching morphogenic program downstream from the Met Δ Grb2 receptor mutant (Figure 7, C and D). This identifies for the first time a requirement for Grb2-independent recruitment for Gab1 biological function.

The failure of Gab2 to rescue the Met-dependent morphogenic program in MDCK cells could therefore be attributed to the absence of the Gab1 Met binding sequence, and hence

the low levels of Gab2 phosphorylated downstream of a Met Δ Grb2 mutant (Figure 4, A–E). However, the inability of Gab2 to rescue morphogenesis cannot solely be attributed to this difference. A Gab2 protein containing the Gab1 Met binding sequence does not rescue the morphogenic program (Gab2/1/2*; Figure 7, C–E), in spite of the increased tyrosine phosphorylation of the Gab2/1/2* protein (Figure 6E). Hence, although we cannot rule out the possibility that the signal downstream from the Gab2/1/2* protein is below a threshold required to rescue the morphogenic response, in spite of their high homology and similar domain structure, Gab1 and Gab2 may not be functionally redundant in MDCK cells.

Gab1 and Gab2 have 15 conserved tyrosines, although only 8–10 of the tyrosines in Gab1 have been shown to be phosphorylated by either the insulin, EGF, or Met receptors (Lehr *et al.*, 1999; Gual *et al.*, 2000; Lehr *et al.*, 2000). The Met receptor may phosphorylate different tyrosine residues in Gab1 or Gab2 and/or other kinases, such as Src family kinases, activated downstream of Met, may participate in Gab1 phosphorylation and not Gab2. However, pretreatment of cells with a Src family kinase inhibitor (PP2) did not alter Gab1 or Gab2 phosphorylation after Met stimulation (our unpublished data). Moreover, when overexpressed and phosphorylated after activation of Met, Gab2 can associate with the same signaling proteins as Gab1, suggesting that Gab2 can be extensively phosphorylated by Met (Figure 2B). Two potentially phosphorylated tyrosines (Y307 and Y373) are absent in Gab2. However, although these tyrosines contain a consensus binding site for Crk/PLC γ , Gab2 retains the ability to bind these signaling proteins (Figure 2B) (Gual *et al.*, 2000; Lamorte *et al.*, 2000; Sakkab *et al.*, 2000; Schaeper *et al.*, 2000). Alternatively, tyrosines 307 and 373 in Gab1 may be required for the association with an unidentified protein that is critical for the morphogenic response. In addition, Gab1 but not Gab2 binds to and is a substrate of Erk2 MAPK (Roshan *et al.*, 1999; Yu *et al.*, 2001), whereas Gab2, but not Gab1 has been shown to be negatively regulated by serine phosphorylation by PKB/Akt (Lynch and Daly, 2002). The possibility that the distinct biological functions of Gab1 and Gab2 reflect recruitment of distinct signaling proteins, or different mechanisms for feedback inhibition, is currently being evaluated using Gab1/Gab2 chimeric proteins.

The Grb2-independent recruitment of the Gab1 MBD to Met is phosphotyrosine dependent and requires the presence of two tyrosines in the Met receptor (Weidner *et al.*, 1996), although the amino acid motif required for Gab1 recruitment is not known. The Gab1 MBD has no known homology with other phosphotyrosine binding modules, nor was any significant homology found with other proteins. From structure–function analysis, we show that Pro 491, 492, and Arg 499 are required for Grb2-independent recruitment of Gab1 to Met (Figures 5E and 6B). A critical arginine in the MBD is reminiscent of SH2 domains, where a conserved arginine is required to directly participate in binding the phosphotyrosine residue (Marengere and Pawson, 1992; Waksman *et al.*, 1993). Thus, although the Gab1 MBD seems to be unique, similarity may only be identified by three-dimensional structure analysis. For instance, the Shc and IRS-1 PTB domains share little primary sequence homology, yet adopt a similar three-dimensional structure and have similar binding specificities (Wolf *et al.*, 1995; Eck

et al., 1996; Zhou *et al.*, 1996). Although the MBD seems to function as a phosphotyrosine binding domain, one major difference from other such domains is the presence of additional protein binding sites within the Gab1 MBD. These include the p85 SH2 domain, the Grb2 SH3 domain, and Erk1 or 2 (Holgado-Madruga *et al.*, 1997; Rocchi *et al.*, 1998; Roshan *et al.*, 1999; Lock *et al.*, 2000; Schaeper *et al.*, 2000; Lewitzky *et al.*, 2001). From our studies, the binding sites for these proteins, and the amino acids required for Grb2-independent recruitment of Met are distinct; hence, it is possible that the Gab1 MBD contains multiple ligand-binding surfaces.

To date, the MBD of Gab1 interacts in a Grb2-independent manner with only the Met receptor and not with other receptors tested, including EGF receptor, platelet-derived growth factor- β v-Sea, TrkA, c-Ros, the insulin receptor DDR, c-Ret, Sek-1, c-Kit, c-Abl, CSF-1R, and keratinocyte growth factor receptor (Weidner *et al.*, 1996; Lock *et al.*, 2000). Similarly, the PTB domain of FRS2/SNT2 shows differential binding specificity for receptors. It binds to canonical NPXpY sequences on TrkA and Ret receptors (Meakin *et al.*, 1999; Kurokawa *et al.*, 2001), whereas it interacts with the fibroblast growth factor receptor through a novel sequence in a nonphosphotyrosine-dependent manner (Xu *et al.*, 1998; Ong *et al.*, 2000). In addition, the Grb7/Grb10/Grb14 family of adapter proteins contains novel receptor-specific interaction domains (BPS/IPS/PIR) in addition to their SH2 domains (He *et al.*, 1998; Kasus-Jacobi *et al.*, 2000; Stein *et al.*, 2001). Moreover, whereas both IRS-1 and IRS-2 contain PTB and PH domains required for efficient association with the insulin receptor, only IRS-2 contains a kinase regulatory loop-binding domain that interacts with the phosphorylated regulatory loop of the insulin receptor β -subunit (Sawka-Verhelle *et al.*, 1996, 1997). The presence of domains 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 different biological responses downstream from receptor tyrosine kinases. We have shown a distinct mechanism of recruitment and distinct function for Gab1 and Gab2 downstream from the Met receptor. It remains to be established whether Gab1 and Gab2 are functionally redundant downstream from other receptors that recruit Gab1 in a Grb2-dependent manner only, and underscores the importance of further studies to understand these differences.

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REFERENCES

Bardelli, A., Longati, P., Gramaglia, D., Stella, M.C., and Comoglio, P.M. (1997). Gab1 coupling to the HGF/Met receptor multifunc-

tional docking site requires binding of Grb2 and correlates with the transforming potential. *Oncogene* 15, 3103–3111.

Birchmeier, C., and Gherardi, E. (1998). Developmental roles of HGF/SF and its receptor, the c-Met tyrosine kinase. *Trends Cell Biol.* 8, 404–410.

Bouscary, D., *et al.* (2001). Role of Gab proteins in phosphatidylinositol 3-kinase activation by thrombopoietin (Tpo). *Oncogene* 20, 2197–2204.

Carpino, N., Wisniewski, D., Strife, A., Marshak, D., Kobayashi, R., Stillman, B., and Clarkson, B. (1997). p62DOK: a constitutively tyrosine-phosphorylated, GAP-associated protein in chronic myelogenous leukemia progenitor cells. *Cell* 88, 197–204.

Crouin, C., Arnaud, M., Gesbert, F., Camonis, J., and Bertoglio, J. (2001). A yeast two-hybrid study of human p97/Gab2 interactions with its SH2 domain-containing binding partners. *FEBS Lett.* 495, 148–153.

Eck, M.J., Dhe-Paganon, S., Trub, T., Nolte, R.T., and Shoelson, S.E. (1996). Structure of the IRS-1 PTB domain bound to the juxtamembrane region of the insulin receptor. *Cell* 85, 695–705.

Fixman, E., Holgado-Madruga, M., Nguyen, L., Kamikura, D., Fournier, T., Wong, A., and Park, M. (1997). Efficient cellular transformation by the Met oncoprotein requires a functional Grb2 binding site and correlates with phosphorylation of the Grb2-associated proteins, Cbl and Gab1. *J. Biol. Chem.* 272, 20167–20172.

Fixman, E.D., Fournier, T.M., Kamikura, D.M., Naujokas, M.A., and Park, M. (1996). Pathways downstream of Shc and Grb2 are required for cell transformation by the Tpr-Met oncoprotein. *J. Biol. Chem.* 271, 13116–13122.

Fournier, T.M., Kamikura, D., Teng, K., and Park, M. (1996). Branching tubulogenesis, but not scatter of Madin-Darby canine kidney cells requires a functional Grb2 binding site in the Met receptor tyrosine kinase. *J. Biol. Chem.* 271, 22211–22217.

Garcia-Guzman, M., Dolfi, F., Zeh, K., and Vuor, K. (1999). Met-induced JNK activation is mediated by the adapter protein Crk and correlates with the Gab1-Crk signaling complex formation. *Oncogene* 18, 7775–7786.

Gherardi, E., and Stoker, M. (1991). Hepatocyte growth factor-scatter factor: mitogen, motogen, and met. *Cancer Cells* 3, 227–232.

Grimm, J., Sachs, M., Britsch, S., Di Cesare, S., Schwarz-Romond, T., Alitalo, K., and Birchmeier, W. (2001). Novel p62dok family members, dok-4 and dok-5, are substrates of the c-Ret receptor tyrosine kinase and mediate neuronal differentiation. *J. Cell Biol.* 154, 345–354.

Gu, H., Pratt, J.C., Burakoff, S.J., and Neel, B.G. (1998). Cloning of p97/Gab2, the major SHP2-binding protein in hematopoietic cells, reveals a novel pathway for cytokine-induced gene activation. *Mol. Cell* 2, 729–740.

Gu, H., Saito, K., Klamann, L.D., Shen, J., Fleming, T., Wang, Y., Pratt, J.C., Lin, G., Lim, B., Kinet, J.P., and Neel, B.G. (2001). Essential role for Gab2 in the allergic response. *Nature* 412, 186–190.

Gual, P., Giordano, S., Williams, T.A., Rocchi, S., Van Obberghen, E., and Comoglio, P.M. (2000). Sustained recruitment of phospholipase C- γ to Gab1 is required for HGF-induced branching tubulogenesis. *Oncogene* 19, 1509–1518.

He, W., Rose, D.W., Olefsky, J.M., and Gustafson, T.A. (1998). Grb10 interacts differentially with the insulin receptor, insulin-like growth factor I receptor, and epidermal growth factor receptor via the Grb10 Src homology 2 (SH2) domain and a second novel domain located between the pleckstrin homology and SH2 domains. *J. Biol. Chem.* 273, 6860–6867.

Herbst, R., Carroll, P.M., Allard, J.D., Schilling, J., Raabe, T., and Simon, M.A. (1996). Daughter of sevenless is a substrate of the

phosphotyrosine phosphatase corkscrew and functions during sevenless signaling. *Cell* 85, 899–909.

Holgado-Madruga, M., Emllet, D.R., Moscatello, D.K., Godwin, A.K., and Wong, A.J. (1996). A Grb2-associated docking protein in EGF- and insulin-receptor signaling. *Nature* 379, 560–564.

Holgado-Madruga, M., Moscatello, D.K., Emllet, D.R., Dieterich, R., and Wong, A.J. (1997). Grb2-associated binder-1 mediates phosphatidylinositol 3-kinase activation and the promotion of cell survival by nerve growth factor. *Proc. Natl. Acad. Sci. USA* 94, 12419–12424.

Isakoff, S.J., Cardozo, T., Andreev, J., Li, Z., Ferguson, K.M., Abagyan, R., Lemmon, M.A., Aronheim, A., and Skolnik, E.Y. (1998). Identification and analysis of P.H. domain-containing targets of phosphatidylinositol 3-kinase using a novel in vivo assay in yeast. *EMBO J.* 17, 5374–5387.

Itoh, M., Yoshida, Y., Nishida, K., Narimatsu, M., Hibi, M., and Hirano, T. (2000). Role of gab1 in heart, placenta, and skin development and growth factor- and cytokine-induced extracellular signal-regulated kinase mitogen-activated protein kinase activation. *Mol. Cell. Biol.* 20, 3695–3704.

Jeffers, M., Rong, S., and Vande Woude, G.F. (1996). Hepatocyte growth factor/scatter factor-Met signaling in tumorigenicity and invasion/metastasis. *J. Mol. Med.* 74, 505–513.

Jones, N., and Dumont, D.J. (1998). The Tek/Tie2 receptor signals through a novel Dok-related docking protein, Dok-R. *Oncogene* 17, 1097–1108.

Kasus-Jacobi, A., Bereziat, V., Perdereau, D., Girard, J., and Burnol, A.F. (2000). Evidence for an interaction between the insulin receptor and Grb7. A role for two of its binding domains, PIR and SH2. *Oncogene* 19, 2052–2059.

Komada, M., and Kitamura, N. (1993). The cell dissociation and motility triggered by scatter factor/hepatocyte growth factor are mediated through the cytoplasmic domain of the c-Met receptor. *Oncogene* 8, 2381–2390.

Kong, M., Mounier, C., Wu, J., and Posner, B.I. (2000). Epidermal growth factor-induced phosphatidylinositol 3-kinase activation and DNA synthesis. Identification of Grb2-associated binder 2 as the major mediator in rat hepatocytes. *J. Biol. Chem.* 275, 36035–36042.

Kouhara, H., Hadari, Y.R., Spivak-Kroizman, T., Schilling, J., Bar-Sagi, D., Lax, I., and Schlessinger, J. (1997). A lipid-anchored Grb2-binding protein that links FGF-receptor activation to the Ras/MAPK signaling pathway. *Cell* 89, 693–702.

Kurokawa, K., Iwashita, T., Murakami, H., Hayashi, H., Kawai, K., and Takahashi, M. (2001). Identification of SNT/FRS2 docking site on RET receptor tyrosine kinase and its role for signal transduction. *Oncogene* 20, 1929–1938.

Lamorte, L., Kamikura, D.M., and Park, M. (2000). A switch from p130Cas/Crk to Gab1/Crk signaling correlates with anchorage independent growth and JNK activation in cells transformed by the Met receptor oncoprotein. *Oncogene* 19, 5973–5981.

Lehr, S., *et al.* (1999). Identification of tyrosine phosphorylation sites in human Gab-1 protein by EGF receptor kinase in vitro. *Biochemistry* 38, 151–159.

Lehr, S., Kotzka, J., Herkner, A., Sikmann, A., Meyer, H.E., Krone, W., and Muller-Wieland, D. (2000). Identification of major tyrosine phosphorylation sites in the human insulin receptor substrate Gab-1 by insulin receptor kinase in vitro. *Biochemistry* 39, 10898–10907.

Lemay, S., Davidson, D., Latour, S., and Veillette, A. (2000). Dok-3, a novel adapter molecule involved in the negative regulation of immunoreceptor signaling. *Mol. Cell. Biol.* 20, 2743–2754.

Lewitzky, M., Kardinal, C., Gehring, N.H., Schmidt, E.K., Konkol, B., Eulitz, M., Birchmeier, W., Schaeper, U., and Feller, S.M. (2001).

The C-terminal SH3 domain of the adapter protein Grb2 binds with high affinity to sequences in Gab1 and SLP-76 which lack the SH3-typical P-x-x-P core motif. *Oncogene* 20, 1052–1062.

Lock, L.S., Royal, I., Naujokas, M.A., and Park, M. (2000). Identification of an atypical Grb2 carboxyl-terminal SH3 domain binding site in Gab docking proteins reveals Grb2-dependent and -independent recruitment of Gab1 to receptor tyrosine kinases. *J. Biol. Chem.* 275, 31536–31545.

Lynch, D.K., and Daly, R.J. (2002). PKB-mediated negative feedback tightly regulates mitogenic signaling via Gab2. *EMBO J.* 21, 72–82.

Marengere, L.E., and Pawson, T. (1992). Identification of residues in GTPase-activating protein Src homology 2 domains that control binding to tyrosine phosphorylated growth factor receptors and p62. *J. Biol. Chem.* 267, 22779–22786.

Maroun, C., Holgado-Madruga, M., Royal, I., Naujokas, M.A., Fournier, T.M., Wong, A.J., and Park, M. (1999a). The Gab-1 PH domain is required for localization of Gab-1 at sites of cell-cell contact and epithelial morphogenesis downstream from the Met receptor tyrosine kinase. *Mol. Cell. Biol.* 19, 1784–1799.

Maroun, C.R., Moscatello, D.K., Naujokas, M.A., Holgado-Madruga, M., Wong, A.J., and Park, M. (1999b). A conserved inositol phospholipid binding site within the pleckstrin homology domain of the Gab1 docking protein is required for epithelial morphogenesis. *J. Biol. Chem.* 274, 31719–31726.

Maroun, C.R., Naujokas, M.A., Holgado-Madruga, M., Wong, A.J., and Park, M. (2000). The tyrosine phosphatase SHP-2 is required for sustained activation of extracellular signal-regulated kinase and epithelial morphogenesis downstream from the met receptor tyrosine kinase. *Mol. Cell. Biol.* 20, 8513–8525.

Meakin, S.O., MacDonald, J.I., Gryz, E.A., Kubu, C.J., and Verdi, J.M. (1999). The signaling adapter FRS-2 competes with Shc for binding to the nerve growth factor receptor TrkA. A model for discriminating proliferation and differentiation. *J. Biol. Chem.* 274, 9861–9870.

Michalopoulos, G.K. (1995). HGF in liver regeneration and tumor promotion. *Prog. Clin. Biol. Res.* 391, 179–185.

Nguyen, L., Holgado-Madruga, M., Maroun, C., Fixman, E.D., Kamikura, D., Fournier, T., Charest, A., Tremblay, M.L., Wong, A.J., and Park, M. (1997). Association of the multisubstrate docking protein Gab1 with the hepatocyte growth factor receptor requires a functional Grb2 binding site involving tyrosine 1356. *J. Biol. Chem.* 272, 20811–20819.

Nishida, K., *et al.* (1999). Gab-family adapter proteins act downstream of cytokine and growth factor receptors and T- and B-cell antigen receptors. *Blood* 93, 1809–1816.

Ong, S.H., Guy, G.R., Hadari, Y.R., Laks, S., Gotoh, N., Schlessinger, J., and Lax, I. (2000). FRS2 proteins recruit intracellular signaling pathways by binding to diverse targets on fibroblast growth factor and nerve growth factor receptors. *Mol. Cell. Biol.* 20, 979–989.

Ponzetto, C., Bardelli, A., Zhen, Z., Maina, F., dalla Zonca, P., Giordano, S., Graziani, A., Panayotou, G., and Comoglio, P.M. (1994). A multifunctional docking site mediates signaling and transformation by the hepatocyte growth factor/scatter factor receptor family. *Cell* 77, 261–271.

Pratt, J.C., Igras, V.E., Maeda, H., Baksh, S., Gelfand, E.W., Burakoff, S.J., Neel, B.G., and Gu, H. (2000). Cutting edge: gab2 mediates an inhibitory phosphatidylinositol 3'-kinase pathway in T cell antigen receptor signaling. *J. Immunol.* 165, 4158–4163.

Raabe, T., Riesgo-Escovar, J., Liu, X., Bausenwein, B.S., Deak, P., Maroy, P., and Hafen, E. (1996). DOS, a novel pleckstrin homology domain-containing protein required for signal transduction between sevenless and Ras1 in *Drosophila*. *Cell* 85, 911–920.

- Rocchi, S., Tartare-Deckert, S., Mordaca, J., Holgado-Madruga, M., Wong, A.J., and Van Obberghen, E. (1998). Determination of Gab1 (Grb2-associated binder-1) interaction with insulin receptor-signaling molecules. *Mol. Endocrinol.* 12, 914–923.
- Rodrigues, G.A., Falasca, M., Zhang, Z., Ong, S.H., and Schlessinger, J. (2000). A novel positive feedback loop mediated by the docking protein gab1 and phosphatidylinositol 3-kinase in epidermal growth factor receptor signaling. *Mol. Cell. Biol.* 20, 1448–1459.
- Rodrigues, G.A., Naujokas, M.A., and Park, M. (1991). Alternative splicing generates isoforms of the met receptor tyrosine kinase which undergo differential processing. *Mol. Cell. Biol.* 11, 2962–2970.
- Rosen, E.M., Nigam, S.K., and Goldberg, I.D. (1994). Scatter factor and the c-met receptor: a paradigm for mesenchymal/epithelial interaction. *J. Cell Biol.* 127, 1783–1787.
- Roshan, B., Kjelsberg, C., Spokes, K., Eldred, A., Crovello, C.S., and Cantley, L.G. (1999). Activated Erk2 interacts with and phosphorylates the docking protein Gab1. *J. Biol. Chem.* 274, 36362–36368.
- Sachs, M., Brohmann, H., Zechner, D., Muller, T., Hulsken, J., Walther, I., Schaeper, U., Birchmeier, C., and Birchmeier, W. (2000). Essential role of Gab1 for signaling by the c-Met receptor in vivo. *J. Cell Biol.* 150, 1375–1384.
- Sakkab, D., Lewitzky, M., Posern, G., Schaeper, U., Sachs, M., Birchmeier, W., and Feller, S.M. (2000). Signaling of hepatocyte growth factor/scatter factor (HGF) to the small GTPase rap1 via the large docking protein gab1 and the adapter protein CRKL. *J. Biol. Chem.* 275, 10772–10778.
- Sawka-Verhelle, D., Baron, V., Mothe, I., Filloux, C., White, M.F., and Van Obberghen, E. (1997). Tyr624 and Tyr628 in insulin receptor substrate-2 mediate its association with the insulin receptor. *J. Biol. Chem.* 272, 16414–16420.
- Sawka-Verhelle, D., Tartare-Deckert, S., White, M.F., and Van Obberghen, E. (1996). Insulin receptor substrate-2 binds to the insulin receptor through its phosphotyrosine-binding domain and through a newly identified domain comprising amino acids 591–786. *J. Biol. Chem.* 271, 5980–5983.
- Schaeper, U., Gehring, N.H., Fuchs, K.P., Sachs, M., Kempkes, B., and Birchmeier, W. (2000). Coupling of Gab1 to c-Met, Grb2, and Shp2 mediates biological responses. *J. Cell Biol.* 149, 1419–1432.
- Schutzman, J.L., Borland, C.Z., Newman, J.C., Robinson, M.K., Kokel, M., and Stern, M.J. (2001). The *Caenorhabditis elegans* EGL-15 signaling pathway implicates a DOS-like multisubstrate adaptor protein in fibroblast growth factor signal transduction. *Mol. Cell. Biol.* 21, 8104–8116.
- Stein, E.G., Gustafson, T.A., and Hubbard, S.R. (2001). The BPS domain of Grb10 inhibits the catalytic activity of the insulin and IGF1 receptors. *FEBS Lett.* 493, 106–111.
- Trusolino, L., Bertotti, A., and Comoglio, P.M. (2001). A signaling adapter function for $\alpha 6 \beta 4$ integrin in the control of HGF-dependent invasive growth. *Cell* 107, 643–654.
- Waksman, G., Shoelson, S.E., Pant, N., Cowburn, D., and Kurlyan, J. (1993). Binding of a high affinity phosphotyrosyl peptide to the src SH2 domain: crystal structures of the complexed and peptide-free forms. *Cell* 72, 779–790.
- Weidner, K.M., Di Cesare, S., Sachs, M., Brinkmann, V., Behrens, J., and Birchmeier, W. (1996). Interaction between Gab1 and the c-Met receptor tyrosine kinase is responsible for epithelial morphogenesis. *Nature* 384, 173–176.
- Weidner, K.M., Sachs, M., and Birchmeier, W. (1993). The met receptor tyrosine kinase transduces motility, proliferation, and morphogenic signals of scatter factor/hepatocyte growth factor in epithelial cells. *J. Cell Biol.* 121, 145–154.
- Wickrema, A., *et al.* (1999). Engagement of Gab1 and Gab2 in erythropoietin signaling. *J. Biol. Chem.* 274, 24469–24474.
- Wigler, M., Pellicer, A., Silverstein, S., Axel, R., Urlaub, G., and Chasin, L. (1979). DNA-mediated transfer of the adenine phosphoribosyltransferase locus into mammalian cells. *Proc. Natl. Acad. Sci. USA* 76, 1373–1376.
- Wolf, I., Jenkins, B.J., Liu, Y., Seiffert, M., Custodio, J.M., Young, P., and Rohrschneider, L.R. (2002). Gab3, a new DOS/Gab family member, facilitates macrophage differentiation. *Mol. Cell. Biol.* 22, 231–244.
- Wolf, G., Trub, T., Ottinger, E., Groninga, L., Lynch, A., White, M., Miyazaki, M., Lee, J., and Shoelson, S.E. (1995). The PTB domains of IRS-1 and Shc have distinct but overlapping binding specificities. *J. Biol. Chem.* 270, 27407–27410.
- Xu, H., Lee, K.W., and Goldfarb, M. (1998). Novel recognition motif on fibroblast growth factor receptor mediates direct association and activation of SNT adapter proteins. *J. Biol. Chem.* 273, 17987–17990.
- Yamanashi, Y., and Baltimore, D. (1997). Identification of the Abl- and rasGAP-associated 62 kDa protein as a docking protein, Dok. *Cell* 88, 205–211.
- Yenush, L., and White, M.F. (1997). The IRS-signaling system during insulin and cytokine action. *Bioessays* 19, 491–500.
- Yu, C.F., Roshan, B., Liu, Z.X., and Cantley, L.G. (2001). Erk regulates the hepatocyte growth factor-mediated interaction of gab1 and the phosphatidylinositol 3-kinase. *J. Biol. Chem.* 276, 32552–32558.
- Zarnegar, R., and Michalopoulos, G.K. (1995). The many faces of hepatocyte growth factor: from hepatopoiesis to hematopoiesis. *J. Cell Biol.* 129, 1177–1180.
- Zhao, C., Yu, D.H., Shen, R., and Feng, G.S. (1999). Gab2, a new pleckstrin homology domain-containing adapter protein, acts to uncouple signaling from ERK kinase to Elk-1. *J. Biol. Chem.* 274, 13649–13654.
- Zhou, M.M., Huang, B., Olejniczak, E.T., Meadows, R.P., Shuker, S.B., Miyazaki, M., Trub, T., Shoelson, S.E., and Fesik, S.W. (1996). Structural basis for IL-4 receptor phosphopeptide recognition by the IRS-1 PTB domain. *Nat. Struct. Biol.* 3, 388–393.
- Zhu, H., Naujokas, M.A., Fixman, E.D., Torossian, K., and Park, M. (1994a). Tyrosine 1356 in the carboxyl-terminal tail of the HGF/SF receptor is essential for the transduction of signals for cell motility and morphogenesis. *J. Biol. Chem.* 269, 29943–29948.
- Zhu, H., Naujokas, M.A., and Park, M. (1994b). Receptor chimeras indicate that the Met tyrosine kinase mediates the motility and morphogenic responses of hepatocyte growth/scatter factor. *Cell Growth Diff.* 5, 359–366.