Recruitment specificity of Gab family docking proteins and implications for Met receptor-mediated epithelial morphogenesis

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Abstract

Activation of cell-surface receptors by extracellular signals can generate distinct biological responses. Many of these signals are coordinated through docking proteins, including those in the Gab family (Gab1, Gab2 and Gab3). Following activation of receptor tyrosine kinases (RTKs), cytokine or antigen receptors, docking proteins are recruited to the receptor complex and become phosphorylated on tyrosine residues, providing binding sites for multiple proteins involved in signal transduction. In this manner, they act to diversify and localize signals downstream from receptors by virtue of their ability to assemble multiprotein complexes.

The recruitment of docking proteins to RTKs depends on the ability of the protein to interact directly or indirectly with the receptor. In chapter II, I established that Gab1 and Gab2 can be recruited to RTKs indirectly, through constitutive association of Gab1 or Gab2 with the C-terminal SH3 domain of the adapter protein Grb2. This requires two highly conserved Grb2 binding sites in Gab proteins. One site corresponds to a canonical SH3 domain binding motif, whereas the second contains an atypical PXXXRXXKP motif that I also identified in the unrelated Grb2-binding protein, Slp-76.

In contrast to the other Gab proteins, Gab1 can also interact in a Grb2-independent manner with the Met/Hepatocyte growth factor receptor. In chapter IV, I established that this interaction requires the structural integrity of the Met receptor, phosphorylation of tyrosine 1349 in the Met C-terminus, and a 13 amino acid Met binding motif (MBM) in Gab1. Instead of the expected interaction of a phosphotyrosine-binding domain in Gab1 with a phosphotyrosine-containing motif in the Met receptor, I propose that the activated kinase domain of Met and the negative charge of phosphotyrosine 1349 engage the Gab1 MBM as an extended peptide ligand.

In response to Met receptor stimulation, Gab1 overexpression promotes an invasive morphogenic program in epithelial cells. In contrast, I have shown in chapter III that Gab2 overexpression fails to induce this response. Mutation of the MBM in Gab1 abolishes the ability of Gab1 to promote morphogenesis, whereas its insertion into Gab2 increases Gab2 association with Met, but does not confer on Gab2 the ability to promote morphogenesis. This indicates that the Grb2-independent recruitment of Gab proteins to

Met is necessary but not sufficient to promote epithelial morphogenesis. Overall, these studies have identified both common and unique mechanisms through which receptor tyrosine kinases can recruit Gab docking proteins, and have established that Gab1 and Gab2 do not share redundant biological functions in epithelial cells.

Résumé

L'activation des récepteurs transmembranaires, qui sont présents à la surface cellulaire, par différents signaux extracellulaires, génère des réponses biologiques distinctes. Les protéines d'arrimage, dont fait partie la famille de protéines Gab (Gab1, Gab2 et Gab3), jouent un rôle important dans la coordination des signaux en aval des récepteurs transmembranaires. Suite à l'activation de récepteurs tyrosine kinase (RTK), de récepteurs cytokine ou de récepteurs antigène, les protéines d'arrimage sont recrutées au récepteur et certaines tyrosines des protéines d'arrimage deviennent phosphorylées, formant des sites de liaison pour de multiples protéines impliquées dans la signalisation cellulaire. De cette manière, les protéines d'arrimage diversifient et localisent les signaux en aval des récepteurs par leur capacité d'assembler des complèxes de protéines.

Le recrutement des protéines d'arrimage au RTK dépend de leur capacité d'interagir directement ou indirectement avec le récepteur. Dans le deuxième chapitre de la thèse, j'ai établi que les protéines Gab1 et Gab2 peuvent être recrutées aux RTK indirectement, grâce à l'association constitutive de Gab1 et Gab2 avec le domaine SH3 C-terminal de la protéine Grb2. Cette interaction nécessite deux sites de liaisons pour Grb2, qui sont conservés dans les protéines Gab. Le premier site de liaison correspond à un motif canonique de liaison de domaine SH3 alors que le second site de liaison contient un motif atypique PXXXRXXKP. J'ai aussi identifié ce motif atypique dans la protéine Slp-76, qui s'associe aussi à Grb2, mais qui ne fait pas partie de la famille de protéines Gab.

Contrairement aux autres protéines Gab, Gab1 peut aussi intéragir avec le récepteur Met/Hepathocyte growth factor indépendamment de la protéine Grb2. Dans le quatrième chapitre, j'établis que cette intéraction nécessite l'intégrité structurelle du récepteur Met, la phosphorylation de la tyrosine 1349 située dans la partie C-terminale du récepteur Met, ainsi qu'un motif de 13 acides aminés présent dans Gab1 que j'ai nommé le "Met Binding Motif" (MBM). Au lieu de l'intéraction anticipée d'un domaine de liaison aux phosphotyrosines présent dans Gab1 avec un motif contenant une phosphotyrosine dans le récepteur Met, je propose que le domaine kinase actif et la charge négative de la phosphotyrosine 1349 du récepteur Met intéragissent avec le domaine MBM de Gab1, qui agirait tel un ligand peptidique linéaire.

En réponse à la stimulation du récepteur Met, la surexpression de Gab1 induit un programme d'invasion morphogénique dans les cellules epitheliales. Dans le troisième chapitre, j'ai démontré que, contrairement à Gab1, la surexpression de Gab2 n'induit pas ce programme d'invasion morphogénique. La mutation du domaine MBM dans Gab1 abolie la capacité de Gab1 à induire la morphogénèse épithéliale, tandis que l'insertion du domaine MBM de Gab1 dans Gab2 augmente l'association de Gab2 avec le récepteur Met. En revanche, cette intéraction ne confère pas à Gab2 la capacité d'induire la morphogénèse épitheliale. Ceci indique que l'association des protéines Gab au récepteur Met indépendamment de Grb2 est nécessaire, mais pas suffisante pour induire la morphogénèse épitheliale.

Dans l'ensemble, ces études m'ont permis d'identifier à la fois un mécanisme commun ainsi qu'un mécanisme unique par lequel les RTK recrutent les protéines d'arrimage Gab. J'ai, de plus, établi que Gab1 et Gab2 ne partagent pas de fonctions biologiques redondantes dans les cellules épithéliales.

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Preface

The guidelines concerning thesis preparation issued by the Faculty of Graduate Studies and Research at McGill University reads as follows:

As an alternative to the traditional thesis format, the dissertation can consist of a collection of papers of which the student is an author or co-author. These papers must have a cohesive, unitary character making them a report of a single program of research. The structure for the manuscript-based thesis must conform to the following:

1. Candidates have the option of including, as part of the thesis, the text of one or more papers submitted, or to be submitted, for publication, or the clearly-duplicated text (not the reprints) of one or more published papers. These texts must conform to the "Guidelines for Thesis Preparation" with respect to font size, line spacing and margin sizes and must be bound together as an integral part of the thesis. (Reprints of published papers can be included in the appendices at the end of the thesis.)

2. The thesis must be more than a collection of manuscripts. All components must be integrated into a cohesive unit with a logical progression from one chapter to the next. In order to ensure that the thesis has continuity, connecting texts that provide logical bridges preceeding and following each manuscript are mandatory.

3. The thesis must conform to all other requirements of the "Guidelines for Thesis Preparation" in addition to the manuscripts.

I have chosen to write my thesis according to these guidelines, with two published papers and manuscript in preparation. The thesis is organized into six chapters: (I) a general introduction and literature review, (II-IV) manuscripts, each with their own abstract, introduction, methods, results and references, (V) a general discussion of all results with references, and (VI) claims to original research.

Publications arising from work of the thesis

Lock, L.S., Royal, I., Naujokas, M.A., and Park, M. (2000) *Identification of an atypical Grb2 carboxy-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.

Lock, L.S., Maroun, C.R., Naujokas, M.A., and Park, M. (2002) *Distinct recruitment and function of Gab1 and Gab2 in Met-receptor mediated epithelial morphogenesis.* Mol. Biol. Cell, 13, 2132-2146.

Lock, L.S., Frigault, M., Saucier, C. and Park, M. *Grb2-independent recruitment of Gab1 requires the C-terminal lobe and structural integrity of the Met receptor kinase domain.* (Submitted to Molecular and Cellular Biology, December 2002).

The specific contributions of various authors to these manuscripts are as follows:

In manuscript one, I.R. performed the microinjections of MBD constructs into MDCK cells, and took the photographs. M.A.N. generated stable Gab1(Grb2 MDCK cell lines, and performed morphogenesis assays.

In manuscript two, C.R.M. initially analyzed the ability of Gab2-overexpressing MDCK cell lines to form branching tubules, while M.A.N. generated stable MDCK cell lines of various Gab1 and Gab2 mutants, and performed morphogenesis assays.

In manuscript three, M.F. helped in cloning of the Met C-terminal truncation mutants (Figure 3D), and the Tyr to Glu mutants (Fig. 5). In addition, M.F. performed the GST-Grb2 pulldown, Gab1 pTyr, and transformation experiments with Tpr-Met Tyr to Glu mutants (Fig. 5 B and C), and created the Met receptor tyrosine kinase used in the model in Fig.7. C.S. created the Tpr Met A and B mutants used in Fig. 3C.

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1. Introduction

One way in which cells respond to changes in their extracellular environment is through the reversible phosphorylation of proteins. The simplicity and flexibility of phosphorylation, coupled with the ready availability of ATP as a phosphoryl donor, explains its selection as the most general regulatory device adopted by eukaryotic cells. Phosphorylation and dephosphorylation, catalyzed by protein kinases and proteins phosphatases, can modify the function of a protein in almost every conceivable way, and has been shown to regulate nearly every aspect of cell life.

Cells respond to changes in the amount of growth factors in the extracellular environment through activation of transmembrane receptor tyrosine kinases (RTKs). RTKs are important regulators of intracellular signaling pathways involved in normal cellular processes. Their activity is normally tightly controlled and regulated. However, when mutated or altered structurally, RTKs can become potent onco-proteins, and it is well established that deregulated activation of RTKs is associated with human tumorigenesis.

2. Receptor tyrosine kinases

A large family of cell surface receptors, termed receptor tyrosine kinases (RTKs), possess intrinsic protein tyrosine kinase activity, and catalyze the transfer of the γ-phosphate of ATP to hydroxyl groups of tyrosines on target proteins, including themselves (Hunter, 1998). RTKs play an critical role in the regulation of fundamental processes including initiation of cell cycle, cell migration, cell metabolism, survival, cell proliferation and differentiation (Schlessinger, 2000).

2.1 Structure of receptor tyrosine kinases

At present, more than 520 protein kinases are encoded in the human genome, including 90 genes for protein tyrosine kinases. Of these, 32 encode cytoplasmic, non-receptor tyrosine kinases, while 58 encode transmembrane RTKs. RTKs can be divided into 20 subfamilies, based on the ligands they recognize, the biological responses they induce, and according to their primary structures (Fig. 1) (Blume-Jensen and Hunter,

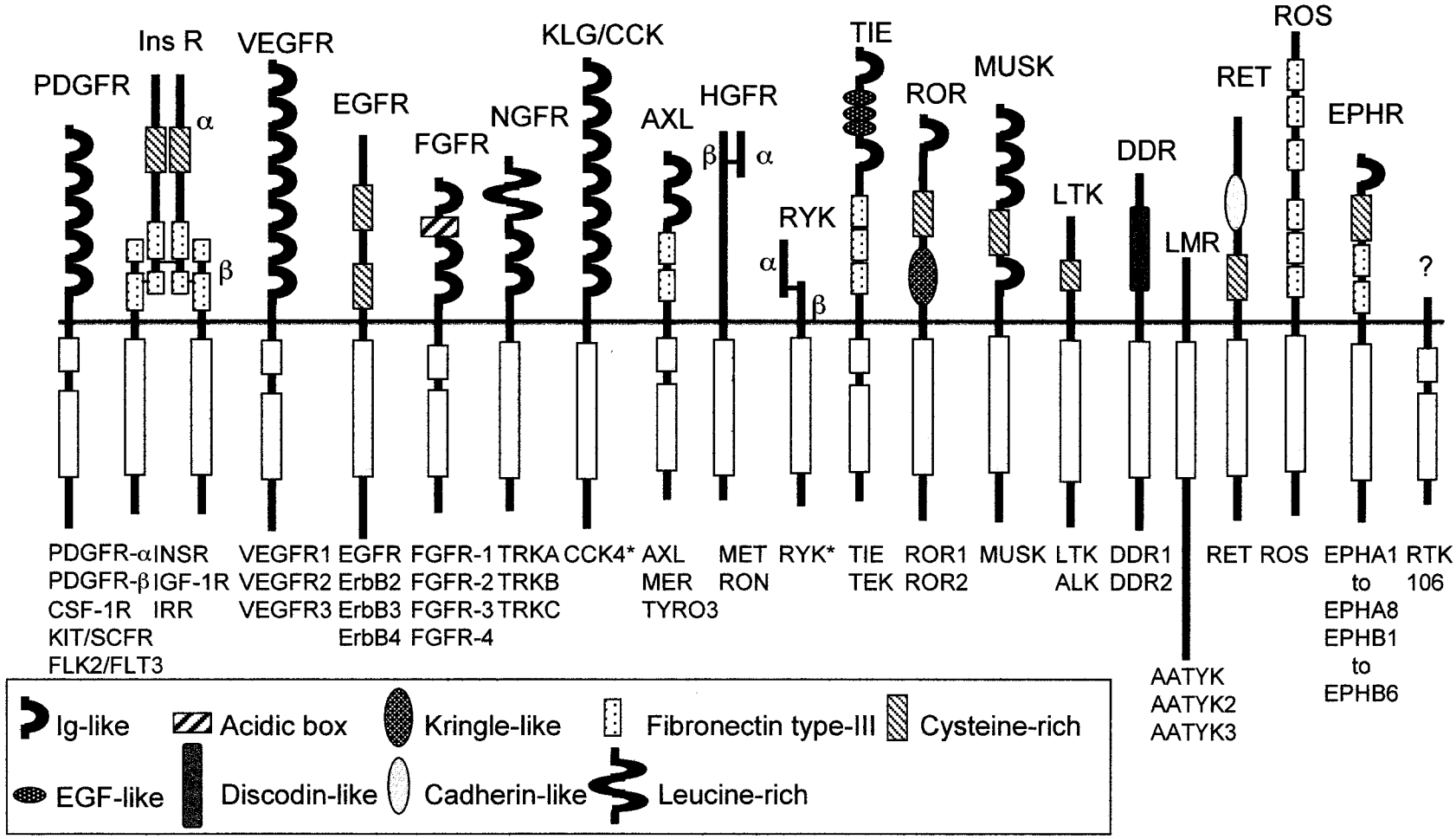

Fig. 1. Human receptor protein-tyrosine kinases. Abbreviations: PDGFR, platelet-derived growth factor; InsR, Insulin receptor; VEGFR, vascular endothelial growth factor receptor; EGFR, epidermal growth factor receptor; FGFR, fibroblast growth factor receptor; NGFR, nerve growth factor receptor; KLG/CCK, colon carcinoma kinase; AXL, a Tyro3 PTK; HGFR, hepatocyte growth factor receptor; RYK, receptor related to tyrosine kinases; TIE, tyrosine kinase receptor in endothelial cells; ROR, receptor orphan; MuSK, muscle-specific kinase; LTK, leukocyte tyrosine kinase; DDR, discoidin domain receptor; LMR, Lemur; RET, rearranged during transfection; ROS, RPTK expressed in some endothelial cell types; EphR, ephrin receptor. An asterisk indicates a lack of intrinsic tyrosine kinase activity.

2001). All RTKs consist of an extracellular ligand-binding domain, a single transmembrane helix and an intracellular domain. The intracellular domain contains a highly conserved protein tyrosine kinase domain, as well as multiple potential sites for tyrosine phosphorylation.

The protein kinase domain is extremely well conserved among serine/threonine and tyrosine kinases, and consists of two subdomains. The N-terminal lobe is composed of a five-stranded β-sheet (β1-5) and one α-helix (helix αC), while the larger C-terminal lobe contains at least two β strands (β7/β8) and 7-8 α helices (αD, αE, αEF, αF -αI/αJ). ATP is coordinated primarily by the N-terminal lobe, while substrate peptide binding and catalysis are performed by residues in the C-terminal lobe (reviewed in (Johnson *et al.*, 1996)).

2.2 Activation of receptor tyrosine kinases

The activation of RTKs is a multi-step process in which the receptor must be converted from an inactive to an active state. This can be separated into four events: (1) binding of ligand to the receptor, (2) dimerization/oligomerization of receptor molecules, (3) transphosphorylation of tyrosine residues within the kinase domain of dimerized/oligomerized receptors, and (4) phosphorylation of tyrosine residues outside of the kinase domain (Fig. 2).

2.2.1 Ligand binding and dimerization

With the exception of the insulin receptor (IR) family of RTKs, all known RTKs are monomers in the cell membrane. Ligand binding to the extracellular domains of receptors induces dimerization or oligomerization, resulting in receptor activation through auto-phosphorylation of their cytoplasmic domains (Schlessinger, 1988; Lemmon and Schlessinger, 1994; Jiang and Hunter, 1999).

Different ligands employ different strategies for inducing the active dimeric state of receptors. For example, vascular endothelial growth factor (VEGF) is a disulfide-linked homodimer that binds two VEGF receptor molecules (Wiesmann *et al.*, 1997), while stable dimerization of fibroblast growth factors receptors (FGFRs) requires two

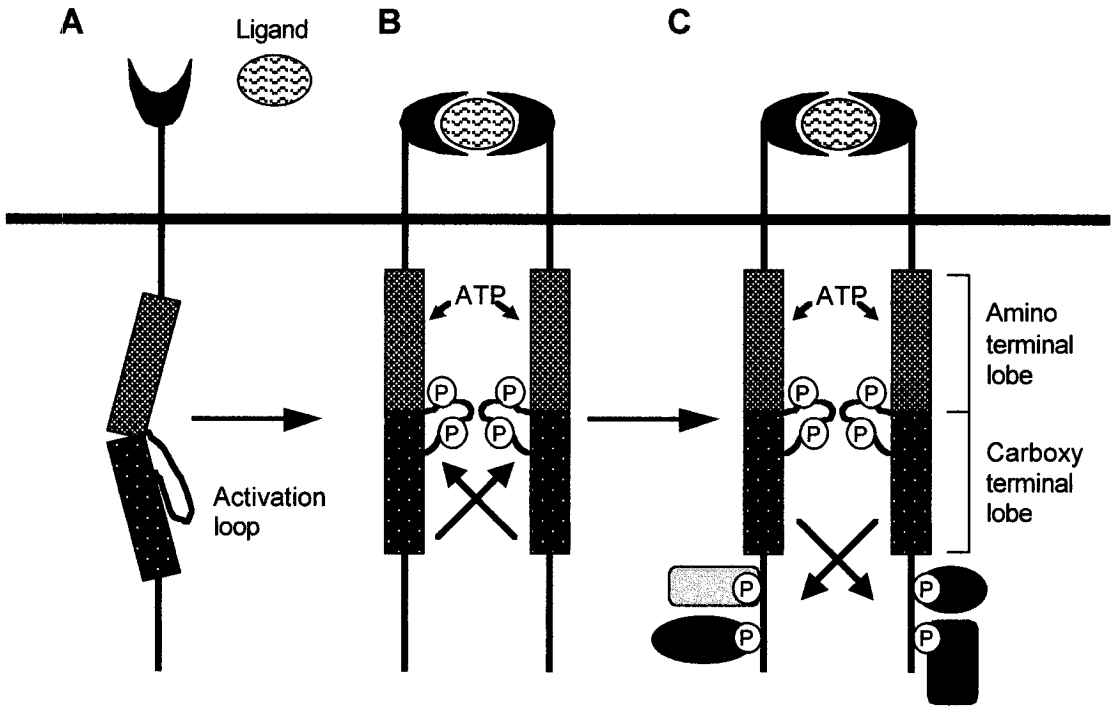

Fig.2. Mechanism of activation of receptor tyrosine kinases. (A) Ligand binding to the receptor tyrosine kinase leads to a conformational change, leading to phosphorylation of active loop tyrosines and stabilization of this loop in a non-inhibiting conformation. (B) Once in the active conformation, the receptor is able to accommodate Mg^{2+}ATP and substrate binding. (C) Phosphorylation occurs on additional tyrosine residues, providing binding sites for signaling proteins.

monomeric FGF proteins in addition to an accessory molecule, heparin sulfate proteoglycan (HSPG) (Spivak-Kroizman *et al.*, 1994; Schlessinger *et al.*, 2000). Dimerization of the epidermal growth factor (EGF) receptor appears to occur via a "receptor-mediated" mechanism, where the binding of ligand to EGFR induces a conformational change that exposes a receptor-receptor interaction site in the extracellular domain. This results in a 2:2 EGF:EGFR complex formed from stable intermediates of 1:1 EGF:EGFR complexes (Lemmon *et al.*, 1997; Garrett *et al.*, 2002; Ogiso *et al.*, 2002). In contrast, members of the insulin receptor family exist as disulfide-linked dimers of two polypeptide chains, forming an $\alpha_2\beta_2$ heterodimer (Van Obberghen, 1994). Binding of ligand induces a conformational change that leads to increased autophosphorylation of the cytoplasmic portion of the receptor, allowing for its activation (Soos and Siddle, 1989) (Hubbard *et al.*, 1998).

Activation of receptor tyrosine kinases has also been shown to occur in the absence of ligand binding, through overexpression of the receptor, or with inhibitors of protein tyrosine phosphatases. This suggests that inactive and active receptor dimers may exist in equilibrium with receptor monomers, and ligand binding may act to stabilize the active dimers and subsequent kinase activity (Schlessinger, 2000).

2.2.2 Kinase activation

Receptor dimerization is followed by receptor "autophosphorylation", which mainly occurs by one receptor molecule phosphorylating the other in the dimer (Ullrich and Schlessinger, 1990). Phosphorylation of tyrosine residues within the kinase domain has been shown to be required for stimulation of kinase activity and biological function of many receptors. A large flexible loop in the N-terminal lobe, called the activation loop, is believed to regulate kinase activity through its ability to undergo large conformational changes when the kinase switches between inactive and active states (Johnson *et al.*, 1996). In almost all kinases, the conformation of the activation loop is controlled by autophosphorylation of specific residues within the loop (Johnson *et al.*, 1996). Autophosphorylation of the activation loop stabilizes it in an open and extended conformation via interactions with positively charged residues. This permits access to ATP and substrates, and enables phosphotransfer from Mg^{2+}-ATP to tyrosines on the

receptor itself and on cellular proteins involved in signal transmission (Hubbard *et al.*, 1994; Hubbard, 1997; Hubbard *et al.*, 1998).

Although the catalytic domains of all active protein kinases adopt strikingly similar structures, different classes of kinases have evolved distinct autoinhibitory conformations (Huse and Kuriyan, 2002). For example, the activation loop of the inactive insulin receptor kinase collapses into the active site, blocking binding of both ATP and peptide substrate. Phosphorylation of three tyrosine residues within the activation loop moves it away from the catalytic center, allowing substrate binding and catalysis (Hubbard *et al.*, 1994). In contrast, residues in the activation loop of the FGF-1 receptor interfere with substrate, but not ATP binding (Mohammadi *et al.*, 1996). The crystal structure of the inactive EphB2 receptor shows that the juxtamembrane region adopts a helical conformation that impinges upon the N-terminal lobe of the kinase domain, stabilizing it in a catalytically inactive conformation (Wybenga-Groot *et al.*, 2001). Activation of the EphB2 receptor requires phosphorylation of two tyrosines in the juxtamembrane region, in addition to the activation loop tyrosines (Binns *et al.*, 2000; Dodelet and Pasquale, 2000).

The distinct conformations of different inactive kinases have provided potential targets for therapeutic application. For example, the anti-cancer drug Gleevec binds selectively to an inactive form of Abl kinase, stabilizing the activation loop in a conformation that mimics bound substrate (Schindler *et al.*, 2000).

2.2.3 Activation of downstream signaling pathways

In addition to its central role in the control of protein tyrosine kinase activity, tyrosine autophosphorylation of RTKs is also crucial for the recruitment and activation of a variety of signaling proteins. Most tyrosine autophosphorylation sites are located in non-catalytic regions of the receptor and function as binding sites for SH2 (Src Homology 2) or PTB (Phosphotyrosine Binding) domains. Hence, RTKs must also be considered as platforms for the co-localization of signaling pathways (Pawson and Schlessinger, 1993).

2.2.3.1 Specific outcomes from general signals

Stimulation of different RTKs leads to unique biological responses. For example, NGF (Nerve growth factor) induces neuronal differentiation in PC12 cells, while EGF induces proliferation (Marshall, 1995). However, many studies have shown that different RTKs stimulate similar collections of intracellular signaling pathways. There are two basic models for how unique outcomes could be generated in response to activation of similar signaling pathways downstream of different RTKs. The first model postulates that there are intrinsic differences in the intracellular signaling pathways activated by various RTK that are either quantitative (strength or duration of the signal) or qualitative (a different combination of intracellular pathways being activated) (Simon, 2000). This model is supported by evidence that NGF produces a sustained activation of Map kinase, while EGF induces a transient Map kinase response in PC12 cells. Importantly, experimental manipulations that lengthen the response of Map kinase signaling to EGF can cause EGF signaling to induce neuronal differentiation instead of proliferation (Marshall, 1995).

The second model postulates that specificity is determined by combination of a general RTK signal plus inputs from other signaling pathways and from pre-existing cell or tissue-specific transcription factors or effector proteins. This is supported by studies showing that expression of a constitutively activated Ras protein can substitute for the absence of RTK function in many developmental settings (Tan and Kim, 1999). This infers that the primary role of RTKs is to provide a 'go' signal, and that the biological outcome is strongly dependent on cellular context. For example, FGFR1 plays an important role in control of cell migration in early development. In contrast, stimulation of FGFR1 in neuronal cells leads to cell survival and differentiation, while stimulation of FGFR1 in fibroblasts leads to cell proliferation (Sahni *et al.*, 1999; Schlessinger, 2000). In this model, the primary reason for the existence of so many ligands and RTKs is to allow the temporally and spatially appropriate activation of general RTK intracellular signaling pathways (Simon, 2000).

Two of the most common growth factor-activated signaling pathways are the MAP kinase and PI3K pathways, which will be discussed in further detail below.

2.2.3.2 Ras/MAP kinase signaling cascade

Cells respond to extracellular signals by transmitting intracellular instructions to coordinate appropriate responses. Among the pathways often used to transduce these signals is the highly conserved Ras/mitogen-activated protein kinase (MAPK) cascade. Ras signal transduction pathways link activation of RTKs to changes in gene expression (Marshall, 1995). All RTKs and other cell surface receptors stimulate the exchange of GDP for GTP on the small G-protein Ras. Both biochemical and genetic studies have demonstrated that Ras is activated by the guanine nucleotide exchange factor, Sos, and that the adapter protein Grb2 plays a key role in this activation. Grb2 forms an SH3 domain-dependent complex with Sos that can be recruited to activated RTKs through binding of the Grb2 SH2 domain to specific phosphotyrosines on the receptor. This leads to the translocation of Sos to the plasma membrane, where it is in close proximity with Ras, and can stimulate exchange of GDP for GTP (reviewed in (Schlessinger, 2000)). Membrane recruitment of Sos can also be accomplished by binding of Grb2/Sos to Shc, which can bind to phosphoytrosines on activated receptors through its PTB domain (Margolis, 1999), or through binding of Grb2/Sos complexes to membrane-linked docking proteins such as IRS-1 or FRS2 (Sun *et al.*, 1993; Kouhara *et al.*, 1997). Once in the active GTP-bound state, Ras interacts with several effector proteins such as Raf or PI3K to stimulate numerous intracellular processes, including activation of the MAPK cascade.

The MAPK cascade is a three-kinase module that is found in all eukaryotic organisms and consists of a MAP-kinase-kinase-kinase (MEK kinase, MEKK) that stimulates a MAP-kinase-kinase (MAPKK, MEK) by phosphorylating a key Ser residue in the activation loop. MAPKK then phosphorylates MAPK on a threonine and a tyrosine residue in the activation loop, leading to its activation. The first and best-characterized MAPK cascade consists of Raf (MEKK), MEK1/2, and ERK1/2, and is regulated by Ras (reviewed in (Robinson and Cobb, 1997). The MAPK family also includes the c-Jun NH_2-terminal kinases (JNKs/SAPKs) and p38 MAPKs. While Erk is activated mainly by growth factors and phorbol esters, JNKs and p38 are primarily activated by extracellular stresses such as UV irradiation and osmotic stress, and by inflammatory cytokines. Activated MAP kinases phosphorylate a variety of cytoplasmic

and membrane-linked substrates, and can translocate into the nucleus to phosphorylate and activate transcription factors, leading to a multiplicity of signal-transducing functions. These highly conserved cassettes play an important role in the control of metabolic processes, cell cycle, cell migration and cell shape, as well as in cell proliferation and differentiation (Davis, 2000).

2.2.3.3 Phosphatidylinositol Metabolism and PI3K signaling

Cell surface receptors also initiate intracellular signaling pathways by recruiting cytosolic signaling enzymes such as phosphoinositide 3-kinases (PI3Ks) to the membrane. Class 1A PI3Ks are heterodimers composed of a p110 catalytic subunit and p85 regulatory subunit. The SH2 domains of the regulatory subunit bind to phosphotyrosines on activated receptors or tyrosine phosphorylated docking proteins such as Gab1, leading to activation of the catalytic subunit through a conformational change. Activated PI3K phosphorylates the 3' position of the phosphoinositides, phosphatidylinositol 4-phosphate (PtdIns(4)P) and PtdIns(4,5)P_2 to generate the second messengers PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 (Vanhaesebroeck and Waterfield, 1999).

PtdIns(3,4,5)P_3 mediates membrane translocation of a variety of pleckstrin homology (PH) domain-containing signaling proteins, including the non-receptor protein tyrosine kinases Btk and Itk, the Ser/Thr kinases PDK1 and PKB/Akt, the Arf exchange factor Grp1, the docking protein Gab1, and PLCγ1, among many others (Rameh and Cantley, 1999; Czech, 2000). The binding of proteins to PI3K products ultimately leads to regulation of multiple cellular processes including cell cycle progression, cell growth, cytoskeletal changes, vesicular transport, and cell survival (Vanhaesebroeck and Waterfield, 1999).

2.3 Oncogenic activation of RTKs

Just over half of all known RTKs have been repeatedly found in either mutated or overexpressed forms associated with human malignancies, leading to enhanced or constitutive kinase activity with qualitatively or quantitatively altered downstream signaling. There are four main mechanisms that promote deregulation of RTK activity:

amplification, formation of an autocrine loop, chromosomal translocation and point mutations. These mechanisms all result in relief or pertubation of autoinhibitory mechanisms that ensure the normal repression of catalytic domains (Blume-Jensen and Hunter, 2001).

In human tumors, RTKs are frequently overexpressed through gene amplification or other potential mechanisms that enhance protein translation or stability. Overexpression is thought to increase the local concentration of receptors at the cell surface, leading to amplification of the signal in response to physiological levels of ligand, or to ligand-independent receptor clustering and kinase activation. Overexpression of growth factors can also promote deregulated and excessive activation of RTKs. Significantly, ErbB2, a member of the EGFR family, is amplified in 10 to 30% of breast (Slamon *et al.*, 1987; Slamon *et al.*, 1989), gastric (Houldsworth *et al.*, 1990; Nakajima *et al.*, 1999), and ovarian cancers (Slamon *et al.*, 1989). The overexpression of growth factors or RTKs is frequently associated with poor prognosis (Lamorte and Park, 2001).

A second mechanism of oncogenic activation occurs through the creation of an autocrine loop, where a receptor and its ligand are co-expressed in the same cell, leading to persistent receptor activation. Autocrine loops involving the PDGF receptor and its ligand, PDGF, have been identified in gliomas and astrocytomas (Hermanson *et al.*, 1992), while transforming growth factor-α (TGF-α) or hepatocyte growth factor (HGF) are secreted by carcinomas that express high levels of their respective receptors, EGFR or Met (Derynck *et al.*, 1987; Rong *et al.*, 1993; Jin *et al.*, 1997; Koochekpour *et al.*, 1997).

Oncogenic activation through chromosomal translocation has been reported in human cancers for many RTKs, including TrkA and C, Ret (glial-derived neurotrophic factor), PDGFRβ (platelet-derived growth factor receptor), FGFR1, and FGFR3 (reviewed in (Lamorte and Park, 2001). RTKs activated by gene rearrangements generally encode chimeric proteins, where an unrelated sequence encoding a dimerization motif is fused in frame with the kinase domain of the receptor, leading to constitutive dimerization and activation of the kinase (Rodrigues and Park, 1994). For the Ret receptor, at least 8 common somatic rearrangements result in fusions between the N-terminus of various proteins and the kinase domain of Ret, resulting in papillary thyroid carcinomas (Jhiang, 2000; Blume-Jensen and Hunter, 2001).

A fourth mechanism for oncogenic activation occurs through point mutations that promote ligand-independent dimerization or kinase activation. For example, the loss of a single cysteine residue in the extracellular domain of Ret in the inherited multiple endocrine neoplasia (MEN) type 2A syndrome promotes disulfide bond formation between receptors due to an uneven number of cysteine residues which are normally involved in intracellular disulfide bond formation (Donis-Keller *et al.*, 1993; Mulligan *et al.*, 1993). In addition, more than 30 gain-of-function mutations, either single amino acid changes or small deletions, have been identified in the Kit/Stem cell factor receptor, and are associated with several highly malignant tumors in humans (Blume-Jensen and Hunter, 2001). Mutations associated with gastrointestinal stromal tumors tend to occur in the juxtamembrane region (Blume-Jensen and Hunter, 2001), and likely relieve the repressive effect of this domain on kinase activity (Hubbard *et al.*, 1998). In mast cell/myeloid leukemias and seminomas/dysgerminomas, Asp816 of Kit is frequently mutated to Val or His, respectively. This residue is located within the kinase domain, and is highly conserved between RTKs. Importantly, mutation of the corresponding residue in Met or Ret results in papillary renal and thyroid carcinomas, respectively (Schmidt *et al.*, 1997). This mutation seems to shift the equilibrium of the activation loop in unstimulated RTKs toward the active conformation (Hubbard *et al.*, 1998).

3. Modular interaction domains in signal transduction

Cytoplasmic proteins that convey information from the cell surface to the interior of the cell are often composed of multiple modular domains that have a catalytic function, or serve to mediate interactions with proteins or other molecules including phospholipids, and nucleic acids (Pawson, 1995). Interaction domains play a critical role in the selective activation of signaling pathways, through their ability to recruit target proteins to activated receptors, and to regulate the subsequent formation of signaling complexes at appropriate subcellular locations (Pawson and Scott, 1997) (Kuriyan and Cowburn, 1997). In addition to regulating signaling from cell surface receptors, interaction domains are also important in the regulation of other cellular processes such as protein trafficking and degradation, cell-cycle progression, cell survival, polarity, gene expression and DNA repair (Pawson *et al.*, 2002).

The earliest interaction domains described included the SH2, SH3, PTB and PH domains, which will be discussed below. These are often found in conjunction with other modular domains, and are embedded in functionally different proteins including enzymes, docking proteins, adapters, transcription factors, and regulators (Pawson *et al.*, 2001). Possible outcomes after interaction of a domain with a ligand include the formation of new signaling complexes, alteration of the subcellular localization of a protein and possible binding partners, alteration of the activity of a protein, as well as induction of a conformational change.

Modular domains have several common features. They are generally 40 to 150 amino acids in length and form compact units that maintain their structures in isolation. The N- and C-termini are generally close together in space and on the opposite side of the domain from the ligand-binding surface, presumably facilitating their integration into surface-exposed regions of their host proteins, while preserving their ligand binding ability. Conserved residues tend to be directly involved in contacts with the ligand or in maintaining the structure of the domain. Importantly, interaction domains are designed to interact with exposed features of their binding partners (Pawson, 1995).

3.1 SH2 domains

Src Homology 2 (SH2) domains are compact modules of approximately 100 amino acids that recognize phosphorylated tyrosines within short, specific peptide sequences. The discovery that SH2 domains provide phosphorylation-dependent and sequence-specific contacts for assembly of receptor signaling complexes provided a breakthrough in understanding signal transduction (Cantley *et al.*, 1991; Koch *et al.*, 1991; Sachs *et al.*, 2000). It is estimated that 111 SH2 domains are present in the human genome, found in proteins with diverse functions such as regulation of protein/lipid phosphorylation, transcriptional regulation, cytoskeletal organization and phospholipid metabolism (Pawson *et al.*, 2002).

SH2 domains were first observed to bind to phosphotyrosine-containing motifs in activated growth factor receptors and cytoplasmic phosphoproteins. The specificity for this interaction is provided by recognition of three to five residues C-terminal to the tyrosine (reviewed in (Pawson *et al.*, 2002)). The majority of the binding energy comes

from association with the phosphotyrosine, although the C-terminal amino acids can increase the affinity by three orders of magnitude (Felder *et al.*, 1993; Panayotou *et al.*, 1993; Piccione *et al.*, 1993). Sequence specific motifs that are selected by various SH2 domains have been identified through oriented peptide-library screening (Songyang *et al.*, 1993; Songyang *et al.*, 1994).

Three-dimensional structures of multiple SH2 domains has revealed a common fold of a seven-stranded β-meander comprised of a three- stranded β-sheet and a four-stranded β-sheet that are linked by a single β-strand (Fig.3A). The four-stranded β-sheet is flanked on either end by α-helices (reviewed in (Yaffe, 2002). The phosphotyrosine moiety projects into a deep pocket on one side of the sheet, where it is coordinated by a bidentate interaction with an invariant arginine residue. An extended surface on the other side of the sheet binds the residues C-terminal to the tyrosine (Eck *et al.*, 1993; Waksman *et al.*, 1993). Variations in the extended surface are responsible for ligand-binding specificity, as shown by mutation of a threonine residue to a tryptophan in the Src SH2 domain. This converted the binding specificity from the Src-like pY-E-E-I to Grb2-like pY-X-N-X. The mutant Src SH2 domain behaves biologically like a Grb2 SH2 domain, indicating that biological activity correlates with biochemical binding specificity (Kimber *et al.*, 2000).

3.2 Phosphotyrosine binding (PTB) domains

The PTB domain was the second described phosphotyrosine interaction module and was identified in the adapter protein Shc as a binding region for phosphorylated tyrosines on activated receptor tyrosine kinases (Blaikie *et al.*, 1994; Kavanaugh and Williams, 1994; van der Geer *et al.*, 1995). This region lacked sequence similarity with SH2 domains and had a different binding specificity for residues surrounding the phosphotyrosine. A second PTB domain was later identified on the basis of a phosphotyrosine-binding function in the docking protein IRS-1 (Gustafson *et al.*, 1995). In all, 24 PTB domain-containing proteins are present in the human genome (Yaffe, 2002).

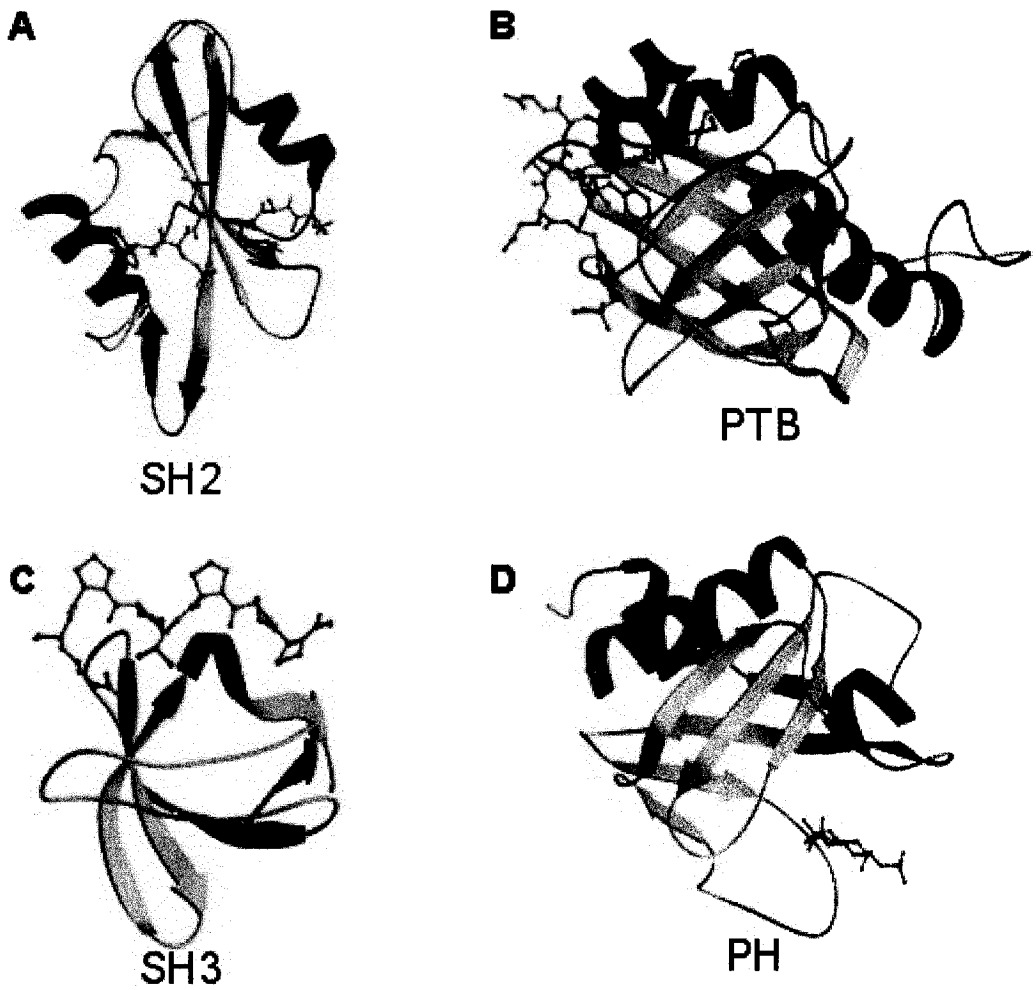

Fig. 3: Structures of modular signaling domains.

(A) The SH2 domain of v-src bound to a pYEEI peptide ligand, (B) the PTB domain of Shc complexed to a HIIENPQpYFS peptide, (C) the Sem5 C-terminal SH3 domain complexed to the mSos-derived sequence PPPVPPRRR, (D) the PH domain of Phospholipase C-δ and inositol-(1,4,5)-trisphosphate.

PTB domains can be divided into two categories: Shc-like and IRS-1 like. These subfamilies have little primary sequence homology, yet adopt a remarkably similar structure. The three-dimensional structures of all PTB domains determined thus far adopt a Pleckstrin homology (PH)-like fold (Fig. 3B). The PH domain is a membrane-targeting domain that binds to phospholipid head groups and will be discussed further in section 3.3. The PH-like fold consists of a β-sandwich containing two nearly orthogonal, antiparallel β-sheets capped at one end by a C-terminal α-helix (reviewed in (Margolis, 1999) (Yaffe, 2002; Yan *et al.*, 2002). The major ligand-binding site is located within an elongated cleft formed by β-5 and α-3. Differences in ligand binding specificity among PTB domains are due to variations of structural features that lie outside of the basic scaffold. The PH-like fold is also seen in other functionally distinct protein interaction domains including the EVH1 domain that binds proline-rich sequences, the Ran binding domain that interacts with Ran-GTPase, and lobe F3 of the FERM domain that functions in protein localization to the plasma membrane (reviewed in (Yan *et al.*, 2002).

In contrast to SH2 domains that recognize sequences C-terminal to the phosphotyrosine, the binding specificity of PTB domains is conferred by sequences N-terminal to the phosphotyrosine. The PTB domains of Shc and IRS-1 specifically interact with phosphopeptides containing an NPXpY motif (where N is asparagine, P is proline, X is any amino acid, and pY is phosphotyrosine) (Batzer *et al.*, 1995; Kavanaugh *et al.*, 1995; Trub *et al.*, 1995). The NPXpY motif of the phosphopeptide forms a type I β-turn that is a crucial component in the interaction. Residues amino terminal to the NPXpY motif form an antiparallel β-strand that undergoes hydrogen bonding with the β-5 strand of the PTB domain in a hydrophobic groove between β-5 and the carboxy-terminal α-helix (Trub *et al.*, 1995). Unlike SH2 domains, arginine residues that participate in phosphotyrosine recognition are conserved only in three-dimensional space, and not in the primary sequences of the Shc and IRS-1 PTB subfamilies.

Interestingly, several PTB domains have been found to specifically bind targets independent of tyrosine phosphorylation or even the canonical NPXY motif. For example, the PTB domains of the neuron-specific X11 proteins and Fe65 both bind to a

peptide derived from a region of the β-amyloid precursor protein (β-APP) containing a non-phosphorylated NPTY sequence (Borg *et al.*, 1996; Zambrano *et al.*, 1997). The PTB domain of the *Drosophila melanogaster* cell fate-determinant protein, Numb, has a broad binding specificity, recognizing the sequence GFSNMSFEDFP in the Ser/Thr protein kinase, Nak, a GPY motif identified through peptide library screening, in addition to binding NPXY-containing sequences (Li *et al.*, 1997; Chien *et al.*, 1998; Dho *et al.*, 1998; Li *et al.*, 1998; Zwahlen *et al.*, 2000). Consequently, the name "phosphotyrosine binding" is an inaccurate representation of a family with such broad ligand binding specificities and the domain is instead considered a generalized protein interaction module.

3.3 SH3 domains

Src homology 3 (SH3) domains are modules of approximately 60 amino acids that were first noted as regions of similarity between divergent signaling proteins such as the Src family tyrosine kinases, the Crk adapter protein and phospholipase C-γ (Mayer *et al.*, 1988; Stahl *et al.*, 1988). This small domain was found to bind to proline-rich sequences, specifically those containing a core conserved binding motif, PxxP, where P is proline and x is any amino acid (Ren *et al.*, 1993).

All SH3 domains consist of two small β-sheets that are packed against each other at approximately right angles (Fig. 3C). The ligand-binding surface of the SH3 domain is relatively flat and hydrophobic, consisting of three shallow pockets containing conserved aromatic residues. The PxxP-containing ligand adopts an extended left-handed helical conformation termed the polyproline II (PP-II) helix, with the two critical prolines located on the same face of the helix. The PPII helix is roughly triangular in cross section and the base sits on the ligand-binding surface of the SH3 domain. Two of the pockets accommodate a proline residue plus a hydrophobic residue, whereas the third pocket generally interacts with a positively charged residue in the ligand distal to the PxxP core. This residue provides some specificity for the interaction (reviewed in (Kay *et al.*, 2000; Mayer, 2001).

Peptide ligands can bind in two orientations, based on the location of the positively charged residue, which forms a salt bridge with residues in the third pocket.

Class I peptides have the consensus +xxPxxP, where + generally represents a basic residue, and bind in an N to C orientation, whereas Class II peptides have the consensus xPxxPx+, and bind in a C to N orientation (reviewed in (Mayer and Eck, 1995)).

Recently, however, multiple SH3 domains have been identified that do not interact with canonical PXXP motifs. These include the Pix SH3-binding site in PAK (PPPVIAPRPETKS) (Manser *et al.*, 1998), the Eps8 SH3 binding consensus (PxxDY) (Mongiovi *et al.*, 1999), and the Grb2/Gads C-terminal SH3 domain binding consensus in the Gab family of docking proteins (Px(V/I)(D/N)RxxKP (Lock *et al.*, 2000; Schaeper *et al.*, 2000; Lewitzky *et al.*, 2001).

In comparison to the phosphorylation-dependent regulation of SH2 domain interactions, SH3 domain-ligand interactions are generally considered to be constitutive (Ren *et al.*, 1993), although serine/threonine phosphorylation of the ligand can regulate such interactions (Zhao *et al.*, 2000). In addition, the affinity of SH3 domains for their ligands is quite low, relative to SH2 domains. Fast on and off-rates may be an advantage in this case, allowing for rapid remodeling of interactions in response to changes in the environment. Indeed, SH3 domain-proline interactions are commonly found in situations requiring the rapid recruitment or interchange of several proteins, such as during the initiation of transcription, signaling cascades, and cytoskeletal rearrangements (Kay *et al.*, 2000). Hence, the principle role of SH3 domain is not to provide a structurally defined complex, but rather to bring proteins together in such a way that subsequent interactions are more probable. This can occur at defined subcellular sites, frequently in conjunction with other modular domains (Cohen *et al.*, 1995; Pawson, 1995).

The most prominent example of a biologically-significant SH3-ligand interaction comes from the adapter protein Grb2. Grb2 is composed of an SH2 domain that binds to phosphorylated tyrosines on activated receptors or cytoplasmic proteins, while one of the binding partners for its SH3 domains is Sos, the guanine-nucleotide-exchange factor (GEF) for the small GTPase Ras. These SH2 and SH3-mediated interactions lead to the recruitment of Sos to the plasma membrane where Ras is localized, thereby coupling the activation of Ras to changes in tyrosine phosphorylation (McCormick, 1993). Genetic evidence has shown that these interactions are crucial for receptor tyrosine kinases to activate the Ras/Map kinase pathway in vivo, and that this is required for normal

embryonic development, postnatal function and tumor formation (Clark *et al.*, 1992; Olivier *et al.*, 1993; Simon *et al.*, 1993; Cheng *et al.*, 1998; Saxton *et al.*, 2001).

3.4 PH domains

Pleckstrin homology (PH) domains were first noted in multiple intracellular signaling proteins as regions containing limited sequence similarity to the protein pleckstrin (Mayer *et al.*, 1993) (Haslam *et al.*, 1993). These modules of approximately 120 amino acids have been identified in at least 100 proteins of diverse function, and are often present in conjunction with other interaction domains including SH2, PTB, and SH3.

PH domains bind to phosphoinositides, which are minor constituents (10%) of cell membranes (Toker, 2002). They function to target their host proteins to membranes, through specific, high-affinity recognition of phosphoinositide head groups (Kavran *et al.*, 1998). The concerted action of phosphoinositide kinases and phosphatases generates multiple polyphosphoinositides phosphorylated at the 3, 4 and 5 positions either alone or in combination. PH domains can be divided into four categories based on their phosphoinositide binding affinities.

PH domains that bind with high specificity to PtdIns(3,4,5)P_3 (PIP_3) or PtdIns(3,4)P_2 have been of particular interest in recent years because of their role in transmitting growth and survival signals downstream of PI3K. Phosphatidylinositol 3-kinases (PI3Ks) function to generate intracellular second messengers by phosphorylating the 3'-position of the inositol ring of phosphoinositides (Toker and Cantley, 1997; Vanhaesebroeck *et al.*, 1997; Downward, 1998). Their major lipid product is PIP_3, which is produced during cell stimulation by various mitogens (Leevers *et al.*, 1999; Rameh and Cantley, 1999). The serine/threonine protein kinase Akt/PKB is activated by PI3K and has been shown to mediate the anti-apoptotic response by PI3K. Akt is regulated through binding of its PH domain to the PI3K products, PIP_3 or PtdIns(3,4)P_2, which relocalizes it from the cytosol to the plasma membrane, where it can be phosphorylated and activated by the protein kinase 3-phosphoinositide-dependent kinase (PDK1) (Alessi *et al.*, 1997; Dudek *et al.*, 1997; Downward, 1998; Stephens *et al.*, 1998).

While PH domains have limited amino acid homology (often less than 10%) and ligand-binding specificity, their core three-dimensional structures are very similar. They are composed of seven β-strands, which form two almost orthogonal β-sheets capped by an α-helix (Fig. 3D). The loop regions between β1/β2, β3/β4 and β6/β7 are of variable length and sequence, and are critical for determining the phosphoinositide-binding specificity. For example, a 20-residue insertion within the β6/β7 loop in the Grp1 PH domain accounts for the ability of this PH domain to selectively bind PtdIns(3,4,5)P_3, but not PtdIns(3,4)P_2 with high affinity (Ferguson *et al.*, 2000; Lietzke *et al.*, 2000). As mentioned previously, this type of fold is quite common in nature, being found in other functionally distinct interaction domains including the PTB and EVH1 domains.

3.5 Other recognition domains

Post-translational modification appears to be appears to be a rather general means of regulating protein-protein interactions. In addition to phosphotyrosine-interacting domains, recognition domains for phosphoserine/threonine (14-3-3 proteins; FHA domains) (Muslin *et al.*, 1996; Durocher *et al.*, 1999; Durocher *et al.*, 2000; Tzivion *et al.*, 2001); as well as acetylated or methylated lysine (bromo and chromo domains)(Owen *et al.*, 2000; Bannister *et al.*, 2001; Marmorstein, 2001) have also recently been identified.

Due to the accessibile nature of proline-rich sequences in proteins, domains that interact with proline-rich ligands are also quite common. WW domains are small modules of 35 to 40 residues that commonly bind to proline-rich motifs, with the consensus PPXY or PPLP (Macias *et al.*, 1996; Sudol, 1996), while the GYF domain of CD2BP2 forms a compact domain with a β-β-α-β-β topology that recognizes a proline-rich sequence in the cytoplasmic tail of the T cell surface glycoprotein CD2 (Freund *et al.*, 1999).

In addition to PH domains, there is a growing family of domains that recognize phospholipids. These include the PX, Tubby, ENTH and Ferm domains (Cullen PJ, 2001 Curr Biol; Santagata, S, 2001 Science), as well as the FYVE domain. This domain is composed of two small β-sheets, with two zinc coordination centers. It specifically recognizes PtdIns(3)-P, and is found in multiple proteins (Stenmark *et al.*, 1996).

The number of known interaction domains is still increasing, and the remainder fall out of the scope of this review.

4. Hepatocyte growth factor/Met receptor signaling

Hepatocyte growth factor/scatter factor (HGF/SF) is produced mainly by mesenchymal cells, and is an effector of cells expressing its high-affinity cell surface receptor, the Met tyrosine kinase (Stoker *et al.*, 1987; Sonnenberg *et al.*, 1993). As its name implies, HGF/SF promotes the growth and/or scattering of various cells. Signaling via this ligand-receptor pair has also been shown to mediate other biological functions including survival, invasiveness, and morphogenesis. *In vivo*, induction of these biological responses is required to integrate complex biological processes including embryogenesis, angiogenesis, tissue regeneration and malignant progression of tumor cells (reviewed in (Gherardi and Stoker, 1991; Michalopoulos, 1995; Zarnegar and Michalopoulos, 1995)).

4.1 Hepatocyte growth factor/ Scatter factor

Hepatocyte growth factor (HGF) was originally identified in rat serum as a potent mitogen for cultured hepatocytes and was considered to act primarily as a humoral mediator of liver regeneration after partial hepatectomy or hepatic injury (Nakamura *et al.*, 1986; Zarnegar and Michalopoulos, 1989; Gohda *et al.*, 1990). However, HGF was also independently identified as scatter factor (SF), a fibroblast-derived effector of dissociation and motility events ("scattering activity") in polarized epithelial cells (Stoker *et al.*, 1987; Gherardi *et al.*, 1989; Stoker, 1989; Weidner *et al.*, 1990), in addition to its identification as a tumor cytotoxic factor (Shima *et al.*, 1991) and a fibroblast-derived epithelial growth factor (Rubin *et al.*, 1991). After biochemical purification and cDNA cloning (Miyazawa *et al.*, 1989; Nakamura *et al.*, 1989), HGF and SF were shown to be the same molecule (Naldini *et al.*, 1991b). It is a mesenchymally-derived multifunctional factor that affects a number of cell targets including epithelium, endothelium, myoblasts, spinal motor neurons and hematopoietic cells.

HGF derives from a single-chain, biologically inert glycoprotein precursor (pro-HGF), which is secreted and then sequestered by cell surface and matrix proteoglycans. Under appropriate conditions, it is converted to its bioactive form through limited intramolecular proteolysis (Trusolino *et al.*, 1998). Several proteases are reported to activate HGF in vitro, including urokinase-type (uPa) and tissue-type (tPa) plasminogen activators, a serine protease isolated from serum and homologous to coagulation factor XII, and coagulation factor XII itself (Comoglio and Trusolino, 2002). Mature HGF has 38% overall sequence similarity with plasminogen, and is a heterodimer consisting of a 62-kDa α and a 32-kDa β chain held together by a disulfide bond. The α chain contains a N-terminal hairpin loop followed by four kringle domains (80 amino acid double-looped structures formed by three internal disulfide bridges), whereas the β chain contains an enzymatically inactive serine protease domain (Gherardi and Stoker, 1991) (Fig. 4). The lack of proteolytic activity in HGF is due to the replacement of the histidine and serine residues contained within the catalytic site of serine proteases with glutamine and tyrosine, respectively (Nakamura *et al.*, 1989). Deletion mutants of HGF have shown that the receptor binding domain resides in the first kringle domain, whereas the functional domain responsible for activation of the motogenic response resides within the amino terminus and first two kringle domains. The β chain, although not required for receptor binding, contributes to receptor activation (Lokker *et al.*, 1992; Trusolino *et al.*, 1998).

4.2 The Met receptor

The Met receptor was originally isolated as an oncoprotein in an N-methyl-N'-nitro-N-nitrosoguanidine treated human osteogenic cell line (Cooper *et al.*, 1984). Chromosomal rearrangement fused a leucine zipper dimerization domain from the tpr (translocated promoter region) locus on chromosome 1 to the Met cytoplasmic domain from chromosome 7, generating a constitutively active kinase, Tpr-Met (Vande Woude *et al.*, 1986; Rodrigues and Park, 1993).

The normal cellular counterpart, Met is a receptor tyrosine kinase (RTK) that is synthesized as a single-chain precursor and undergoes intracellular proteolytic cleavage, to yield a mature disulfide-linked heterodimer, comprised of an extracellular 45 kDa α-

subunit and 145 kDa β-subunit that spans the plasma membrane and contains a cytoplasmic region with tyrosine kinase activity (Gonzatti-Haces *et al.*, 1988; Giordano *et al.*, 1989) (Fig. 4). The Met receptor is expressed in normal epithelium of almost every tissue, however other cell types such as melanocytes, endothelial cells, microglial cells,

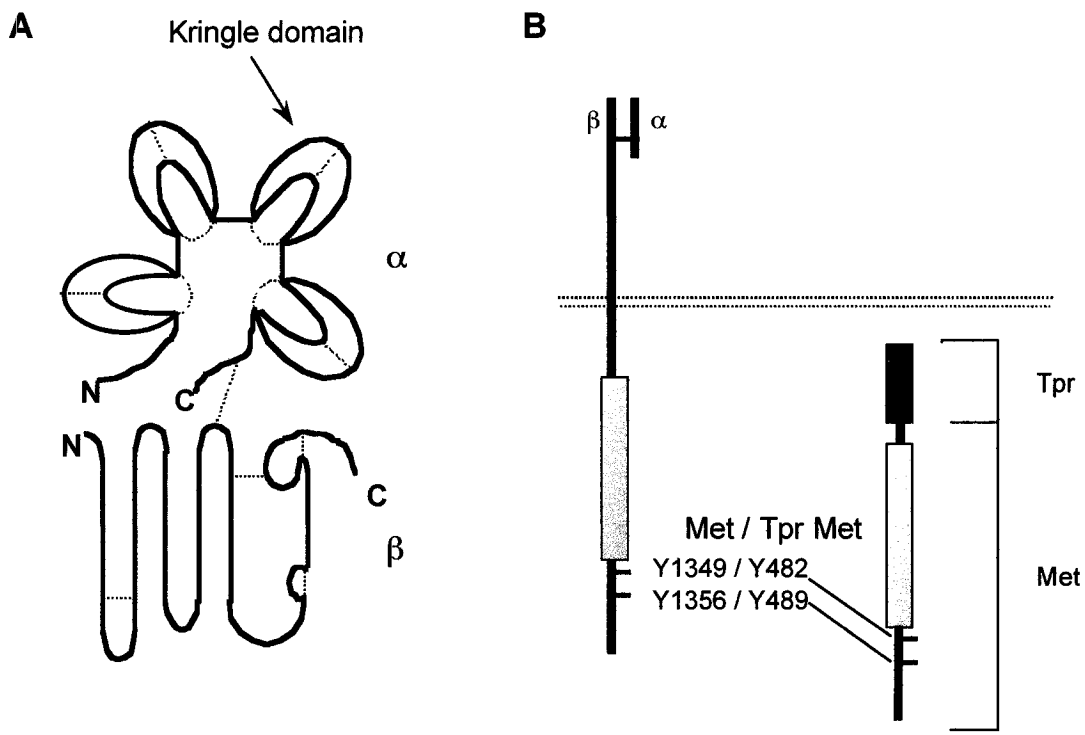

Fig. 4: Structures of HGF, Met recepto r and Tpr Met oncoprotein.

(A) The α-chain of HGF contains four kringle domains, and is disulfide-bonded to the serine protease-like β-chain. (B) Met is a 190 kDa disulfide bonded αβ heterodimer (left). The 65 kDa Tpr Met fusion protein consists of tpr sequences fused to the cytoplasmic portion of the Met receptor (right). Numbering for the conserved tyrosines within the Met receptor are on the left, while numbering used with the Tpr Met oncoprotein is indicated on the right.

neurons, hematopoietic cells and a variety of tumor cell lines of various origins also express this receptor (Zarnegar and Michalopoulos, 1995).

Met belongs to a small subfamily of RTKs that can induce proliferation, cell movement, and morphogenic differentiation. Other members include the mammalian Ron, and the avian Sea receptors. The ligand for Ron is macrophage stimulating protein (MSP) and a chicken homologue of MSP (chMSP) has recently been identified as a ligand for Sea (Gaudino *et al.*, 1994; Wahl *et al.*, 1999). Analogous to HGF/SF-Met signaling, stimulation of Ron with MSP or stimulation of a chimeric Trk-Sea receptor with nerve growth factor can induce mitogenic responses and stimulate cell motility and invasive growth in epithelial cells, suggesting that members of the Met receptor subfamily have a conserved biological function (Medico *et al.*, 1996; Santoro *et al.*, 1996). Interestingly, while Met homologues have been found in several vertebrates, Met family members are noticeably absent from the genomes of the worm *Caenorhabditis elegans* and the fruit fly *Drosphila melanogaster* (Rubin *et al.*, 2000).

The Met receptor has also been found to bind to and become activated by the *Listeria monocytogenes* protein, InlB. Met is required for InlB-dependent entry into mammalian cells, and this interaction represents a novel mechanism of internalization of a microbial pathogen through engagement of a host RTK (Shen *et al.*, 2000).

4.3 Biological functions

4.3.1 Proliferation

The proliferation of cells is critical for a wide variety of physiological processes including embryogenesis, cell growth and wound healing. HGF was originally identified as a potent mitogen for mature hepatocytes in culture (Nakamura *et al.*, 1984), and was later found to induce mitogenesis in a variety of cells *in vitro*, including keratinocytes, tubular epithelial cell lines, osteoblasts, melanoma cells and vascular endothelial cells (Igawa *et al.*, 1991; Kan *et al.*, 1991; Matsumoto *et al.*, 1991a; Matsumoto *et al.*, 1991b; Morimoto *et al.*, 1991; Sato *et al.*, 1995).

The proliferative effect of HGF/Met has also been found to be important in organ regeneration. Following injury of organs such as the liver, lung or kidney, compensatory DNA synthesis occurs due to a sharp increase in HGF levels (Matsumoto and Nakamura, 1993). However, the acceleration of organ reconstruction also depends on the ability of HGF to modulate complex architectural events that contribute to the re-establishment of normal tissue patterning (Trusolino *et al.*, 1998).

4.3.2 Scattering and Motility

Cell motility plays a key role during embryogenesis, angiogenesis, and tissue repair, in addition to pathological conditions of tumor invasion and metastasis. In sheets of epithelial cells, this process can be divided into three phases; cell spreading, breakdown of cell-cell junctions, and cell dissociation resulting in a mesenchymal-type, scattered morphology. Under normal conditions, the assembly and maintenance of intercellular junctions is tightly regulated. HGF/SF is capable of inducing all three responses and was isolated based on its ability to dissociate epithelial sheets (Stoker *et al.*, 1987; Gherardi *et al.*, 1989; Stoker, 1989; Weidner *et al.*, 1990). HGF stimulation of various epithelial and endothelial cell lines in culture results in a mesenchymal-like transition, leading to the breakdown of cell-cell junctions and subsequent cell dispersal (Stoker *et al.*, 1987; Rosen *et al.*, 1990; Matsumoto and Nakamura, 1993). Furthermore, consistent with the scattering activity of HGF, receptor-deficient embryos lack muscles of the limbs, diaphragm, and tip of the tongue, all deriving from migratory precursors (Bladt *et al.*, 1995).

4.3.3 Morphogenesis

The involvement of HGF and Met in epithelial morphogenesis was initially observed by Montesano et al. who noted that the addition of HGF to Madin-Darby canine kidney (MDCK) epithelial cells suspended in a collagen matrix, led to the formation of branching tubules. These effects could be blocked by the addition of antibodies against HGF to the medium (Montesano *et al.*, 1991).

HGF functions as an inductive, rather than instructive, effector of epithelial morphogenesis, inducing inherent, tissue-specific morphogenic activities in a wide variety of epithelial cells. For example, colon carcinoma cells form crypt-like structures, mammary epithelial cells form long branches with end buds that resemble developing mammary ducts, while lung epithelial cells form alveolar-like structures (Brinkmann *et al.*, 1995). HGF-dependent epithelial morphogenesis is based on a finely tuned interplay between related phenomena including cell proliferation, motility, extracellular matrix degradation, and survival (Trusolino *et al.*, 1998).

4.3.4 Survival

The Met signaling system is essential for normal growth and cell survival in several tissues, as shown through gene ablation (Schmidt *et al.*, 1995; Maina *et al.*, 1996) and overexpression studies in transgenic mice (Amicone *et al.*, 1997). *In vitro*, HGF has been found to protect MDCK cells against apoptosis induced by detachment of cells from their substrate (Frisch and Francis, 1994) and can inhibit apoptosis in various cell lines induced by DNA-damaging agents (Fan *et al.*, 1998).

HGF-induced cell survival can occur through intracellular activation of anti-apoptotic signals such as the PI3K-AKT-Bad signaling pathway. However, a novel mechanism for cell survival has also recently been proposed, whereby Met binds to and sequesters the death receptor, Fas, in hepatocytes. This interaction prevents Fas self-aggregation and Fas ligand (FasL) binding, thus inhibiting Fas activation and apoptosis (Wang *et al.*, 2002).

Paradoxically, the HGF-Met signaling system has been reported to induce apoptosis depending on the cell line and setting (Arakaki *et al.*, 1998; Gohda *et al.*, 1998; Arakaki *et al.*, 1999; Conner *et al.*, 1999). Indeed, HGF was independently discovered as a cytotoxic factor that induced death in a sarcoma cell line (Shima *et al.*, 1991).

4.3.5 Development

During embryogenesis, HGF is expressed in mesenchymal tissue while Met is expressed in neighboring epithelial, endothelial or myogenic cells, indicative of a

paracrine mode of action (Sonnenberg *et al.*, 1993; Andermarcher *et al.*, 1996). The importance of HGF and Met in development has been clearly demonstrated by the finding that inactivation of Met or HGF/SF genes in mice lead to an identical phenotype, embryonic lethality between E12.5 and 15.5 caused by severe deficiencies in the development of embryonic liver and placenta (Schmidt *et al.*, 1995; Uehara *et al.*, 1995). Similarly, expression of a dominant negative Met in *Xenopus* embryos resulted in depletion of liver and gross underdevelopment of intestine and kidney (Aoki *et al.*, 1997).

HGF/SF has also been shown to contribute to the development of lung, kidney, muscle, chondrocytes, mammary gland, placenta, and tooth germ, as well as playing an important role in neural development, survival, differentiation and chemoattraction (reviewed in (Kamalati *et al.*, 1999; Maina and Klein, 1999)).

4.3.6 Angiogenesis

The induction of angiogenesis (formation of new blood vessels) occurs during embryogenesis and organ regeneration, and appears to be an important mechanism that permits tumor cell proliferation and eventually metastasis. It is a complex process that requires a delicate balance between the activity of local factors that promote or inhibit neovascularization. Tumor-derived angiogenic factors can stimulate the degradation and invasion of basement membrane, migration of cells to a new site of angiogenesis, and the formation of new blood vessels (Folkman *et al.*, 2001).

It is not surprising then, that the Met receptor and HGF, which are critical regulators of cell mitogenesis, motility and morphogenesis, play an important role in angiogenesis (Bussolino *et al.*, 1992). HGF stimulation of vascular endothelial cells that express the Met receptor promotes migration, proliferation, protease production, invasion, and organization into capillary-like tubes (Bussolino *et al.*, 1992) (Grant *et al.*, 1993; Rosen *et al.*, 1997). Moreover, HGF can also stimulate expression of the angiogenic factors, vascular endothelial growth factor (VEGF) and interleukin (IL)-8 by tumor cells (Dong *et al.*, 2001).

4.3.7 Cancer

In addition to regulating normal functions, Met and HGF are involved in malignant cell transformation. Dysregulation of Met activation in tumor cells has been found to occur through several molecular mechanisms. Met can be activated in a ligand-dependent manner, much as occurs in normal cells. Met-positive tumor cells that do not express HGF may respond to HGF produced by stromal cells in a paracrine fashion, while in some cases, tumor cells express both HGF and its receptor, forming an autocrine loop. Coexpression of Met and HGF in a variety of human and mouse cells leads to highly tumorigenic cells when implanted in nude mice (Bellusci *et al.*, 1994; Rong *et al.*, 1994; Jeffers *et al.*, 1996). Such HGF-Met autocrine loops have been detected in gliomas, osteosarcomas, and mammary, prostate, breast, lung and other carcinomas. They are often associated with malignant progression of tumors, and correlate with poor prognosis (Danilkovitch-Miagkova and Zbar, 2002).

Met may also be activated in a ligand-independent manner, particularly as a result of overexpression. Increased Met expression can be mediated by MET gene amplification, by enhanced transcription, or by post-transcriptional mechanisms. Increased Met expression has been found in papillary carcinomas of the thyroid gland, in carcinomas of colon, pancreas and ovary, in osteogenic sarcomas, and in other types of cancer (Rong *et al.*, 1993; Jin *et al.*, 1997; Koochekpour *et al.*, 1997; Danilkovitch-Miagkova and Zbar, 2002).

Finally, a large class of somatic and inherited mutations in the MET gene can lead to active, typically ligand-independent Met signaling in tumor cells. Missense point mutations in Met have been identified in hereditary and sporadic papillary renal carcinomas, hepatocellular and gastric carcinomas, and head and neck squamous carcinomas (Schmidt *et al.*, 1997; Park *et al.*, 1999; Schmidt *et al.*, 1999). Currently, 21 such mutations have been identified, the majority of which are located within the kinase domain and lead to increased Met kinase activity (Danilkovitch-Miagkova and Zbar, 2002). The identification of overexpressed or deregulated Met receptor molecules in various forms of human tumors suggests that Met may play an important role in the development and progression of human malignancy.

4.4 Met signal transduction

The signaling and biological function of both Tpr-Met and the Met receptor, are dependent on phosphorylation of two tyrosine doublets. Tyrosines 1234 and 1235 are located within the activation loop of the kinase domain and autophosphorylation of these tyrosines functions to greatly enhance the enzymatic activity of the Met receptor (Ferracini *et al.*, 1991; Naldini *et al.*, 1991a; Longati *et al.*, 1994; Rodrigues and Park, 1994). In contrast, tyrosines 1349 and 1356, within the multisubstrate docking site Y^{1349}VHVNATY^{1356}VNV, are localized in the C-terminus of the Met receptor and when phosphorylated, they provide docking sites for signaling proteins that contain SH2 or PTB domains. Although substitution of tyrosines 1349 and 1356 to non-phosphorylatable phenylalanine residues does not affect the kinase activity of the receptor, it abolishes the ability of the receptor to mediate all biological signals both *in vitro* (cell proliferation, motility, invasion and morphogenesis) (Ponzetto *et al.*, 1994; Zhu *et al.*, 1994a; Fixman *et al.*, 1995; Weidner *et al.*, 1995), and *in vivo* (Maina *et al.*, 1996). Mutation of Y1356 alone interferes heavily with all Met mediated events, whereas mutation of Y1349 alone has only a limited effect on transformation and no effect on motility. Chimeric receptors that contain the multisubstrate docking site can induce mitogenic, motogenic and morphogenic responses similar to those observed with the wild-type Met receptor suggesting that this binding site is primarily responsible for Met-mediated signal transduction (Komada and Kitamura, 1993; Weidner *et al.*, 1993; Zhu *et al.*, 1994b).

Phosphorylation of tyrosine 1356 provides a direct binding site for the Grb2 and Shc adaptor proteins, and is required for the recruitment of the p85 subunit of PI3K, Phospholipase C-γ, and the ubiquitin ligase, Cbl (Ponzetto *et al.*, 1994; Fixman *et al.*, 1996; Fournier *et al.*, 1996). In addition, phosphorylation of tyrosines 1349 and 1356 are required for recruitment of the Gab1 docking protein (Weidner *et al.*, 1996; Bardelli *et al.*, 1997; Fixman *et al.*, 1997; Nguyen *et al.*, 1997) (Fig. 5).

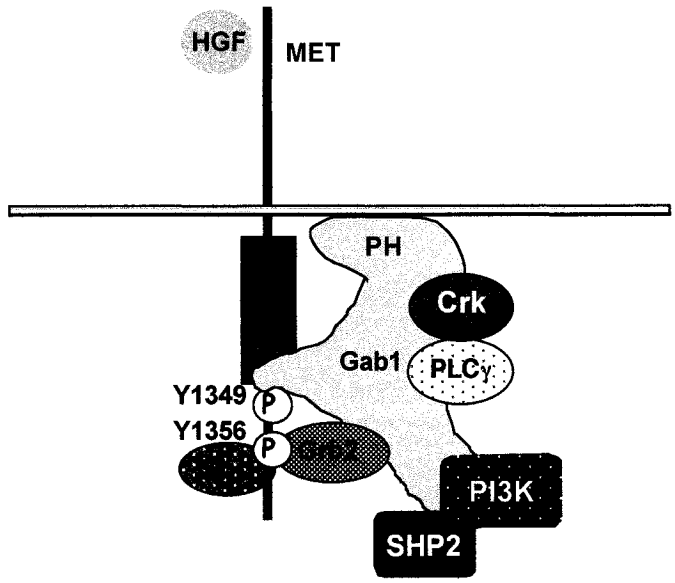

Fig. 5: Recruitment of signaling proteins to phosphotyrosines 1349 and 1356 in the Met receptor C-terminus.

4.4.1 Grb2

The adapter protein Grb2 consists of an SH2 domain sandwiched between two SH3 domains, and is known to recruit Sos to activated RTKs to induce Ras-Mapk signaling (Schlessinger, 1993). In addition, Grb2 plays a key role in the recruitment of the Gab1 docking protein to Met and other RTKs, through the association of its C-terminal SH3 domain with two proline-rich motifs in Gab1 (Bardelli *et al.*, 1997; Nguyen *et al.*, 1997; Lock *et al.*, 2000).

The amino acids surrounding phosphorylated Y1356 in Met (pYVNV) form a consensus binding site for the SH2 domain of Grb2 (pYXNX) (Songyang *et al.*, 1994), and several groups have shown that Grb2 can associate directly with the activated Met receptor (Ponzetto *et al.*, 1994; Fixman *et al.*, 1995; Fixman *et al.*, 1997). While

mutation of Y1356 to F disrupts the association of several signal transducers with Met (Grb2, PI3K, PLCγ, etc), mutation of N1358 to H specifically disrupts only the Grb2-Met association (Fixman *et al.*, 1996; Fournier *et al.*, 1996; Ponzetto *et al.*, 1996). This mutant has been used extensively to elucidate the role of Grb2 in Met receptor signaling and biological responses. A Grb2 binding site on Met is required for efficient cellular transformation by the Tpr Met oncogene (Fixman *et al.*, 1997), promotion of morphogenesis in MDCK epithelial cells (Fournier *et al.*, 1996), but not for the promotion of cell scattering (Fournier *et al.*, 1996; Ponzetto *et al.*, 1996). *In vivo*, using a "knock-in" approach, a direct link of Grb2 with Met has been shown not to be required for development of mice to term. However, these mice had a striking reduction in limb muscles and secondary fibres, indicating that this association is essential for normal development of skeletal muscle (Maina *et al.*, 1996).

4.4.2 Shc

Grb2, in addition to a direct interaction with the Met receptor, can also be recruited to Met indirectly through the SHC adapter protein. The PTB domain of SHC is able to bind to pY1356, however phosphorylation of SHC also requires pY1349 (Pelicci *et al.*, 1995; Fixman *et al.*, 1996). While the Grb2-Sos/ SHC-Grb2-Sos models of Ras activation are well established in other RTK systems, it is not completely clear whether these complexes play an identical role in activating the Ras/MapK pathway following HGF stimulation, and the role of SHC downstream of Met is still undefined (Furge *et al.*, 2000).

4.4.3 Gab1

Gab1 is the major tyrosine phosphorylated protein upon HGF stimulation in epithelial cells (Nguyen *et al.*, 1997) and is an essential component of Met receptor-mediated epithelial morphogenesis (Weidner *et al.*, 1996; Maroun *et al.*, 1999). It is recruited to the Met receptor both indirectly through the adapter protein Grb2, and directly through an undefined mechanism involving Y1349 of the Met receptor and a

proline-rich region in Gab1 (Nguyen *et al.*, 1997; Lock *et al.*, 2000; Schaeper *et al.*, 2000; Lock *et al.*, 2002), and will be discussed further in section 5.

4.4.4 PI3'K and PLCγ

While both the p85 regulatory subunit of PI3K and phospholipase C-γ can associate directly with Y1356 of the Met receptor (Ponzetto *et al.*, 1994), evidence suggests that they associate with Met primarily through the Gab1 docking protein. Indeed, more PI3K activity co-precipitates with Gab1 than co-precipitates with Met (Bardelli *et al.*, 1997; Maroun *et al.*, 1999). The role of their associations with Gab1 downstream of the Met receptor will be discussed further in section 5.6.3.

4.4.5 Cbl

The c-Cbl protein is recruited to the Met receptor indirectly through the adapter protein Grb2, and directly, through the Cbl TKB domain and tyrosine 1003 in the juxtamembrane of the receptor (Peschard *et al.*, 2001). Cbl has been shown to function as an E3-ubiquitin protein ligase (Levkowitz *et al.*, 1998; Joazeiro *et al.*, 1999; Yokouchi *et al.*, 1999) and act as a negative regulator of receptor tyrosine kinases (Yoon *et al.*, 1995; Hime *et al.*, 1997; Meisner *et al.*, 1997). Cbl overexpression promotes ubiquitination of the Met receptor, and mutation of the juxtamembrane Cbl TKB domain binding site converts Met into a transforming protein (Peschard *et al.*, 2001), hence identifying Cbl as a negative regulator of Met receptor signaling. Moreover, it has been proposed that Cbl targets the receptor to clathrin-coated pits by recruiting the Cin85-endophilin complex. This complex drives plasma membrane invagination and vesicle formation, thus resulting in negative modulation of signal transduction and biological responses (Petrelli *et al.*, 2002).

4.5 MDCK cells: In vitro model system to study Met–mediated responses

Numerous biological processes in epithelial cells require coordinated cell movement and changes in cell morphology, including organ development, regeneration following injury, transformation to carcinoma and metastasis. The Madin-Darby canine

kidney (MDCK) cell line treated with HGF serves as an in vitro model system to help define the mechanisms involved with complex epithelial processes such as cell movement and tubulogenesis (Fig. 6), and allows for the elucidation of mechanisms through which Met and HGF mediate these responses (Zhu *et al.*, 1994b; Royal and Park, 1995; Fournier *et al.*, 1996; Maroun *et al.*, 1999).

In culture, MDCK cells form a continuous polarized sheet, one cell thick, that exhibit many of the properties of the normal canine kidney from which it was derived. These cells form a well-polarized monolayer exhibiting apical and basolateral regions and well-defined cell-cell junctional complexes containing tight junctions, adherens junctions and desmosomes. (Simons and Fuller, 1985; Rodriguez-Boulan and Nelson, 1989). The treatment of MDCK cell monolayers with HGF leads to cell scatter (Stoker *et al.*, 1987). The scattering process in response to HGF stimulation requires changes in cytoskeletal reorganization (membrane ruffling and lamellipodium extension, disappearance of peripheral actin bundles at the edges of colonies, and an overall decrease in stress fibres), loss of intercellular junctions and cell migration (Ridley *et al.*, 1995; Royal and Park, 1995).

Interestingly, when cultured in three-dimensional collagen gels, MDCK cells form hollow fluid-filled cysts. Exposure of these preformed epithelial cysts to HGF causes the cysts to develop branching tubules in a process that resembles tubulogenesis in vivo (Montesano *et al.*, 1991; Fournier *et al.*, 1996). Manipulation of this system through pharmacological and genetic means has proven to be a rich source of information on the mechanisms of tubulogenesis, and signaling pathways activated through the Met receptor that regulate this process (Pollack *et al.*, 1998).

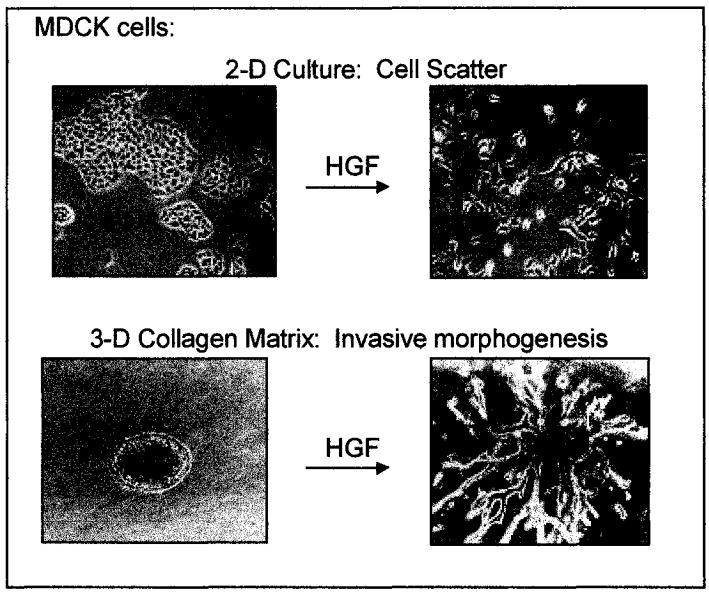

Fig. 6. Cell scatter and invasive morphogenesis upon HGF stimulation in MDCK cells

5. Docking proteins

Docking proteins function as platforms for the co-localization of signaling pathways. Following activation of tyrosine kinase and cytokine receptors, they become phosphorylated on tyrosine residues, thereby 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 multi-protein complexes.

Docking proteins, including those in the IRS, Dok, FRS2 and Gab families have several common features. First, they lack enzymatic activity. Secondly, all contain an amino-terminal membrane-targeting domain. This is usually a PH domain, but in the

case of FRS2, membrane recruitment is achieved through a myristoylation signal. A PTB domain, which functions as a receptor-targeting domain, is usually located after the membrane-targeting signal. This domain, however, is noticeably absent in the Gab family of proteins, and these proteins are recruited to receptors by alternate methods, involving indirect binding through the adapter protein Grb2. Lastly, the C-terminal regions of docking proteins are extremely variable, yet all contain multiple potential tyrosine phosphorylation sites that function as binding sites for SH2 domain-containing proteins.

5.1 Gab Family

The Gab family of docking proteins consists of Gab1, Gab2 and Gab3 in mammals, DOS (Daughter of Sevenless) in *Drosophila*, and SOC-1 (Suppressor of Clear-1) in *C. elegans* (Fig. 7). All Gab family members share a common architecture consisting of a highly conserved N-terminal PH domain, followed by a poorly conserved C-terminus containing a proline-rich region and multiple potential tyrosine phosphorylation sites within consensus binding sites for SH2 domain-containing proteins involved in signal transduction. Binding sites for the C-terminal SH3 domain of Grb2, the SH2 domains of the SHP2 tyrosine phosphatase, and the SH2 domains of the p85 subunit of PI3K are present in all proteins. Gab proteins are tyrosine phosphorylated upon recruitment to multiple RTK and non-TK receptors and provide a link to activation of several downstream signaling pathways including the MapK, PI3K, and JNK pathways. The signals generated through Gab proteins are crucial for normal growth, differentiation and development programs (reviewed in (Liu and Rohrschneider, 2002).

5.1.1 Gab1

Gab1 (Grb2-associated binder 1) was originally identified as a Grb2 binding protein in a cDNA library of glioblastoma tumors (Holgado-Madruga *et al.*, 1996), and later as a direct binding protein for the Met receptor tyrosine kinase (Weidner *et al.*, 1996). It is a widely expressed protein, and is phosphorylated downstream from numerous receptor tyrosine kinases, cytokine receptors, G protein-coupled receptors and antigen receptors.

Gab1 plays a crucial role in linking a broad range of growth factor and cytokine signals to intracellular signaling pathways.

In vivo, Gab1 is critical for embryonic development, as Gab1-deficient mice die in utero, displaying defects in the heart, placenta and skin, as well as reduced liver size. This phenotype is remarkably similar to phenotypes observed in mice lacking signals from the HGF, PDGF, and EGF pathways (Itoh *et al.*, 2000; Sachs *et al.*, 2000). *In vitro*, Gab1 promotes cell survival, neurite outgrowth, and DNA synthesis in neuronal cells downstream from the TrkA receptor (Holgado-Madruga *et al.*, 1997; Korhonen *et al.*, 1999). However, in contrast, Gab1 has been found to inhibit cell survival and DNA repair downstream of the HGF/Met receptor (Fan *et al.*, 2001).

Extensive structure-function studies have been carried out on Gab1 in regards to its role as an essential mediator of Met receptor-induced epithelial morphogenesis. Overexpression of Gab1 in MDCK epithelial cells rescues the ability of a Met receptor mutant to induce branching morphogenesis (Maroun *et al.*, 1999a). This has been shown to require the association of Gab1 with Shp-2, Crk, and PLCγ; as well as an intact PH domain; and the ability to interact with the Met receptor in a Grb2-independent manner (Weidner *et al.*, 1996; Maroun *et al.*, 1999a; Maroun *et al.*, 1999b; Maroun *et al.*, 2000; Schaeper *et al.*, 2000; Lamorte *et al.*, 2002; Lock *et al.*, 2002). Importantly, the morphogenic capacity does not exist in Gab2 (Lock *et al.*, 2002) or Gab3 (Sangwan, V., and Park, M., unpublished results), indicating that these similar proteins are not functionally redundant.

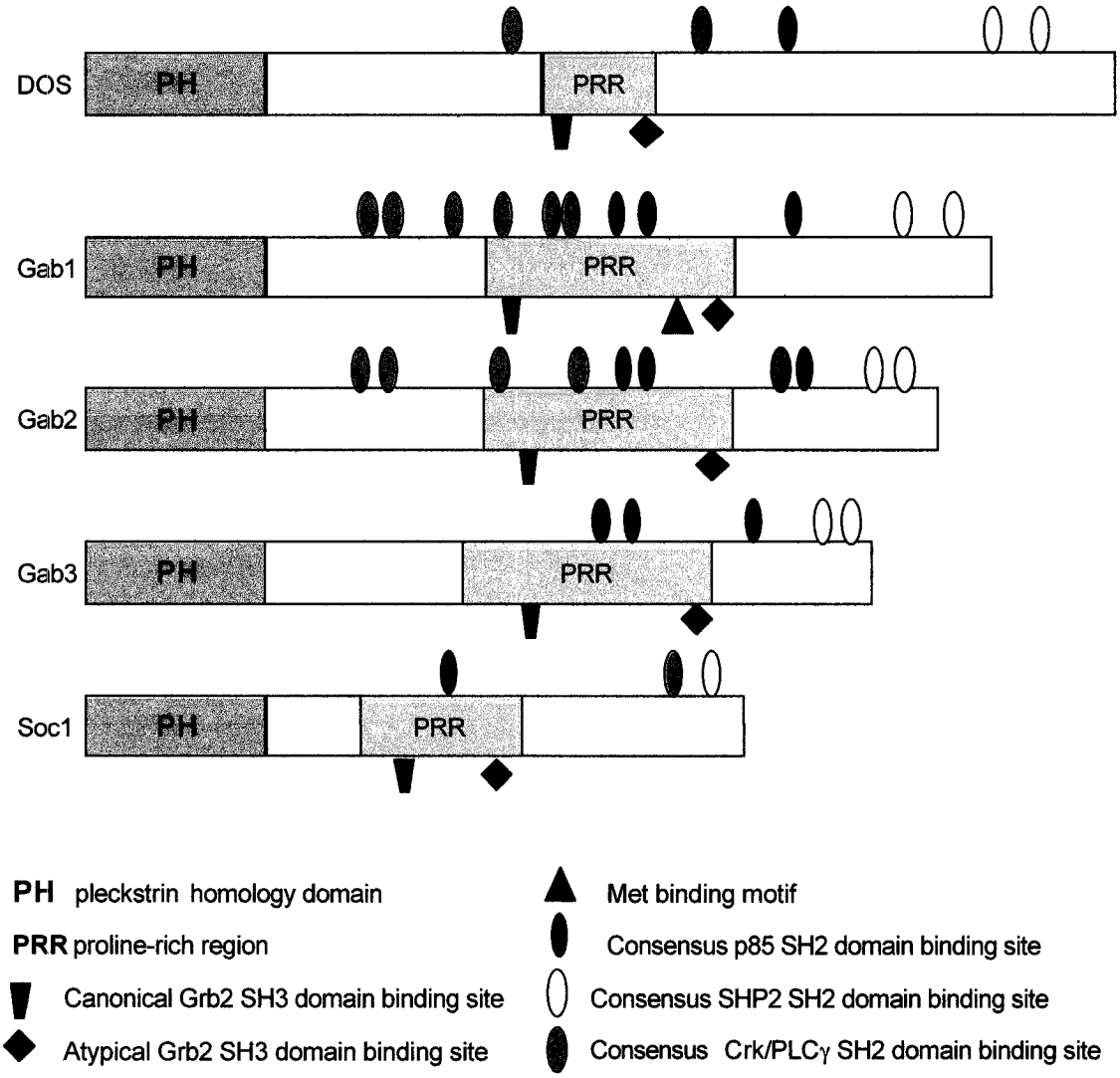

Fig. 7: Gab family proteins

Schematic domain structures of mammalian Gab1, 2, and 3; *Drosophila* DOS and *C. elegans* Soc-1. All Gab family members consist of an N-terminal PH domain, a central proline-rich region, and multiple tyrosines within potential binding motifs favored by various SH2 domain-containing proteins (as indicated in the figure). The binding sites for Grb2 and Met receptor are indicated.

5.1.2 Gab2

Gab2 is a widely expressed protein that was initially cloned as a major binding protein of the SHP2 tyrosine phosphatase in IL-3-stimulated hematopoietic cells (Gu *et al.*, 1998). In contrast to Gab1, mice lacking Gab2 are viable and generally healthy, but have a defect in FcεRI-mediated allergic responses, including passive cutaneous and systemic anaphylaxis, cytokine gene expression and mast cell degranulation, as well as mast cell development (Gu *et al.*, 2001; Nishida *et al.*, 2002). Gab2 is also crucial for the differentiation of hematopoietic cells, particularly, macrophage differentiation triggered by M-CSF, and megakaryocytic differentiation of human K562 chronic myelogenous leukemia cells (Liu *et al.*, 2001; Dorsey *et al.*, 2002).

Gab2 is tyrosine phosphorylated in response to many stimuli including IL-2, IL-3, erythropoeitin, thrombopoeitin, stem cell factor, Flt-3 ligand, B-cell receptor, T-cell receptor, Bcr-Abl, EGF, heregulin and HGF (Gu *et al.*, 1998; Nishida *et al.*, 1999; Wickrema *et al.*, 1999; Zhao *et al.*, 1999; Gu *et al.*, 2000; Bouscary *et al.*, 2001; Lock *et al.*, 2002; Lynch and Daly, 2002), and has been found to have contrasting roles in cell signaling. Gab2 plays a positive role in multiple cytokine and growth factor signaling pathways (Gu *et al.*, 1998; Nishida *et al.*, 1999; Zhao *et al.*, 1999; Gu *et al.*, 2000; Dorsey *et al.*, 2002), as well as in β_1-integrin-mediated hematopoietic cell adhesion and migration (Yu *et al.*, 2002). In contrast, Gab2 plays an inhibitory role in T-cell receptor signaling (Pratt *et al.*, 1996; Yamasaki *et al.*, 2001), and EGF-mediated Elk-1 dependent transcription (Zhao *et al.*, 1999).

To date, Gab2 has been most extensively studied in hematopoietic cells and cytokine-mediated signaling. However, in epithelial cells, Gab2 was found to be tightly regulated downstream of heregulin (HRG)-activated ErbB receptors by Akt/PKB-mediated negative feedback via phosphorylation of Ser159 on Gab2. Release from this negative constraint leads to potent amplification of mitogenic signaling pathways, hence identifying Gab2 as a novel proto-oncogene (Lynch and Daly, 2002). In addition, Gab2 has been found to be overexpressed in a subset of breast cancer cell lines, and appears to be a link between steroid and growth factor signaling in breast cancer (Daly *et al.*, 2002).

5.1.3 Gab3

Gab3 is the most recent mammalian family member identified, and has a more restricted pattern of expression than Gab1 or Gab2, primarily in hematopoietic tissues (Wolf *et al.*, 2002). Gab3 is important for macrophage differentiation in response to M-CSF stimulation, and this process appears to require the early phosphorylation of Gab2, followed by induction and subsequent phosphorylation of Gab3 (Wolf *et al.*, 2002). The association of Gab3 with Mona, a Grb2-like adapter protein appears to play a specific role in mediating the M-CSFR differentiation signal (Bourgin *et al.*, 2002).

5.1.4 DOS

Daughter of Sevenless (DOS) is the only identified homolog in *Drosophila melanogaster*, and was cloned as a potential substrate for the protein tyrosine phosphatase Corkscrew, Csw (the homolog of mammalian SHP2). DOS is required for signaling downstream of receptor tyrosine kinases including Sevenless, Torso, and DER (homolog of mammalian EGF receptor), and is essential for normal development throughout the fly (Herbst *et al.*, 1996; Raabe *et al.*, 1996; Bausenwein *et al.*, 2000).

5.1.5 SOC-1

SOC-1 (Suppressor of Clear-1) was recently identified in *C. elegans*, and is essential for development in the nematode. SOC-1 functions in conjunction with the C.elegans SHP2 homologue, PTP2, to mediate a portion of the EGL-15 (homologue of mammalian fibroblast growth factor receptor) signal transduction cascade. (Schutzman *et al.*, 2001).

5.1.6 Functional motifs and domains in Gab family members

Structure-function analysis of Gab family members has identified multiple regions that are critical for the biological responses initiated downstream of activated receptor complexes. These include motifs or domains that have been found to interact with other signaling proteins, or are involved in subcellular localization.

5.1.6.1 PH domain

The PH domain is the most conserved structural element among Gab family members. Indeed, a Gab1 PH domain is able to functionally replace the DOS PH domain (Isakoff *et al.*, 1996). The importance of this domain has been shown in several biological systems. Gab1 mutants with a deletion of the PH domain, or mutations at a conserved phospholipid binding site (W26A/C, R29A/C) were unable to mediate Met receptor-induced epithelial morphogenesis (Maroun *et al.*, 1999a). PH domain mutants of DOS failed to function in Sevenless signaling and to rescue the lethal phenotype of DOS loss-of-function mutant flies (Bausenwein *et al.*, 2000), while a PH domain mutant of SOC-1 was unable to rescue the suppressor of clear phenotype in the Egl-15 signaling pathway (Schutzman *et al.*, 2001). These results are consistent with a conserved and specific function of the PH domains from different docking proteins in regulating receptor tyrosine kinase mediated signaling processes (Bausenwein *et al.*, 2000).

PH domains bind to phosphoinositides and function to target their host proteins to membranes. The Gab1 and DOS PH domains have been shown to bind specifically to the PI3K product, phosphatidylinositol-3,4,5-P_3 (PIP3) (Isakoff *et al.*, 1998; Maroun *et al.*, 1999b; Rodrigues *et al.*, 2000), and are necessary and sufficient for their localization at sites of cell-cell contact (Maroun *et al.*, 1999a; Bausenwein *et al.*, 2000). Although the phosphoinositide binding specificity of Gab2 has not been determined, it also localizes to cell-cell junctions in MDCK epithelial cells (Lock *et al.*, 2002).

Importantly, replacement of the Gab1 PH domain with a myristoylation tag was sufficient to allow Gab1 to mediate epithelial morphogenesis, indicating that the PH domain of Gab1 functions predominantly as a localization module, rather than having additional roles in regulating intracellular signaling (Maroun, C.R., et al, in press).

5.1.6.2 Proline-rich region

The central region of Gab proteins is rich in proline, and contains multiple PXXP motifs, which are potential binding sites for SH3 domain containing proteins. However, the only SH3 domain-containing proteins found to bind thus far are the adapter proteins Grb2 and the Grb2-like Gads/Mona. All Gab family members associate constitutively

with Grb2 (Holgado-Madruga *et al.*, 1996; Gu *et al.*, 1998; Bourgin *et al.*, 2002; Wolf *et al.*, 2002), and contain two conserved binding sites for the Grb2 C-terminal SH3 domain. One is a canonical PXXP-containing motif, whereas the other is atypical, with a $P(X)_3R(X)_2KP$ consensus sequence (Nguyen *et al.*, 1997; Lock *et al.*, 2000; Schaeper *et al.*, 2000; Lewitzky *et al.*, 2001)..

Gab proteins do not contain PTB domains that bind directly to activated receptors. Instead, the recruitment of Gab proteins to activated receptor complexes appears to depend on an indirect mechanism involving Grb2. While the C-terminal SH3 domain associates constitutively with Gab proteins, the SH2 domain of Grb2 binds to specific phosphotyrosine-containing motifs in a number of receptor tyrosine kinases, including the EGF receptor. Mutation of the Grb2 binding sites on either Gab1 or the EGF receptor abolishes the association between Gab1 and the EGF receptor (Lock *et al.*, 2000; Rodrigues *et al.*, 2000), while an absence of Gab1 tyrosine phosphorylation in response to EGF is found in fibroblast cells isolated from mice expressing a null/ hypomorphic mutant of Grb2 (Saxton *et al.*, 2001). These results provide both biochemical and genetic evidence for a Grb2-dependent mechanism of recruitment of Gab1 to the EGF receptor. Recruitment of Gab proteins to receptors that do not contain a Grb2 binding site may require a fourth protein to mediate the interaction between the receptor and the SH2 domain of Grb2. For example, Gab2 is recruited to the interleukin-3 receptor beta common chain (βc) through a Shc-Grb2-Gab2 mechanism (Gu *et al.*, 2000), while an FRS2-Grb2-Gab1 interaction is utilized for the recruitment of Gab1 to the FGFR1 receptor (Ong *et al.*, 2001).

Gab1 is unique from the other Gab proteins in that it was found to bind directly to the Met receptor tyrosine kinase, but not to other receptor tyrosine kinases tested. This Grb2-independent interaction requires phosphorylation of tyrosines in the Met receptor C-terminus, and a proline-rich region in Gab1 termed the Met binding domain (MBD), as identified through yeast 2-hybrid analysis (Weidner *et al.*, 1996). A 13 amino acid sequence, GMQVPPPAHMGFR, within the MBD, termed the Met binding motif (MBM), has been identified as the critical component of the MBD-Met interaction (Schaeper *et al.*, 2000; Lock *et al.*, 2002). Although the MBD has been proposed to be a

phosphotyrosine binding (PTB)-like domain, the mechanism of interaction between the Gab1 MBD and Met is unclear.

5.1.6.3 Phosphotyrosine-dependent interactions

Upon stimulation, Gab proteins are rapidly tyrosine phosphorylated, providing binding sites for SH2 domain containing proteins including the tyrosine phosphatase SHP2, the p85 subunit of PI3K, the adapter protein Crk, and phospholipase C-γ. Association with these proteins was found to be critical for the function of Gab proteins in mediating intracellular signaling pathways from activated receptor complexes. Other proteins such as Shc, SHIP, Src, and ras gap have been reported to associate with some Gab proteins, however it is not clear whether these associate directly, as binding sites have yet to be identified.

5.1.6.3.1 Gab1-SHP2 interactions

Upon tyrosine phosphorylation, all Gab family members, including DOS and SOC-1, have been shown to bind SHP2 (or its homologs). SHP2 contains two tandem SH2 domains followed by a phosphatase domain, and binds to one or two YXXV/I/L-containing motifs at the extreme C-terminus of Gab proteins (Raabe *et al.*, 1996; Gu *et al.*, 1998; Lehr *et al.*, 1999; Nishida *et al.*, 1999; Schutzman *et al.*, 2001; Wolf *et al.*, 2002).

The biological significance of the Gab-SHP2 interaction has been extensively studied using mutants of Gab family members containing tyrosine to phenylalanine mutations in the SHP2 binding sites. In mammalian cells, a SHP2-binding mutant of Gab1 is unable to mediate Met receptor-induced epithelial morphogenesis (Maroun *et al.*, 2000; Schaeper *et al.*, 2000), while a mutant of Gab2 unable to bind SHP2 blocked M-CSF-induced macrophage differentiation (Liu *et al.*, 2001). Moreover, a SHP2-binding mutant of DOS is unable to function during Sevenless signaling and fails to rescue the lethality associated with *dos* loss of function mutations (Herbst *et al.*, 1999; Bausenwein *et al.*, 2000), while a mutant SOC-1 unable to bind SHP2 fails to function in Egl-15 signaling (Schutzman *et al.*, 2001). Importantly, mutation of all other tyrosine residues in

DOS or SOC-1 has no effect on the biological functions mediated by these proteins, illustrating the critical nature of this interaction (Bausenwein *et al.*, 2000; Schutzman *et al.*, 2001).

Accumulating evidence has suggested that both SHP2 and Gab proteins function as positive regulators of Map kinase activity downstream from receptor tyrosine kinases (Bennett *et al.*, 1994; Tang *et al.*, 1995; Bennett *et al.*, 1996; Neel and Tonks, 1997; Cleghon *et al.*, 1998; O'Reilly and Neel, 1998) (Weidner *et al.*, 1996; Takahashi-Tezuka *et al.*, 1998; Korhonen *et al.*, 1999; Nishida *et al.*, 1999; Itoh *et al.*, 2000; Rodrigues *et al.*, 2000). In accordance, it has been found that downstream of various stimuli, the binding of SHP2 to Gab proteins can lead to activation of MAP kinase signaling (Gu *et al.*, 1998; Cunnick *et al.*, 2000; Maroun *et al.*, 2000; Schaeper *et al.*, 2000; Cunnick *et al.*, 2001; Liu *et al.*, 2001). The activation of MAP kinase downstream of Gab has been shown to require the phosphatase activity of SHP2 (Maroun *et al.*, 2000; Schaeper *et al.*, 2000; Shi *et al.*, 2000). Recent evidence indicates that binding of the SH2 domains of SHP2 to phosphotyrosine(s) in Gab proteins is an essential part of the mechanism that upregulates the phosphatase activity of SHP2 (Cunnick *et al.*, 2001).

The precise mechanism of MAP kinase activation through the association of Gab proteins with SHP2 has not been defined. Importantly, use of a fusion protein consisting of the Gab1 PH domain and an active SHP2 has suggested that the primary role of Gab proteins is to target an activated SHP2 to the membrane, in proximity to its substrates (Cunnick *et al.*, 2002). It is not known yet which substrates of SHP2 are required for activation of the MAP kinase pathway. Although Gab2 and DOS were originally identified as substrates of SHP2, and in vitro phosphatase assays support that Gab1 is a substrate of SHP2 (Herbst *et al.*, 1996; Gu *et al.*, 1997; Nishida *et al.*, 1999; Cunnick *et al.*, 2001), there is no evidence to link dephosphorylation of any phosphotyrosines on Gab proteins to activation of MAP kinase. A p90 protein that can serve as a substrate of SHP2 has been identified in a complex with Gab1, and thus could play a role in this pathway (Shi *et al.*, 2000).

5.1.6.3.2 Gab-p85 interactions

The mammalian Gab proteins all contain 3 YXXM-containing motifs for binding the p85 subunit of PI3K, while DOS and SOC-1 contain only one site each. The physical interaction of Gab1 or Gab2 with p85 has been shown to be crucial in activation of the PI3K/Akt signaling pathway induced by a variety of stimuli *in vitro*, including HGF, EGF, NGF, FGF, and IL-2 (Holgado-Madruga *et al.*, 1997; Korhonen *et al.*, 1999; Laffargue *et al.*, 1999; Maroun *et al.*, 1999a; Gu *et al.*, 2000; Rodrigues *et al.*, 2000; Ong *et al.*, 2001; Yart *et al.*, 2001). The interaction of Gab proteins with p85 is predicted to form a positive feedback loop, since activation of PI3K leads to production of PIP_3, which in turn can bind the PH domain of Gab1 and promote further activation of PI3K (Rodrigues *et al.*, 2000).

Direct recruitment of PI3K by Gab1 is required for the enhancement of NGF-induced cell survival *in vitro* (Holgado-Madruga *et al.*, 1997), while *in vivo*, direct recruitment of PI3K by Gab2 is required for activation of the PI3K/Akt signaling pathway, and subsequent production of PIP_3 induced by the high affinity immunoglobulin-ε (IgE) receptor, FcεRI (Gu *et al.*, 2001). Moreover, c-Kit-mediated Akt activation is reduced in bone marrow mast cells derived from Gab2-deficient mice (Nishida *et al.*, 2002). However, the importance of the Gab-p85 interaction appears to depend on the context in which it occurs. Mutation of the p85 binding sites in DOS or SOC-1 does not lead to functional abnormalities (Bausenwein *et al.*, 2000; Schutzman *et al.*, 2001), nor does mutation of the sites in Gab1 affect the ability of Gab1 to rescue Met receptor-mediated morphogenesis (Maroun *et al.*, 1999a).

The above results suggest that while the Gab-SHP2 interaction is important for MAP kinase activation, the Gab-p85 interaction plays a key role in activating the PI3K/Akt pathway in mammalian cells. However, the association of p85 with Gab1 has also been shown to be required for activating the JNK pathway upon EGF stimulation (Rodrigues *et al.*, 2000). Moreover, these pathways are not necessarily mutually exclusive, as it has been proposed that a PI3K-dependent pathway involving Gab1 and SHP2 is essential for Ras activation upon EGF stimulation (Yart *et al.*, 2002), while SHP2 is suggested to regulate the strength and duration of PI3K activation in a receptor-

specific manner, through dephosphorylation of p85 binding sites on Gab1 (Zhang *et al.*, 2002).

5.1.6.3.3 Gab-Crk/PLC-γ interactions

Gab1 and Gab2 contain multiple YXXP motifs, which are involved in binding to the SH2 domains of the adapter protein Crk and PLCγ (Gual *et al.*, 2000; Lamorte *et al.*, 2000; Sakkab *et al.*, 2000; Crouin *et al.*, 2001). Both Crk and PLCγ have been found to associate with Gab1 in response to Met receptor activation, and this association is essential for Met-receptor mediated epithelial morphogenesis (Gual *et al.*, 2000; Lamorte *et al.*, 2002). Gab1-Crk association has also been found to correlate with anchorage-independent growth and JNK activation in cells transformed by the Met receptor oncoprotein (Garcia-Guzman *et al.*, 1999; Lamorte *et al.*, 2000). However, the functional significance of the association of Gab2 with Crk and PLC-γ has not yet been established. Gab3 noticeably lacks the multiple tyrosines involved in Crk/PLC-γ binding, providing a possible mechanism for functional differences between family members.

5.2 IRS family

The insulin receptor substrate (IRS) family contains 4 members, IRS1-4, that all contain an N-terminal PH domain, followed by a PTB domain and a C-terminus of varying length containing multiple potential tyrosine phosphorylation sites, some of which are distinctive for a particular IRS (Van Obberghen *et al.*, 2001).

These proteins play a major role in generation of biological signals downstream from both the insulin and IGF-1 receptor tyrosine kinases. The PTB domains of IRS proteins bind directly to the NPXpY sequence surrounding tyrosine-960 of the insulin receptor, and to the corresponding tyrosine-950 of the IGF-1 receptor (Tartare-Deckert *et al.*, 1995). Upon tyrosine phosphorylation, they become platforms for several SH2 domain-containing proteins including Grb2, SHP2, and PI3K, leading to activation of specific signaling cascades (White, 1994, 1998).

IRS-1 was the first of these proteins to be discovered, and its major role appears to be in embryonic and post-natal somatic cell growth. Mice lacking IRS-1 have a

reduced body size and are mildly insulin-resistant (Araki *et al.*, 1994). IRS-2 on the other hand, appears to play a key role in the regulation of carbohydrate metabolism and reproduction. Deletion of IRS-2 leads to insulin resistance associated with a defect in pancreatic β-cell development resulting in diabetes (Withers *et al.*, 1998), as well as female infertility (Burks *et al.*, 2000). IRS-1 and 2 are widely expressed, whereas IRS-3 is found predominantly in adipose tissue, and IRS-4 is expressed in the thymus, brain and kidney (Lavan *et al.*, 1997a; Lavan *et al.*, 1997b). IRS-3 and IRS-4 deficient mice have no apparent phenotype (Liu *et al.*, 1999; Fantin *et al.*, 2000), although there is evidence that these proteins may act as negative regulators of the IGF-1 signaling pathway by suppressing the function of other IRS proteins (Tsuruzoe *et al.*, 2001).

5.3 Dok Family

The Dok (Downstream of kinase) family of proteins contains five members (Dok-1 to 5), all of which contain amino-terminal PH domains, followed by a PTB domain, as well as tyrosine residues in the C-terminus within consensus binding sites for SH2 domains and several PXXP SH3 binding motifs. The carboxy-terminal sequences vary in length and show only limited sequence identity (Grimm *et al.*, 2001). Dok-1 to 3 are mainly expressed in hematopoietic tissues (Carpino *et al.*, 1997; Nelms *et al.*, 1998; Lemay *et al.*, 2000), while Dok-4 is broadly expressed in many tissues, and Dok-5 is specifically expressed in the brain (Grimm *et al.*, 2001).

Dok-1 (generally called p62dok) is a target for many tyrosine kinases (Yamanashi and Baltimore, 1997), while Dok-2 (Dok-R) binds to Tek/Tie2, Ret and weakly to the EGF receptor (Ellis *et al.*, 1990; Moran *et al.*, 1991; Jones and Dumont, 1998; Grimm *et al.*, 2001). These proteins have long carboxy-terminal regions and have been shown to associate in a phosphotyrosine-dependent manner with RasGap, Nck, and c-Abl (Yamanashi and Baltimore, 1997), and are suggested to play a negative role in the regulation of MAP kinase signaling (Jones and Dumont, 1999; Tamir *et al.*, 2000; Yamanashi *et al.*, 2000). Dok-3 is a negative regulator of immune receptor and v-Abl signaling and binds SHIP and Csk, but not RasGap (Cong *et al.*, 1999; Lemay *et al.*, 2000). In contrast, dok-4 and dok-5 bind to the c-Ret receptor, contain short carboxy-

terminal sequences with fewer tyrosines, do not associate with RasGap, or Nck. and play a positive role in activation of the Map kinase pathway (Grimm *et al.*, 2001).

5.4 FRS-2 (SNT) family

FGF Receptor Substrate-2 (FRS-2) proteins (α and β), (also known as Suc1-associated neurotrophic factor-induced tyrosine-phosphorylated target, SNT) are targeted to the plasma membrane by myristylation at the N-terminus, and contain a PTB domain that mediates interactions with the fibroblast growth factor (FGF), nerve growth factor (NGF) and Ret receptor tyrosine kinases (Meakin *et al.*, 1999; Dhalluin *et al.*, 2000; Ong *et al.*, 2000; Kurokawa *et al.*, 2001; Melillo *et al.*, 2001; Xu and Goldfarb, 2001). Interestingly, the PTB domain of FRS2α is capable of interacting with canonical phosphotyrosine-containing NPXpY sequences in NGF receptors, and with a unique, non-phosphorylated region in the juxtamembrane of FGFRs that contains no tyrosine or arginine residues (Xu *et al.*, 1998; Ong *et al.*, 2000).

Multiple tyrosine phosphorylation sites are present in the C-terminus of the FRS2s, including four binding sites for the adapter protein Grb2, and two for the protein tyrosine phosphatase, SHP2 (Kouhara *et al.*, 1997; Hadari *et al.*, 1998). Recruitment of both Grb2 and SHP2 to FRS2α are required for FGF-mediated Map kinase activation, proliferation of fibroblasts, neuronal differentiation of PC12 cells (Kouhara *et al.*, 1997; Hadari *et al.*, 1998).

Targeted disruption of the FRS2α gene causes severe impairment in mouse development, resulting in embryonic lethality (Hadari *et al.*, 2001), as well as disrupting FGF-dependent cell proliferation, Map kinase activation, PI3K activation, and chemotactic response (Hadari *et al.*, 2001). FRS2α also functions as an adapter protein. In response to FGF stimulation, FRS2α associates with Gab1 indirectly via Grb2, resulting in tyrosine phosphorylation of Gab1 and activation of the PI3K/AKT survival pathway (Hadari *et al.*, 2001; Ong *et al.*, 2001).

Unlike the other families of docking proteins, little is known yet about the differences between FRS2 α and β, although they have only 49% sequence identity (Kouhara *et al.*, 1997).

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Chapter II: Identification of an atypical Grb2 carboxy-terminal SH3 domain binding site in Gab docking proteins reveals Grb2-dependent and independent recruitment of Gab1 to receptor tyrosine kinases.

Preface

The Gab1 docking protein is phosphorylated in response to multiple growth factors and cytokines, and functions as a platform for the co-localization of signaling proteins upon receptor stimulation. However, the mechanism of recruitment of Gab1 to activated receptor complexes was unclear.

Gab1 was originally identified as a Grb2-binding protein, and requires binding sites for the SH2 domain of Grb2 on the Met and EGF receptors for its recruitment, implicating a Grb2-dependent, indirect mode of recruitment. In contrast, yeast two-hybrid analysis identified Gab1 as a direct binding partner for the Met receptor. This interaction required phosphorylation of tyrosines 1349 and 1356 in the C-terminus of the Met receptor, and a proline-rich Met binding domain (MBD) in Gab1 that may represent a distinct phosphotyrosine binding domain. The significance of either mode of recruitment had not been examined. We sought to determine the role of Grb2 in recruitment to tyrosine kinases, through identification of the Grb2 binding sites in Gab1.

Abstract

The Gab family of docking proteins are phosphorylated in response to various growth factors and cytokines and serve to recruit multiple signaling proteins. Gab1 acts downstream from the Met-hepatocyte growth factor receptor, and Gab1 overexpression promotes Met-dependent morphogenesis of epithelial cells. Recruitment of Gab1 to Met or EGF receptors requires a receptor binding site for the Grb2 adapter protein and a proline rich domain in Gab1, defined as the Met binding domain. To determine the requirement for Grb2 in Gab1 recruitment, we have mapped two Grb2 C-terminal SH3 domain binding sites conserved in Gab1 and related protein Gab2. One corresponds to a canonical Grb2 binding motif, whereas the second, located within the Gab1 Met binding domain, requires the proline and arginine residues of an atypical PXXXR motif. The PXXXR motif is required but not sufficient for Grb2 binding, whereas an extended motif, $PX_3RX_2KPX_7PLD$, conserved in Gab proteins as well as the Grb2/Gads- docking protein, Slp-76, efficiently competes binding of Grb2 or Gads adapter proteins. The association of Gab1 with Grb2 is required for Gab1 recruitment to the EGF receptor but not the Met receptor. Hence different mechanisms of Gab1 recruitment may reflect the distinct biological functions for Gab1 downstream from the EGF and Met receptors.

Introduction

Receptor tyrosine kinases regulate diverse biological processes ranging from cell proliferation and survival to cell motility, metabolism and differentiation. The initiation of receptor signaling involves ligand-induced activation of the catalytic domain of the enzyme and the phosphorylation of specific tyrosine residues on the receptor. These provide binding sites for proteins containing Src homology 2 (SH2) and phosphotyrosine binding domains (PTB) that act to transduce signals to the interior of the cell (reviewed in (1)). The biological responses elicited by each receptor are thus controlled by the repertoire of signaling proteins recruited to the receptor. Recently, docking proteins have been identified that act to diversify the signal downstream from receptor tyrosine kinases. These include Insulin Receptor Substrate-1 (IRS-1), IRS-2, IRS-3, Daughter of Sevenless (DOS), Downstream of Kinases (Dok), FGF receptor substrate 2 (FRS2) and the recently identified proteins Gab1 and Gab2 (2-9). These proteins lack enzymatic activities, but following activation of tyrosine kinase or cytokine receptors, they are recruited to the receptor complex and become phosphorylated on tyrosine residues, providing binding sites for multiple proteins involved in signal transduction. In this manner they act to diversify and localize signals downstream from receptors by virtue of their ability to assemble multiprotein complexes.

Hepatocyte growth factor (HGF), also known as scatter factor (SF), is a multifunctional factor that stimulates a wide variety of cellular responses including mitogenesis, motility and invasiveness of carcinoma cells, but induces the inherent morphogenic program of kidney, breast, and lung epithelium grown in matrix culture and promotes organ regeneration *in vivo* (10-14). The receptor for HGF, the Met tyrosine kinase, is sufficient to mediate the pleiotropic effects of HGF *in vitro* and *in vivo* (15-17). Tight control of cell proliferation and morphogenesis is required to ensure normal tissue patterning and prevent cancer formation and deregulation of the Met/HGF axis is implicated in human tumorigenesis (18-21). From a search for Met specific substrates that are implicated in modulating epithelial morphogenesis, we have recently identified the Grb2-associated binder (Gab1), as a protein that becomes highly phosphorylated following HGF stimulation of epithelial cells that undergo a morphogenic program (22). Gab1 belongs to a new family of docking proteins that includes DOS and the recently

identified protein Gab2 (3,4,9). Following stimulation of epithelial cells with HGF, Gab1 couples with the p85 subunit of PI3 kinase and associated PI3 kinase activity, PLCγ1, the tyrosine phosphatase SHP-2 (22,23) and the adapter protein Crk (24) and in turn acts to recruit these signaling proteins to the Met receptor (23).

From structure function analyses using Met receptor mutants, the phosphorylation and recruitment of Gab1 to the Met receptor, requires the presence of two tyrosine residues within the carboxyl terminus of Met (Y1349 and Y1356) (22,25,26). These residues are crucial for biological activity and Met-induced branching morphogenesis of epithelial cells (25,27,28). Tyrosine 1356 forms a multisubstrate binding site, coupling the Met receptor directly with the Grb2 and Shc adapter proteins (29). Met receptor mutants lacking a Grb2 binding site, fail to form branching tubules in response to Met activation (28), and the overexpression of Gab1 in these cells rescues the tubulogenesis defect, thereby implicating Gab1 in modulating the Met-dependent morphogenic program (23).

Docking proteins in general contain two distinct domains involved in membrane targeting and interaction with receptor tyrosine kinases. Many docking proteins, including the Gab1 family of proteins, contain an amino terminal pleckstrin homology (PH) domain with the capacity to bind membrane phospholipids. The Gab1 PH domain preferentially associates with $PI(3,4,5)P_3$ (30-32) and this association is required for the localization of Gab1 to cell-cell junctions in the vicinity of the Met receptor (23,30). In addition, the IRS family, FRS2 and Dok proteins contain a phosphotyrosine binding domain that acts to target these proteins to specific tyrosine phosphorylated residues on receptor tyrosine kinases. However, Gab1 lacks an apparent PTB domain. Instead, the interaction of Gab1 with Met is thought to occur both indirectly via the Grb2 adapter protein (22,26,33) and directly through a proline-rich Met binding domain (MBD) in Gab1, that may represent a distinct phosphotyrosine binding domain (25).

Gab1 is not a substrate unique to the Met receptor, but is also phosphorylated downstream from multiple receptor tyrosine kinases including, EGF, Insulin and TrkA receptors, cytokine receptors, T-cell and B-cell antigen receptors, and erythropoietin receptor (8,34-39). The mechanism of recruitment of Gab1 to these receptors is unknown. Interestingly, the association of Gab1 with the EGF receptor requires two

tyrosines in the receptor that act as Grb2 binding sites (31), suggesting that the indirect recruitment of Gab1 via the Grb2 adapter protein may be a common mechanism for Gab1 recruitment to receptor tyrosine kinases.

To address this, we have mapped the domain in Gab1 which interacts with the Grb2 SH3 domain. We show that two proline-rich PXXPXR motifs, previously suggested as potential sites of interaction with the Grb2 adapter protein (8) are not involved in Grb2 binding. Instead, we have identified two Grb2 SH3 domain binding sites that are conserved in Gab1 and the related protein Gab2. One contains an atypical motif, PXXXR in which the proline and arginine residues are essential for Grb2 C-teminal SH3 domain binding. This identifies a new interaction motif for the C-terminal SH3 domains of Grb2-like adapter proteins. We show that a Gab1 mutant that lacks Grb2 binding sites still associates with the Met receptor and rescues the morphogenesis defect of Met receptor mutants, whereas this mutant fails to associate with the EGF receptor.

Experimental Procedures

Plasmids and Mutagenesis - Deletion of proline-rich sites in Gab1 was achieved using the Chameleon Double-Stranded Mutagenesis Kit. All mutants were generated in an HA-tagged Gab1- pCDNA1.1 construct. New restriction sites were engineered in (or deleted) for identification of mutant clones. Primers used to generate the deletion mutants were (all 5' to 3'): ΔPro1/2 (452-PNSPPR-457 and 501-PKTPPR-506), GAACTACGTTCCCATGAACCAACATTCCGGAAGCTTTACCGAGCC and GCTCATATGGGCTTCAGATCTAGCAGGCCAGTTCTTGTTGC; ΔPro3 (158-LPPPY-162), GGGAGTCAGATGGAAGCCTCGAGTGTCGCGCAGGTCATCAG-CCTTGG; ΔPro4 (337-IPDIPPPRPP-346), GAGAGCACACTGGGAC-AGTCATCAAAGCTGGACACCAAGCCACATCCAACTCATGACCGGTCTCCTGT GGAAACG; ΔPro5 (517-PPPVDR-522), GAGGCCAGTTCCT-GTTGCTGACTGTGAAAACCTCAAGCCCGATCGAAAAGTCAAGCCGGCACC; ΔPro6 (566-PMSPRP-571), GGGACTCCTCTAGGTTTGATTCTGTTCATAGT-ACGACATCGG. The ΔPro4/5 deletion mutant was generated by subcloning an Nde I digested fragment of the ΔPro4 mutant containing the desired mutation, into the Nde I site of the ΔPro5 mutant. The generation of the Gab1ΔPH domain mutant was described elsewhere (23). The 6 amino acids deleted in ΔPro5 were individually mutated to alanine in the ΔPro4 mutant construct, and a Pvu I restriction site that does not alter the amino acid composition was added for identification of mutant clones. The following primers were used with the Chameleon mutagenesis kit (all 5' to 3', mutated codon is underlined, Pvu I site is in italics): P517A, CTGTTGCTGACTGTG-AAGCACCCCCTGT*CGATCG*GAACCTCAAGCCAG; P518A, GTTGCTGACTGT-GAACCAGCCCCTGT*CGATCG*GAACCTCAAGCCAG; P519A, GCTGACTGTG-AACCACCCGCTGT*CGATCG*GAACCTCAAGCCAG; V520A, GACTGTGAAC-CACCCCCGGC*CGATCG*GAACCTCAAGCCAG; D521A, GAACCACCCCCGGTG-GCTAGGAACCTCAAGCC*CGATCG*AAAAGTCAAGCCGGCAC; R522A, GAACCACCCCCGGTGGATGCTAACCTCAAGCC*CGATCG*AAAAGTCAAGCCGG CAC. The MBD constructs were PCR-amplified out of WT or ΔPro5 Gab1 using oligos:

forward, 5'-ATCGGATATCGG-ATCCACCGTGGATTTGAACAAGTTG-3', and reverse, 5'-GCATGGGCCCGAATTCTCAC-GGCTTGACTTTTCTGTCTGG-3'. The PCR products were subcloned into the Bam HI/Eco RI sites of both pGEX 2TK and PRK5. All mutants generated were sequenced prior to use.

Cell culture, DNA transfections and Whole cell extracts - For transient transfections, Cos-1 cells were seeded at $8x10^5$/ 100-mm petri dish and transfected 24 h later with 6μg of plasmid DNA by a standard DEAE-dextran precipitation method as described previously (40). 293T cells were seeded at $1x10^6$/ 100-mm petri dish and transfected 24h later with 2 μg of DNA by the calcium phosphate precipitation method (41). Cos-1 and 293T cells were serum starved in 0.1% FBS for 24h, washed twice with ice-cold phosphate-buffered saline (PBS), 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 μg aprotinin/ml, 10 μg leupeptin/ml, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium fluoride, and 1 mM sodium vanadate). Following a 10 min incubation on ice, the lysates were centrifuged at 10,000 rpm for 10 min. The generation of MDCK cell lines expressing wild-type colony-stimulating factor-1 (CSF)-Met receptor and mutants thereof by retroviral infection have been described previously (15,28). The generation of stable cell lines expressing WT and mutant HA-tagged Gab1 was described previously (23).

Immunoprecipitations and Western blotting - Approximately 1/25th of lysate from a 100 mm plate was incubated with the appropriate antibodies for at least 1 h at 4°C with rocking. Protein A or G-Sepharose (20 μL of a 50% solution) was added, and mixing continued for an additional hour at 4°C. Following three washes in 0.5% Triton X-100 lysis buffer, immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis (PAGE), and transferred to a nitrocellulose membrane. The membranes were blocked for 1 h with 1% bovine serum albumin in TBST (10 mM Tris-HCl, pH 7.4, 2.5 mM EDTA, 150 mM NaCl, 0.1% Tween-20) at room temperature, and then with the appropriate antibody for at least an additional hour. Following 5 washes in TBST, the proteins were revealed with secondary anti-mouse (Jackson ImmunoResearch Laboratories, Inc.) or protein A conjugated to horseradish peroxidase (Amersham). The

proteins were visualized with an enhanced chemiluminescence detection system (Amersham).

GST Fusion proteins, in vitro association assays, and peptide competition - Fusion proteins were produced in the DH5 *Escherichia coli* strain, by induction with isopropyl-1-thio-β-D-galactopyranoside. GST fusion proteins (0.5 –1 μg) immobilized on glutathione sepharose beads were incubated with the indicated amount of 293T cell lysate or approximately 1/20 -1/100th of the indicated Cos cell lysate in 0.5% Triton X-100 lysis buffer for at least 2 hours at 4°C with rocking. Bound proteins were washed three times with lysis buffer, and subjected to SDS-PAGE as above. For some experiments, the following peptides were added at the indicated concentrations: Pro5 (CEPPPVDRNL); Pro4 (TIPDIPPPRPPKPH); SOS (VPPPVPPRRR); Pro1 (MNPNSPPRQH); Pro5* (PPPVDRNLKPDRKVKPAPLD), Slp-76 (APSIDRSTKPPLDRSLAPLD), and P518A/R522A (PAPVDANLKPDRKVKPAPLD). The peptides were synthesized at the Sheldon Biotechnology Centre (Montreal, Quebec).

Far Western assay - Approximately 400 μg of the indicated lysates were immunoprecipitated with anti-HA antibody, run on 8% SDS-PAGE and transferred to nitrocellulose. The membrane was blocked in renaturation buffer (5% dry milk, 20 mM Hepes, pH 8.0, 1 mM Kcl, 5 mM DTT, 5 mM $MgCl_2$) for at least 6 hours. Glutathione sepharose-bound GST-Grb2 C-SH3 fusion protein was washed three times with PBS, then eluted two times in 1 ml of 10mM glutathione in 50mM Tris, pH 8.0. Approximately 300 μg of eluted fusion protein was added to the renaturation buffer overnight at 4°C. The membrane was washed extensively, and probed for one hour with anti-GST Antibody. The membrane was stripped and reprobed with anti-HA Ab.

Collagen assays - The ability of MDCK cells to form branching tubules was described previously (27). Briefly, $5X10^3$ cells were resuspended in 500 μL of collagen solution (Vitrogen 100 [Celtrix]) prepared as specified by the manufacturer and layered over 350 μL of the collagen solution in a 24-well plate. The cells were maintained in Liebowitz medium containing 5% FBS and allowed to form cysts for 5 to 7 days, after which HGF

or recombinant CSF (rhCSF-1) (15U/mL) was added to the Liebowitz medium. Tubules were apparent by light microscopy 5 to 10 days after the addition of stimuli. For quantification of the morphogenic response, 60 colonies in each of six independent cultures (wells) were scored for their ability to form branching tubules (structure whose length is five times their diameter).

Microinjection- For microinjection, MDCK cells ($5x10^3$) were seeded on glass coverslips (Bellco Glass Inc.). Small colonies of 10-50 cells were partially microinjected (Eppendorf). For all experiments, 100-200 cells were microinjected over a 15 min-period and data shown are representative of the results obtained in a minimum of 4 experiments. Expression vectors (200 μg/ml) were diluted in PBS and co-injected with rabbit immunoglobulin G (Pierce) (2 mg/ml) to mark injected cells. The cells were incubated 2 h to allow for protein expression and further incubated in the presence of HGF (5 units/ml) for an additional 3-4h before fixation.

Antibodies and Reagents - Antibodies against a C-terminal peptide of the human Met protein were used (40), as well as DL-21 (Upstate Biotechnology Inc., Lake Placid, N.Y.). Anti-phosphotyrosine (4G10) was purchased from Upstate Biotechnology Inc., Lake Placid, N.Y., anti-HA (HA-11) from BABCO, Richmond, Calif., anti-Grb2 and anti-GST from Santa Cruz Biotechnology (Santa Cruz, Calif.). HA-tagged Gab2 was a gift from Dr. Ben Neel. A GST-Gads fusion protein was provided by Dr. Jane McGlade, and GST-Grap was provided by Dr. Gen-Sheng Feng.

Results

Mapping of Grb2 SH3 domain binding sites in Gab1

Gab1 was originally identified as a Grb2 binding protein from a screen of a glial tumor cDNA library (8). Grb2 is a small adapter protein comprised of an SH2 domain flanked by two SH3 domains. Gab1 was shown to bind specifically to the carboxy-terminal SH3 domain and not to the amino-terminal SH3 domain (22,26). SH3 domains bind to proline-rich peptides that adopt a type II, left-handed polyproline helix, characterized by the sequence signature PXXP (42-44). Grb2 has been shown to bind through its SH3 domains to class II proline-rich ligands containing the sequence PXXPXR (45-47), and Gab1 contains two potential binding sites for the SH3 domains of Grb2 based on this PXXPXR motif (8).

To examine whether these sites (452-PNSPPR-457, and 501-PKTPPR-506) were involved in binding the C-terminal SH3 domain of Grb2, we generated a mutant (ΔPro1/2), in which both of these sites were deleted (Fig. 1H). The ability of ΔPro1/2 to associate *in vitro* with a GST-Grb2 fusion protein was assessed by pull down assays of Gab1 proteins expressed by transient transfection in Cos-1 cells. The ΔPro1/2 mutant associates with a GST-Grb2 protein to similar levels as WT Gab1 (Figs. 1B, E and F). Moreover, the ΔPro1/2 mutant coimmunoprecipitates with endogenous Grb2 to the same extent as WT Gab1 (Figs. 1A, E and F). Therefore, although the Pro1/2 sites are consensus binding sites for the Grb2 N-terminal SH3 domain, the Grb2 N-terminal SH3 domain does not associate with Gab1, and these sites are not required for the association of the C-terminal SH3 domain with Gab1.

To define the mode of Grb2 binding with Gab1, mutants were generated that delete additional proline-rich sites in Gab1 (Fig. 1H). Two of these mutants, ΔPro4 and ΔPro5, have significantly reduced association with a GST-Grb2 protein when compared to WT Gab1. A mutant that lacks both of these sites, ΔPro4/5, fails to associate with GST-Grb2 (Fig. 1B). Moreover, the ΔPro4/5 mutant fails to coimmunoprecipitate with endogenous Grb2, even though equal levels of protein are expressed (Figs. 1A, 1E). In contrast, two additional mutants ΔPro3 and ΔPro6 associate with Grb2 to similar levels as

WT Gab1, as does a ΔPH Gab1 construct. To establish that the Gab1 mutants interact with the carboxy-terminal SH3 domain (C-SH3) of Grb2, Gab1 WT and mutant proteins were used in an *in vitro* association assay with GST-Grb2 C-SH3 domain alone, or amino terminal (N-SH3) domain alone. As previously shown for WT Gab1 (22), Gab1 mutant proteins associate predominantly with the GST C-SH3 domain of Grb2 and not with the N-SH3 domain (Figs. 1C and 1D). To confirm that the C-terminal SH3 domain of Grb2 is interacting directly with the ΔPro4 and ΔPro5 sites, a Far Western assay was performed using the GST C-SH3 domain of Grb2 (Fig. 1G). Probing with anti-GST antibody revealed direct binding of the GST-C-SH3 domain fusion protein to WT Gab1 and similar binding to the ΔPro4 mutant. Less binding was observed to the ΔPro5 mutant, and none was observed to the ΔPro4/5 double mutant, indicating that the C-terminal SH3 domain of Grb2 interacts directly with Gab1 and has a preference for the ΔPro5 site.

Grb2 binding requires the proline and arginine of an atypical PXXXR motif in Gab1

The ΔPro4 mutant deletes a 10 amino acid stretch (IPDIPPPRPP) containing 3 potential PXXP motifs, whereas ΔPro5, which is located within the Met binding domain, deletes the sequence PPPVDR, which lacks a consensus PXXP motif (Fig. 1H). To define the requirements for the Grb2 SH3 domain binding to the Pro5 site, we generated alanine scanning mutants (Fig. 2A). Each of the 6 amino acids deleted in ΔPro5 was individually substituted by alanine, and the ability to bind Grb2 was analyzed using an *in vitro* GST-Grb2 association assay, as described in Fig. 1B. These mutations were made in the context of the ΔPro4 mutant to facilitate direct evaluation of the alanine substitutions. Mutants with alanine substitutions of the second or sixth amino acids, (proline 518 or arginine 522) fail to associate with a GST-Grb2 protein, identifying these amino acids as crucial for binding to the Grb2 C-terminal SH3 domain. Hence, Grb2 C-SH3 domain binding requires an atypical proline-rich motif in Gab1, PXXXR (Fig. 2B).

To assess the ability of the ΔPro5 and ΔPro4 proline-rich sites to individually associate with Grb2 and compete Gab1-Grb2 interactions, peptides corresponding to each site were generated and used in a peptide competition assay (Fig. 2C). A proline rich peptide derived from the Grb2 binding site of Sos was used as a positive control. Although Sos binds primarily to the N-terminal SH3 domain of Grb2, the same peptide

was shown previously to compete the association of Gab1 with the Met receptor, through a Grb2-dependent interaction (33). In a similar manner to the Sos derived peptide, the peptide corresponding to ΔPro4 (aa 336-349) competed Gab1-Grb2 interactions at 500 μM. Unexpectedly, a peptide corresponding to ΔPro5 (aa 515-524) failed to compete at 500 μM, whereas, as expected, a peptide corresponding to ΔPro1 (aa 450-459), failed to compete. This may indicate that although deletion of the amino acids PPPVDR at Pro5 is sufficient to disrupt binding between Gab1 and Grb2, a peptide consisting of only these amino acids may not be sufficient to compete for Gab1/Grb2 interactions.

The Pro4 and Pro5 Grb2 binding sites are conserved in Gab2

Gab2, a docking protein with structural and sequence similarity to Gab1, also associates in a constitutive manner with Grb2 (9). Alignment of the Met binding domain of Gab1 and its cognate region in Gab2 (9), demonstrated that Gab2 does not contain the two proline-rich PXXPXR motifs (Pro1/2 in Gab1) corresponding to the predicted Grb2 binding sites in Gab1. This supports our data that Pro1/2 sites are not involved in Gab1-Grb2 binding. Alignment of the ΔPro4 site with Gab2 (Fig. 3A) shows that there are 8 identical, sequential amino acids containing the sequence PPPRPPKP. This corresponds to the last 6 amino acids deleted in ΔPro4, plus two additional amino acids. The ΔPro5 site, which is located within the Met-binding domain, deletes the sequence PPPVDR, which does not contain a PXXP motif. Alignment of Gab1 with Gab2 (Fig. 3B) indicates that this site is almost identical (PPPVNR). Importantly, the alignment of Gab1 and Gab2 revealed that 13 out of 14 amino acids following the ΔPro5 site are homologous in Gab2 (Fig. 3B), possibly indicating a conserved function at this site in both Gab1 and Gab2. A peptide, Pro5*, containing these conserved 20 amino acids in Gab1 that includes Pro5, was generated and assayed for its ability to compete Gab1-Grb2 interactions (Fig. 3C). Significantly, the Pro5* peptide disrupted a Gab1/GST-Grb2 interaction when present at 50 μM (Fig. 3C), whereas a Pro5* peptide with P518 and R522 substituted with alanines (P518A/R522A), was unable to compete the binding of Gab1 from GST-Grb2 (Fig. 3D). This confirms that the proline and arginine of the PXXXR motif in the ΔPro5 mutation are required for Grb2 SH3 domain binding.

Since Gab2 contains the conserved amino acids corresponding to the Gab1-Grb2 SH3 domain binding sites, we first established if Gab2 binds the C-terminal SH3 domain in the same manner as Gab1. GST fusion proteins containing the various domains of Grb2 were incubated with lysates of HA-Gab2 and glutathione sepharose beads, and the amount of Grb2-bound Gab2 was evaluated with anti-HA (Fig. 4A). As demonstrated for Gab1, Gab2 bound predominantly to the C-terminal SH3 domain of Grb2 (compare C-SH3 versus N-SH3) (Fig. 4A). Some binding of Gab2 to the Grb2 N-SH3 domain was observed when the Grb2 SH2 domain was present, and the presence of both Grb2 SH3 domains, or the full length molecule greatly enhanced the association with Gab2. To assess whether the two conserved Grb2 binding sites in Gab1 are also involved in the binding of Gab2 to Grb2, the ability of the Pro4 and Pro5* peptides to compete Gab2/GST-Grb2 interactions was assessed in an *in vitro* association assay. As shown for Gab1, the Pro5*, Pro4, and Sos peptides competed the association of Gab2 from GST-Grb2, whereas the Pro1 or Pro5*P518A/R522A peptide failed to compete (Fig. 4 B, C).

The extended PXXXR Grb2 binding motif is required for association with the Grb2-related protein Gads, and is present in the Grb2 binding protein Slp-76

To determine whether the interaction of the Grb2 C-terminal SH3 domain with the atypical PXXXR motif could be a general mechanism for C-SH3 domains of Grb2-family proteins, we tested the Grb2-like adapter, Gads. GST-Gads fusion protein was incubated with HA-tagged WT Gab1 or mutant Gab1 expressing lysates, and glutathione sepharose beads (Fig. 5A). As in previous experiments, the ability of Gab1 mutant proteins to interact with GST-Gads protein was assessed by Western blotting with anti-HA antibodies. In contrast to Grb2, the association of Gads with Gab1 is dependent only on the ΔPro5 site, and not the ΔPro4 site.

The hematopoietic adaptor protein, Slp-76, which is unrelated to Gab1, also binds to the C-terminal SH3 domains of Grb2 (48), as well as Gads (also known as Grf40 or Grap-2) (49,50). The site of interaction with Grb2/Gads, was mapped to a region containing amino acids 224-244 of Slp-76 (48-51), and does not contain a consensus PXXP site for SH3 domain binding. Alignment of the conserved PXXXR Grb2 binding site in Gab1 and Gab2 with amino acids 232-251 of Slp-76 revealed 50% similarity (Fig.

5B). Significantly, proline 518 and arginine 522 of Gab1, which were shown to be critical for Grb2 binding (Fig. 2A), are conserved. To evaluate whether Grb2 binds to this extended PXXXR motif in Slp-76, a peptide containing amino acids 232-251 of Slp-76 was generated and used in a peptide competition assay, as in Fig. 2C. This peptide was able to disrupt the interaction between Gab1 and Grb2, as well as Gab2 and Grb2 (Fig. 5C), as efficiently as the Pro5* peptide. The consensus sequence between the three proteins is PX(3)RX(2)KPX(7)PLD, which defines a novel, non-PXXP type of interaction domain with C-terminal SH3 domains of Grb2-like adapter proteins.

Association of the EGF receptor with Gab1 is dependent on Grb2 binding sites within Gab1

Yeast-2-hybrid studies demonstrated that Gab1 was capable of interacting with the Met receptor, but not with TrkA, c-Ros, c-Neu, the insulin receptor, DDR, c-Ret, Sek-1, c-Kit, c-Abl, v-Sea, or the receptors for epidermal growth factor, colony-stimulating factor-1 and keratinocyte growth factor (25). Yet Gab1 is phosphorylated following activation of several of these receptors. The interaction with the Met receptor requires tyrosines 1349 and/or 1356 of Met, and the Met binding domain (MBD) of Gab1, which may constitute a novel type of phosphotyrosine interaction domain (25). In addition, the recruitment of Gab1 to the EGF receptor requires the MBD of Gab1 (31, 40). Since we show that the MBD of Gab1 contains a Grb2 binding site (Figs. 1A, B, and G), we assessed the requirement for Grb2 in receptor binding. The ability of a GST fusion protein of the Gab1 MBD and a protein lacking the ΔPro5 site (Gab1 MBDΔGrb2) to associate with the Met and EGF receptors was tested by an *in vitro* association assay. When overexpressed, the Met and EGF receptors are activated in the absence of growth factor stimulation and are tyrosine phosphorylated (Fig. 6B) (31). The Gab1 MBDΔGrb2 fusion protein is unable to bind Grb2 (Fig. 6A). A slight decrease in the association of the Gab1 MBDΔGrb2 fusion protein with both the processed and unprocessed forms of the Met receptor was consistently observed, when compared to the WT MBD protein (Fig. 6B). In contrast, the MBDΔGrb2 fusion protein failed to associate with the EGF receptor, whereas efficient association of the EGFR was observed with a WT MBD protein (Fig. 6B). To establish if a full length Gab1ΔGrb2 (Gab1ΔPro4/5) mutant protein

associates with the Met and EGF receptors, 293T cells were transiently co-transfected with either the full length WT Gab1 or Gab1ΔGrb2 and either the Met or EGF receptors (Fig. 6C). Consistent with the *in vitro* association assays, the Met receptor coimmunoprecipitates with both WT Gab1 and the Gab1ΔGrb2 mutant, whereas the EGF receptor only coimmunoprecipitates with WT Gab1. This suggests that Gab1 association with the EGF receptor is indirect through Grb2, whereas Gab1 is capable of associating with the Met receptor in a Grb2-independent manner.

Gab1-Grb2 association is not required for a Gab1-dependent morphogenic program

Structure-function studies with chimeric Met receptors containing the extracellular domain of the CSF-1 receptor fused to the transmembrane and cytoplasmic domains of Met, revealed that a Met receptor mutated in its Grb2 binding site (N1358H) failed to induce branching tubulogenesis of Madine-Darby canine kidney (MDCK) epithelial cells following stimulation (28). In stable MDCK cell lines, this Met mutant is impaired in its ability to associate with Gab1, and overexpression of WT Gab1 in these cells rescues the branching tubulogenesis defect in a presumably Grb2-independent manner (23). To test this, we determined whether the Gab1ΔGrb2 (ΔPro4/5) mutant is capable of rescuing the tubulogenesis defect when overexpressed in cells expressing the CSF-Met receptor Grb2 mutant (N1358H-17). Notably, whereas the parental cell lines failed to form branching tubules in response to CSF, four independent MDCK cell lines that express similar levels of HA-Gab1ΔGrb2, formed tubules in response to CSF to the same extent as cells expressing WT Gab1 (WT-3), (Figs. 7A and B).

The MBDΔGrb2 fusion protein inhibits HGF-induced cell scatter

A fusion protein encoding the Met binding domain of Gab1 was shown to inhibit HGF-induced cell scattering of MDCK epithelial cell colonies (25), possibly through its ability to compete with the WT Gab1 protein for association with the Met receptor. Alternatively, this inhibitory activity could be due to its ability to soak up the cellular pool of Grb2, hence blocking Grb2 signaling. To test this, we established whether MBDΔGrb2 could inhibit HGF-induced cell scatter of MDCK cells following microinjection. Constructs expressing Gab1 MBD WT, Gab1 MBDΔGrb2, or vector

alone, were microinjected into MDCK cells. Cells were incubated 2h to allow for protein expression and then stimulated with HGF (5 units/ml) for an additional 3-4h to induce cell spreading and dissociation before fixation. Importantly, whereas vector-injected cells spread to the same extent as non-injected cells in response to HGF, injection of Gab1 MBDΔGrb2 inhibits HGF-induced MDCK cell spreading to the same extent as the Gab1 MBD WT (Fig. 7C).

Discussion

Gab1 is a non-enzymatic docking protein that is phosphorylated in response to multiple extracellular signals and is thought to function to diversify and compartmentalize signals downstream from receptor tyrosine kinases, cytokine receptors and G-protein coupled receptors. A significant biological role for Gab1 is supported by observations that overexpression of Gab1 in neuronal cells promotes cell survival, neurite outgrowth, and DNA synthesis downstream from the TrkA receptor (34,39), and that Gab1 promotes epithelial cell morphogenesis downstream from the Met receptor (23). The recruitment of Gab1 to receptor tyrosine kinases and other receptors and its subsequent phosphorylation is poorly understood. Recruitment of Gab1 to the Met or EGFR requires in part a Grb2 binding site in the receptor and the proline rich Met binding domain in Gab1 (22,25,26,31,33). Both Gab1 and Gab2 associate constitutively with the Grb2 adapter protein, supporting a role for Grb2 in the indirect recruitment of Gab1 and Gab2 to receptor tyrosine kinases.

By combining approaches such as deletion analyses, alanine scanning mutagenesis and peptide competition, we have identified two Grb2 C-terminal SH3 domain binding sites in Gab1. One site contains several PXXP motifs, which are requirements for SH3 domain interactions (42-44). The other site contains an atypical non-PXXP motif, including a PXXXR motif critical for binding the Grb2 C-terminal SH3 domain. Binding sites for SH3 domains invariably contain a PXXP motif. Proline-rich sequences favor the formation of the polyproline (PP-II) helix, and form hydrophilic patches on the surface of proteins, enabling the ligand to interact with the elongated patch of aromatic residues in the ligand-binding face of the SH3 domain (reviewed in (52)). Gab1 contains two PXXPXR motifs that are class II, PP-II left-handed helical sequences known to bind to SH3 domains, including the amino terminal SH3 domain of Grb2 (43,45-47). However, deletion of both of these sites, ΔPro1/2, (452-PNSPPR-457, and 501-PKTPPR-506), had no effect on the binding of Gab1 to Grb2 by an *in vitro* pulldown assay (Fig. 1A), or by co-immunoprecipitation (Fig. 1B). Moreover, neither of the predicted PXXPXR Grb2 binding sites are conserved in the Gab1-like protein, Gab2 (9), which associates with the C-terminal SH3 domain of Grb2 (Fig. 3D). Although PXXPXR is considered a general consensus for class II-type ligands of SH3 domains, a

more specific motif is q-P-X-q-P-X-R, where q represents a hydrophobic residue, often proline, valine or leucine. The q-Pro sequence is crucial for intercalation with conserved hydrophobic residues of binding sites 1 and 2 on the surface of SH3 domains (reviewed in (52)). Notably, the two predicted PXXPXR Grb2-binding sites in Gab1 contain relatively hydrophilic, polar residues (Ser, Thr) in the "q" site, which could make these poor binding sites for the Grb2 SH3 domains, and would correlate with the observation that these do not function as Grb2 SH3 domain binding sites.

Further deletions of proline-rich regions in Gab1, demonstrated that deletion of two sites, ΔPro4, (IPDIPPPRPP) or ΔPro5, (PPPVDR), completely eliminated the association of Gab1 with Grb2 (Figs. 1A, B, C, and 2A), whereas deletion of either site alone severely diminished Gab1-Grb2 interactions (Figs. 1A, B, C). Alignment of these sequences with the Gab1-related protein, Gab2 (Fig. 3A) reveals a stretch of 8 identical amino acids, PPPRPPKP, corresponding to Pro4 that contains 3 potential consensus PXXP binding sites for Grb2 SH3 domains. Consistent with this a peptide corresponding to Pro4, (TIPPPRPPKPHP) was able to disrupt the interaction between Gab1 or Gab2 and GST-Grb2 (Figs. 2C and 3E) and supports previous findings that the C-terminal Grb2 SH3 domain can interact with a proline-rich PXXP motif in Sos and other proteins (33,43,53-55).

Although the majority of ligands for SH3 domains contain a conserved PXXP motif, a proline-X-X-aspartate-tyrosine (PXXDY) consensus was recently shown to be indispensable for binding to the SH3 domain of Eps8 (56). Similarly, the ΔPro5 motif (PPPVDR) required for binding the Grb2 C-terminal SH3 domain, which is located within the Met Binding Domain, does not contain a consensus PXXP motif. From alanine scanning mutagenesis we have established that the second or sixth amino acids in this sequence, proline 518, and arginine 522 are crucial for Grb2 binding (Fig. 2A). In addition to Grb2, Gab1 was shown to interact with the C-terminal SH3 domain of the Grb2-like adapter protein, Gads (57), and this is dependent on only the ΔPro5 binding site (Fig. 4A). The alignment of Gab1 with Gab2 demonstrated that a stretch of 20 amino acids that contains the PXXXR motif, is highly conserved between Gab1 and Gab2, (17 out of 20 amino acids) (Fig. 3B). Moreover, the hematopoietic docking protein, Slp-76, which is unrelated to Gab1 or Gab2, also binds to the C-terminal SH3 domains of Grb2

(48), as well as Gads (50,58). The site of interaction with Grb2 and Gads has been mapped to a region that does not contain a consensus PXXP site for SH3 domain binding (48-51). Alignment of this domain in Slp-76 with the conserved 20 amino acids in Gab1 and Gab2 reveals a consensus between the three proteins, PX(3)RX(2)KPX(7)PLD, that contains the PXXXR site. However, this motif does not bind all Grb2 related proteins. The Grb2 related protein Grap, interacted only minimally with Gab2 (59) or Gab1 (data not shown).

The extended PX(3)RX(2)KPX(7)PLD motif may therefore represent a common interaction motif for the C-terminal SH3 domains of Grb2 and Gads proteins. The binding of Gab1 with GST-Grb2 was competed only by a peptide corresponding to the 20 amino acid Pro5* or SLP76 sequence and not by a peptide corresponding to the ΔPro5 sequence (CEPPPVDRNL), (Figs. 3C and E and 4C). Importantly a Pro5* peptide with alanine substitutions for the proline and arginine residues of the PXXXR motif fails to compete. This suggests that the interaction of Gab1 and Gab2 with the Grb2 C-terminal SH3 domain requires the proline and arginine residues plus additional contacts within this conserved region. Consistent with this, residues outside of the minimal SH3 domain binding motif (PXXP) have been shown to contribute significantly to the affinity of interaction (60). For example a full length Nef protein can bind to SH3 domains 300 times more efficiently than a peptide corresponding to the known PXXP region in Nef (61). Since Grb2 also associates with the more typical proline-rich ΔPro4 site, this may indicate that its C-terminal SH3 domain is more promiscuous than that of Gads. This supports the observations that the C-terminal SH3 domain of Grb2 is known to bind typical PXXP motifs found in Sos (43,53,54,62), and a PPPP motif in Vav (55), whereas the Gads C-terminal SH3 domain does not interact with these proteins.

Although Gab1 is phosphorylated in response to multiple factors that activate receptor tyrosine kinases, including HGF, EGF, insulin and NGF (8,25,34), the mechanism of recruitment of Gab1 to receptor tyrosine kinases is unclear. Recruitment of Gab1 to the Met and EGF receptors requires in part a Grb2 binding site in the receptor and the Gab1 MBD (22,23,31,33), suggesting a possible indirect mechanism of recruitment. In addition, a direct association of the Met receptor with the Gab1 MBD was implicated from a yeast two hybrid approach and by Far Western analysis (22,25).

However, the significance of either mode of recruitment had not been examined. Consistent with an indirect mode of recruitment, a Gab1 MBD fusion protein shows efficient association with the EGF receptor, whereas a Gab1 MBDΔGrb2 fusion protein or Gab1DGrb2 mutant fails to associate or coimmunoprecipitate with the EGF receptor (Figs. 5B and C). In contrast to this, the Gab1 MBDΔGrb2 fusion protein or Gab1ΔGrb2 protein is still capable of association or coimmunoprecipitation with the Met receptor (Figs. 5B and C). This supports yeast two hybrid data and demonstrates that the Gab1 MBD and a full length Gab1 protein can associate with the Met receptor independently of Grb2 *in vivo*.

The overexpression of a Gab1 MBDΔGrb2 fusion protein by microinjection, blocked HGF-induced spreading and dispersal of MDCK cells in a similar manner to overexpression of a WT Gab1 MBD fusion protein (Fig. 6C) (25). This indicates that the inhibition of cell scatter is not due to the ability of the Gab1 MBD to compete for the cellular pool of Grb2 and may be due to the ability of the Gab1 MBD to associate with the Met receptor and compete with the endogenous Gab1 protein. Moreover, the ability of the Gab1ΔGrb2 mutant to become phosphorylated downstream from the Met receptor, and rescue the branching tubulogenesis defect of a Met receptor mutant lacking a Grb2 binding site (N1358H) (Figs. 5C and 6A), indicates that Grb2/Gab1 interactions are not essential for this event. This is not unexpected since a Met receptor that lacks a Grb2 binding site is still capable of recruiting Gab1 when overexpressed (23).

It remains unclear as to why Gab1 has two Grb2 SH3 domain binding sites. This may promote the association of two Grb2 SH2 domains with two phosphotyrosine residues. The Met receptor contains only one Grb2 SH2 domain binding site at Y1356, whereas the EGF receptor or the adapter protein, Shc, which is recruited to and phosphorylated by the Met receptor, contain two Grb2 SH2 domain binding sites. This raises the possibility that the formation of a $(Grb2\text{-}SH3)_2$-Gab1 complex may promote an association of the Grb2 SH2 domains with the twin Grb2 binding sites on the EGFR or Shc, as suggested for Grb2-Shc interactions with the inositol phosphatase, SHIP (63).

Taken together, our data indicate that the extended PXXXR containing motif found in, Gab1, Gab2 and Slp-76, is a new binding motif for the C-terminal SH3 domains of Grb2/Gads proteins. Importantly, we show that the recruitment of Gab1 to the EGF

receptor is dependent on this site, whereas the recruitment of Gab1 to the Met receptor can occur through a Grb2 independent mechanism. A direct versus indirect mode of recruitment of Gab1 to the Met and EGFR, may reflect a more stable association with the Met receptor. This is consistent with the prolonged and elevated phosphorylation of Gab1 observed following activation of the Met but not the EGF receptor (Fig. 5C and (23)), and the ability of Gab1 to promote branching tubulogenesis only in response to HGF and not in response to EGF (23). However, it still remains to be determined whether Gab1 can be directly recruited to other receptors, and what role the direct recruitment of Gab1 might play in the biological function of the Met receptor.

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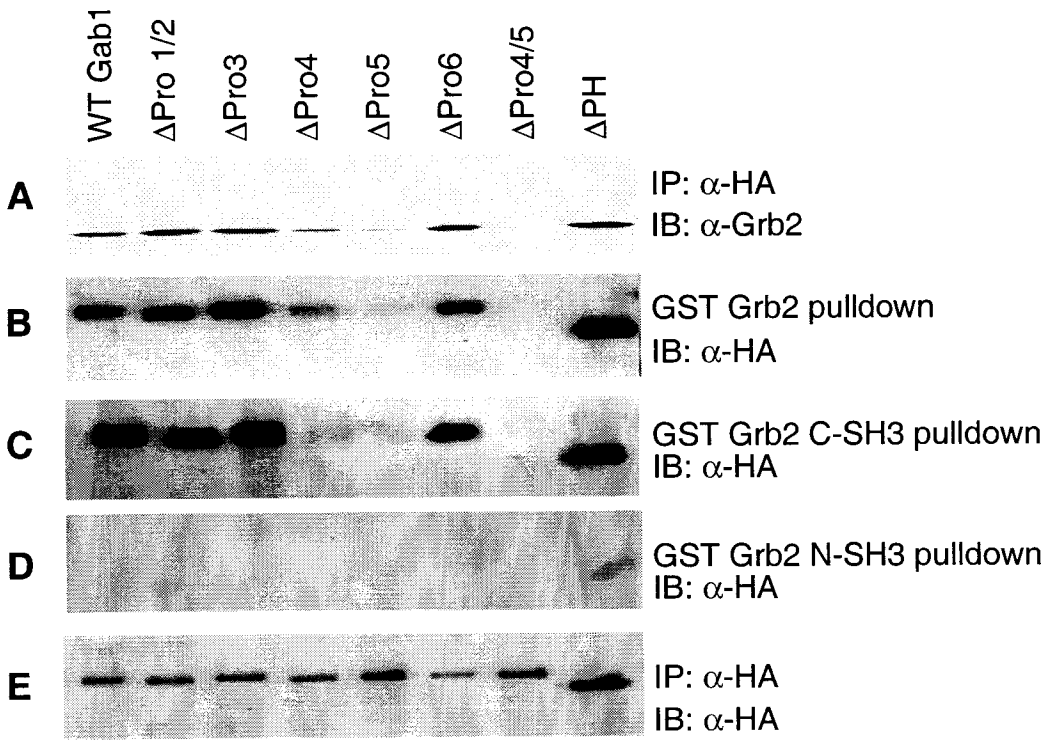
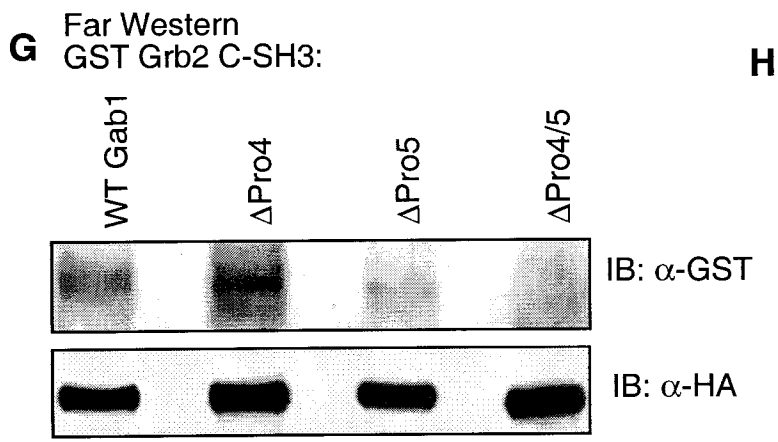
Fig. 1. Mapping of Grb2 SH3 domain binding sites in Gab1.

Cos-1 cells were transiently transfected with HA-tagged WT Gab1 or HA-tagged Gab1 deletion mutants, and lysed in buffer containing 0.5% Triton X-100. (A) Gab1 was immunoprecipitated from the indicated cell lysate with anti-HA, proteins were subjected to Western blot and analyzed for presence of bound Grb2 proteins using anti-Grb2 antibodies. (B) GST-Grb2 fusion protein immobilized on glutathione sepharose beads was incubated with the indicated cell lysate. Bound HA-Gab1 proteins were detected by Western blot analysis using anti-HA antibody. (C) and (D) The ability of Gab1 mutants to associate with GST-Grb2 C-SH3 and N-SH3 fusion proteins was determined, as in (A). (E) The equal expression of Gab1 mutant proteins was determined through immunoprecipitation and Western blot analysis with anti-HA antibody. (F) Summary of interactions of proline deletion mutants with Grb2 through *in vitro* binding and co-immunoprecipitation. (G) Far Western assay. Lysates containing HA-WT Gab1 or mutant Gab1 were immunoprecipitated with anti-HA, as in Fig. 1A, and the membrane was probed overnight with GST Grb2 C-SH3 fusion protein (300 μg). Direct binding of the fusion protein to Gab1 proteins in the membrane was revealed with anti-GST antibody, and levels of Gab1 protein was determined by stripping and reprobing with anti-HA antibody. (H)Schematic representation of Gab1 deletion mutants - amino acids deleted are indicated.



F

Gab1	Grb2	
	In vitro binding	Co-immuno precipitation
ΔPro1/2	+++	+++
ΔPro3	+++	+++
ΔPro4	+	+
ΔPro5	+/-	+/-
ΔPro6	+++	+++
ΔPro4/5	-	-
ΔPH	+++	+++



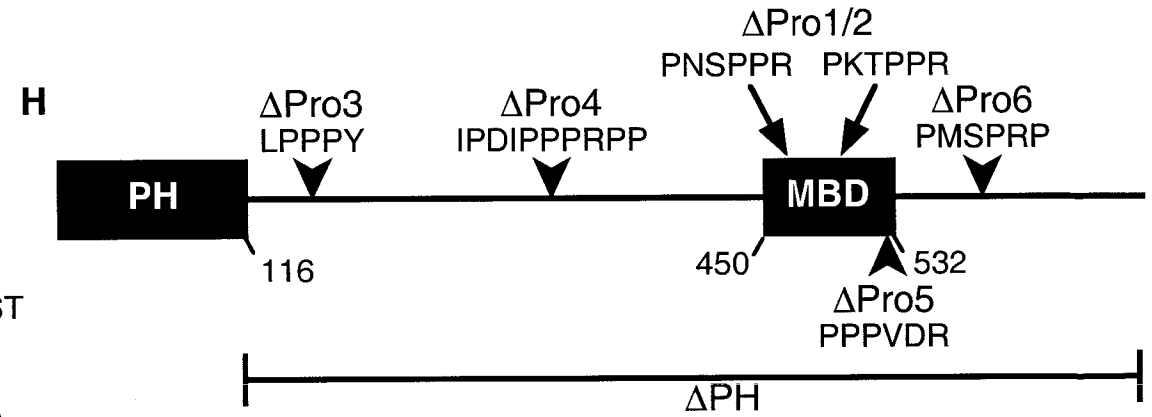
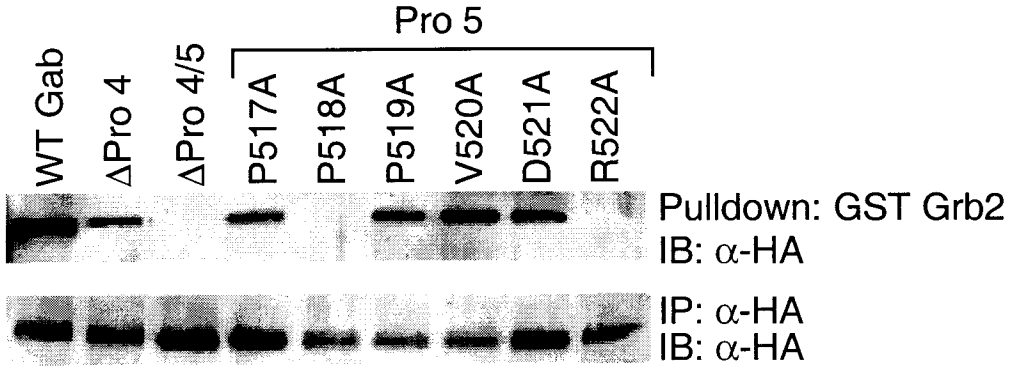


Fig. 2. Grb2 binding requires an atypical PXXXR motif in Gab1.

(A) The six amino acids deleted in ΔPro5 were individually mutated to alanine in the context of the ΔPro4 mutant, and mutant proteins were tested for their ability to associate with a GST-Grb2 fusion protein by *in vitro* association assay. The equal expression of Gab1 mutant proteins was determined through immunoprecipitation and Western blot analysis with anti-HA antibody. (B) Summary of amino acids required for Grb2 association. (C) Competition of Gab1 from Grb2 using peptides of the Grb2-binding sites. Different concentrations of peptides as indicated, were incubated with the GST-Grb2 fusion protein plus glutathione sepharose beads for one hour prior to addition of cell lysate containing Gab1 proteins. The peptides Pro5, Pro4, SOS, and Pro1 are described in Materials and Methods.

A



B

Gab1	Grb2		
	In vitro binding		
P517A	+	X	
P518A	-	P	consensus
P519A	+	X	
V520A	+	X	
D521A	+	X	
R522A	-	R	

C

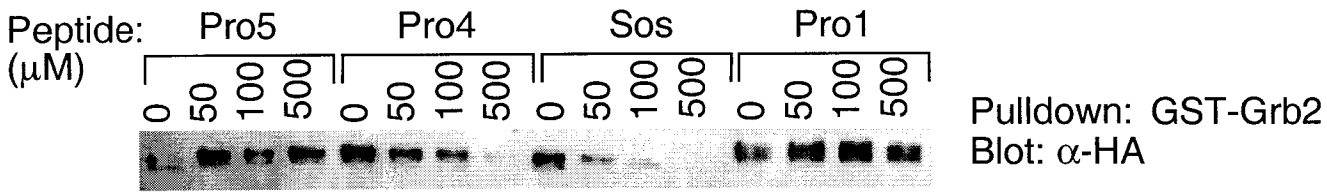
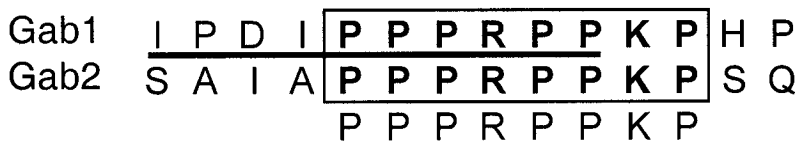


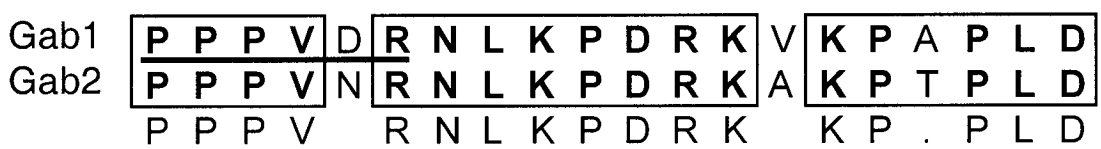
Fig. 3. The Pro4 and Pro5 Grb2 binding sites are conserved in Gab2.

(A) An alignment of ΔPro4 site in Gab1 with Gab2 is provided and the deleted region in Gab1 is underlined (amino acids 337-346). (B) Alignment of conserved region in the Met-binding domain of Gab1 with Gab2 with amino acids deleted in ΔPro5 (517-522) underlined. (C) A peptide, Pro5∗, corresponding to the conserved 20 amino acid region in Gab2 and the MBD of Gab1 competes for Gab1/Grb2 association. The *in vitro* binding assay was carried out with GST-Grb2 or GST alone, using peptide Pro5∗. (D) Different concentrations of the peptides Pro5∗ and Pro5∗ with Pro518 and Arg522 mutated to alanine (P518A/R522A) were used with GST-Grb2 and Gab1 lysates in an *in vitro* binding peptide competition assay.

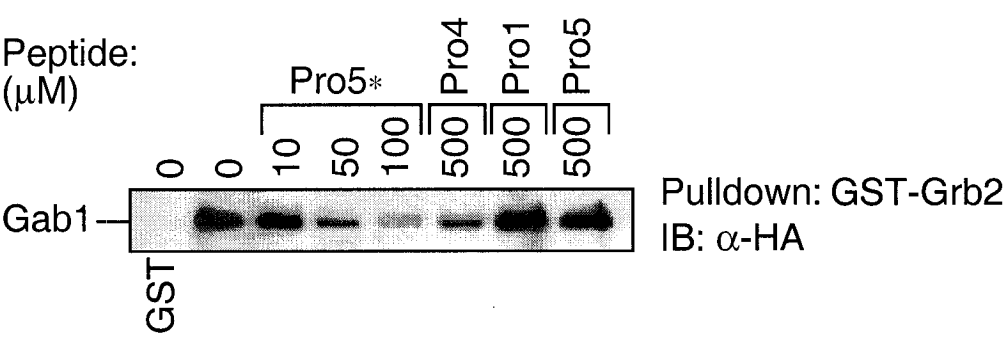
A


ΔPro4
Gab1 I P D I P P P R P P K P H P
Gab2 S A I A P P P R P P K P S Q
P P P R P P K P

B


ΔPro5
Gab1 P P P V D R N L K P D R K V K P A P L D
Gab2 P P P V N R N L K P D R K A K P T P L D
P P P V R N L K P D R K K P . P L D

C


Peptide:
(μM)
Pro5*
Pro4
Pro1
Pro5
0 0 10 50 100 500 500 500
Gab1
GST
Pulldown: GST-Grb2
IB: α-HA

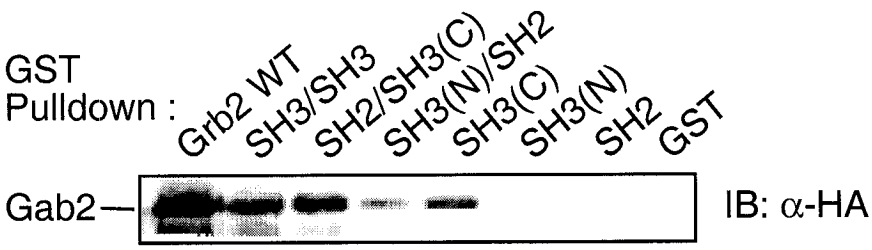
D


Pro5*
Peptide:
(μM)
Pro5*
P518A/R522A
0 0 10 50 100 500 10 50 100 500
Gab1
GST
Pulldown: GST-Grb2
IB: α-HA

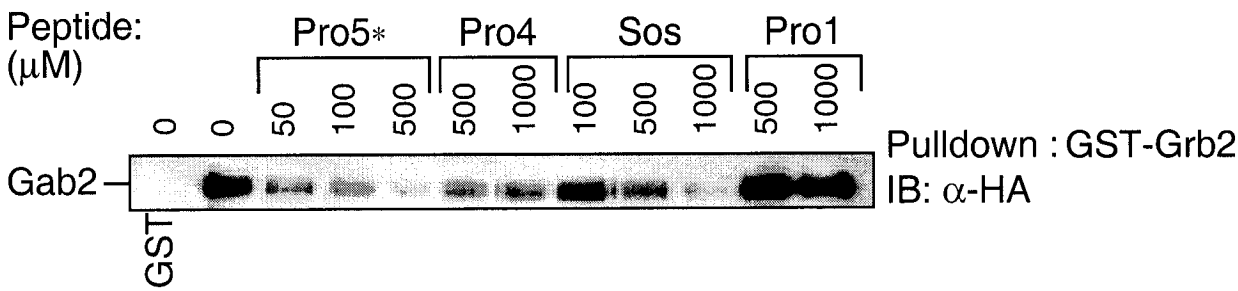
Fig. 4. Recruitment of Grb2 to Gab2 is competed by the extended PXXXR containing peptide.

(A) GST fusion proteins of WT Grb2 or the various domains of Grb2, as indicated, were incubated with cell lysates containing HA-tagged Gab2. Bound HA-Gab2 protein was detected by Western blot analysis using anti-HA antibody. (B and C) Different concentrations of the peptides indicated were incubated with GST-Grb2 fusion protein and glutathione sepharose beads for one hour, prior to addition of the cell lysate containing HA-Gab2 proteins.

A


GST
Pulldown :
Grb2 WT
SH3/SH3
SH2/SH3(C)
SH3(N)/SH2
SH3(C)
SH3(N)
SH2
GST
Gab2
IB: α-HA

B


Peptide:
(μM)
Pro5*
Pro4
Sos
Pro1
0
0
50
100
500
500
1000
100
500
1000
500
1000
Gab2
GST
Pulldown : GST-Grb2
IB: α-HA

C

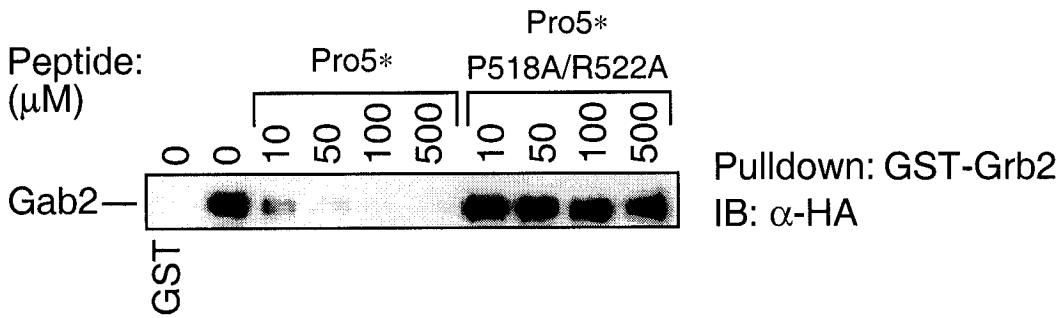
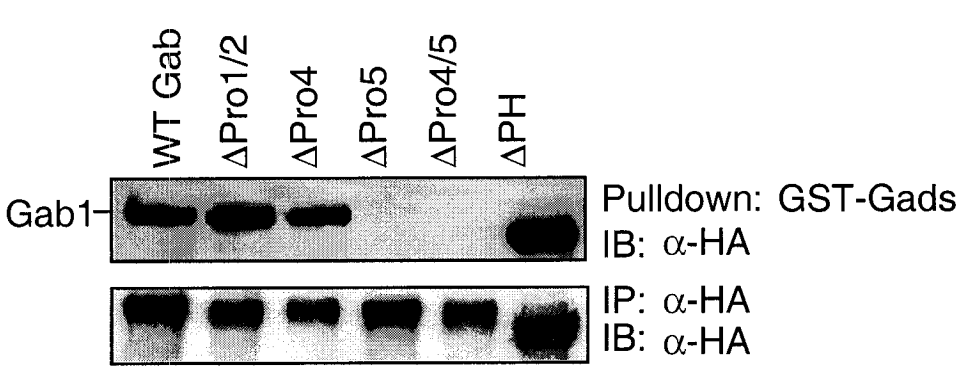

Pro5*
Peptide:
(μM)
Pro5*
P518A/R522A
0
0
10
50
100
500
10
50
100
500
Gab2
GST
Pulldown: GST-Grb2
IB: α-HA

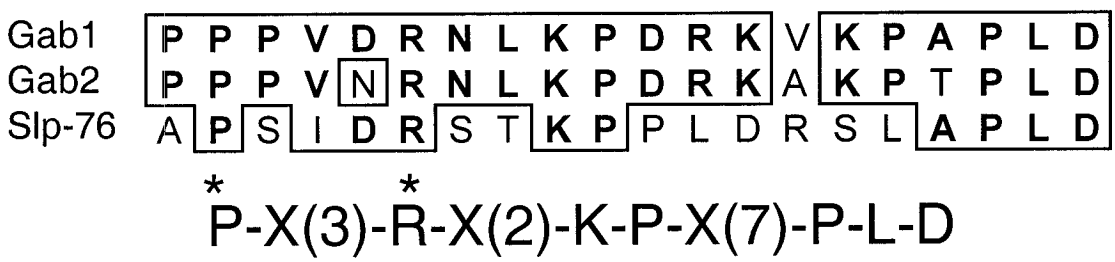
Fig. 5. The extended PXXXR containing Grb2 binding motif is required for association with the Grb2-related protein Gads, and is present in the Grb2 binding motif of Slp-76.

(A) GST-Gads fusion protein (0.5 μg) was incubated with cell lysates expressing the indicated Gab1 mutant proteins, and glutathione sepharose beads. Bound HA-Gab1 protein was detected by Western blot analysis using anti-HA antibody. Equal expression of Gab1 proteins was determined through immunoprecipitation and Western blot analysis with anti-HA. (B) Alignment of the conserved Grb2 binding site in Gab1 and Gab2 with Slp-76 (Genbank™ accession number g1083554). Asterisks indicate the residues shown by alanine scanning to be required for Gab1 association with Grb2. (C) A 20 amino acid peptide from Slp-76 was used to compete the association of Gab1 and Gab2 with Grb2. Different concentrations of peptides as indicated, were incubated with the GST-Grb2 fusion protein plus glutathione sepharose beads for one hour prior to addition of cell lysate containing HA-Gab1 or HA-Gab2 proteins. The peptides Slp-76, Pro4, Pro5*, and Pro1 are described in Materials and Methods.

A


WT Gab
ΔPro1/2
ΔPro4
ΔPro5
ΔPro4/5
ΔPH
Gab1
Pulldown: GST-Gads
IB: α-HA
IP: α-HA
IB: α-HA

B


Gab1 P P P V D R N L K P D R K V K P A P L D
Gab2 P P P V N R N L K P D R K A K P T P L D
Slp-76 A P S I D R S T K P P L D R S L A P L D
* *
P-X(3)-R-X(2)-K-P-X(7)-P-L-D

C

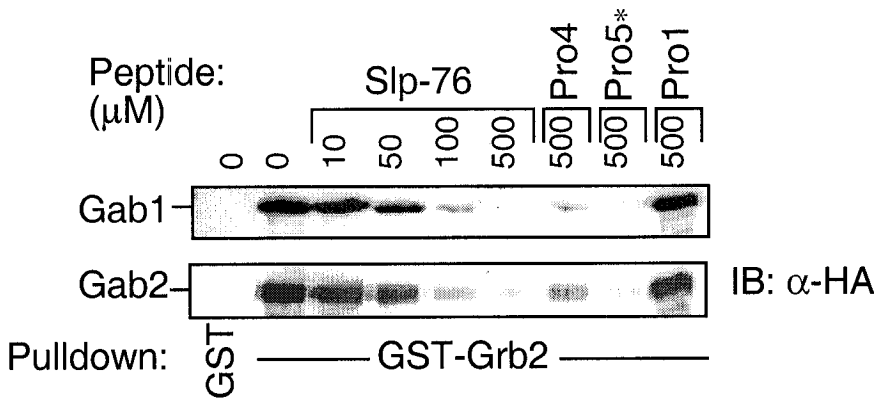

Peptide: (μM)
Slp-76
Pro4
Pro5*
Pro1
0 0 10 50 100 500 500 500 500
Gab1
Gab2
IB: α-HA
Pulldown: GST GST-Grb2

Fig. 6. Association of the EGF receptor with Gab1 is dependent on Grb2 binding sites within Gab1.

(A) A Gab1 MBD fusion protein lacking the ΔPro5 site does not bind Grb2. Lysate from Cos-1 cells transiently transfected with a Grb2 expression construct was incubated with GST protein alone, GST-MBD or GST-MBDΔGrb2 fusion proteins. Proteins collected on glutathione sepharose beads, as well as proteins from total cell lysate, were separated by SDS-PAGE, transferred to nitrocellulose then immunoblotted with anti-Grb2 antibody. (B) A Gab1 MBDΔGrb2 fusion protein still associates with the Met receptor, but not the EGFR. Lysates prepared from cells transiently transfected with either Met receptor (200μg), or EGFR (1 mg) were incubated with GST protein alone, GST-MBD or GST-MBDΔGrb2 fusion proteins. The pulldown reactions were split and run on two separate gels, along with total cell lysates. One gel (top panel) was probed with anti-phosphotyrosine antibody to show amount of receptor bound to fusion proteins, while the other (lower panel) was probed with anti-GST to show equal levels of fusion proteins used in pulldown assays. (C) 293T cells were transiently co-transfected with either the Met receptor or EGF receptor with or without HA-WT Gab1 or HA-Gab1ΔGrb2. Lysates were immunoprecipitated with anti-HA antibody and probed with either anti-Met (DL-21) or anti-pTyr to show amount of receptor able to co-immunoprecipitate with Gab1 or Gab1ΔGrb2. Total cell lysates were probed with either anti-Met, anti-pTyr or anti-HA to show equal expression levels.

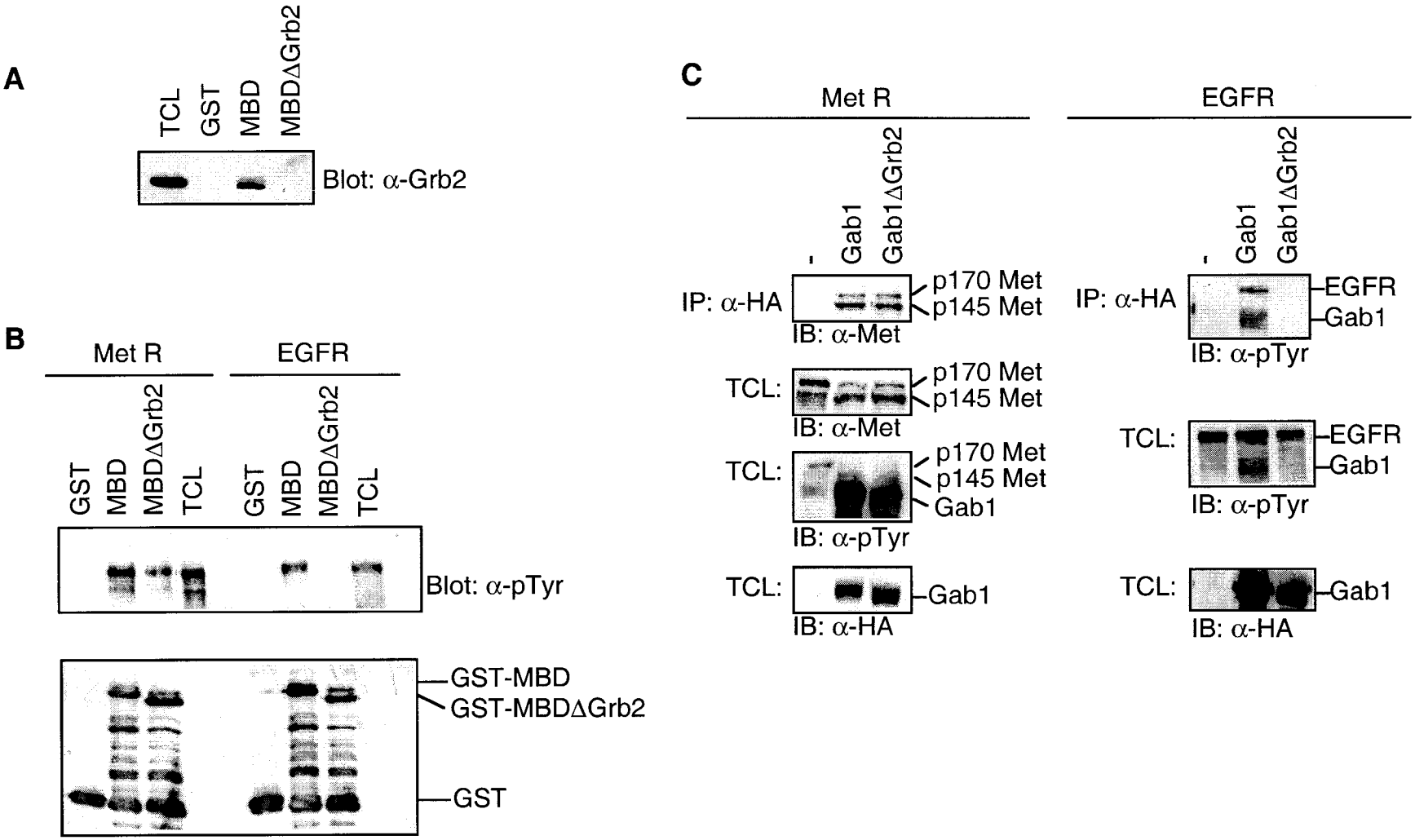
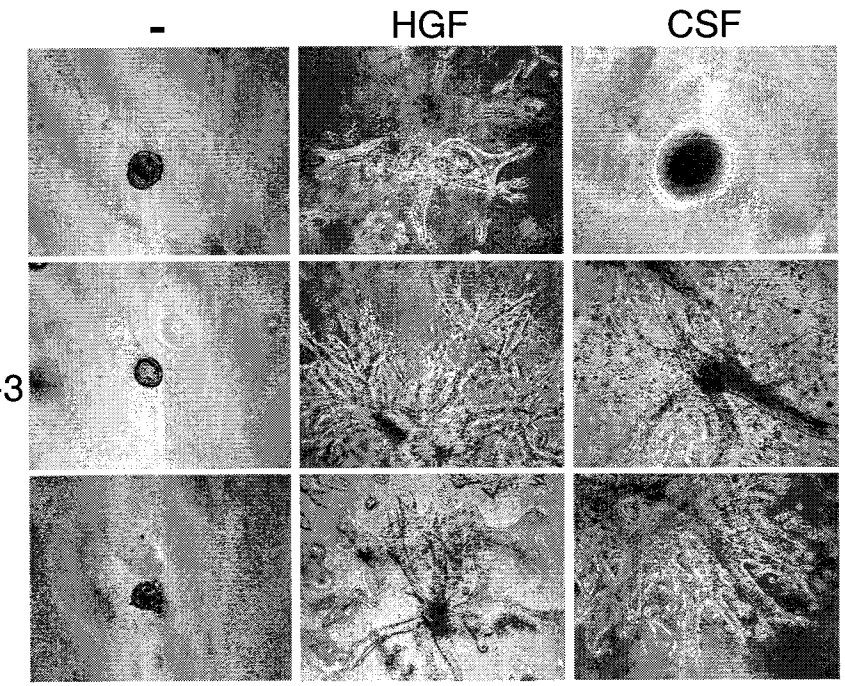

A
TCL
GST
MBD
MBDΔGrb2
Blot: α-Grb2
B
Met R
EGFR
GST
MBD
MBDΔGrb2
TCL
Blot: α-pTyr
GST-MBD
GST-MBDΔGrb2
GST
C
Met R
EGFR
-
Gab1
Gab1ΔGrb2
IP: α-HA
p170 Met
p145 Met
IB: α-Met
TCL:
Gab1
IB: α-pTyr
IB: α-HA
EGFR

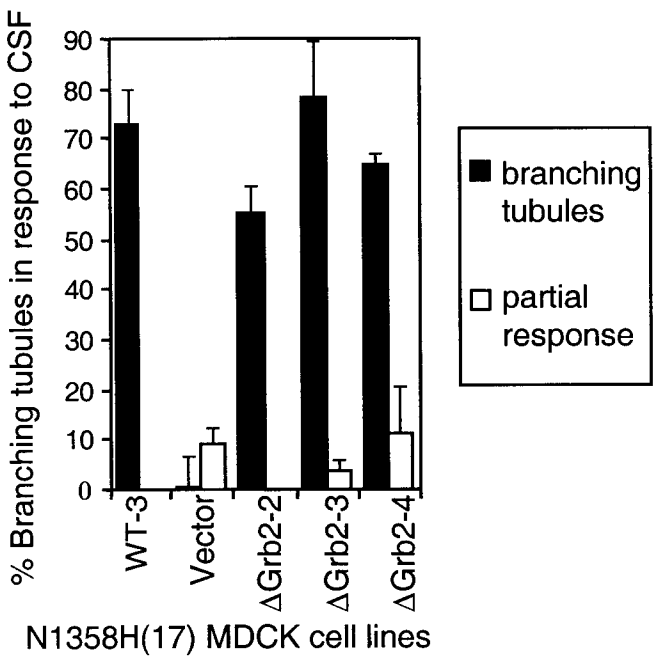
Fig. 7. Gab1-Grb2 association is not required for a Gab1-dependent morphogenic program.

(A) MDCK cell lines co-expressing CSF-Met N1358H-17 and either vector, WT Gab1 or Gab1ΔGrb2 were seeded in collagen and allowed to form cysts for 7 days in Liebowitz medium supplemented with 5% fetal bovine serum, after which, 15 units of rhHGF or rhCSF-1 was added to the media. Tubules were apparent by light microscopy 5 to 10 days after the addition of stimuli. A representative cell line for each group is shown. (B) Quantitation of the tubulogenic response following stimulation with rhCSF-1 was undertaken as described in Materials and Methods. WT Gab1, vector control and three representative lines of Gab1ΔGrb2 are shown. The responses are plotted as the percentage of cysts that have undergone branching tubulogenesis. The values were derived from three independent experiments. None of the cysts formed tubules in the absence of stimulation and all cell lines formed approximately 70% branching tubules upon HGF stimulation. A partial response is intermediate between a cyst and a tubule structure whose length is five times the width. (C) Small colonies of MDCK cells were partially microinjected with expression vectors (200 μg/ml) for MBD, MBDΔPro5 or vector (PRK5) and co-injected with rabbit immunoglobulin G (2 mg/ml) to mark injected cells. The cells were incubated 2 h to allow for protein expression and further incubated in the presence of HGF (5 units/ml) for an additional 3-4h before fixation.

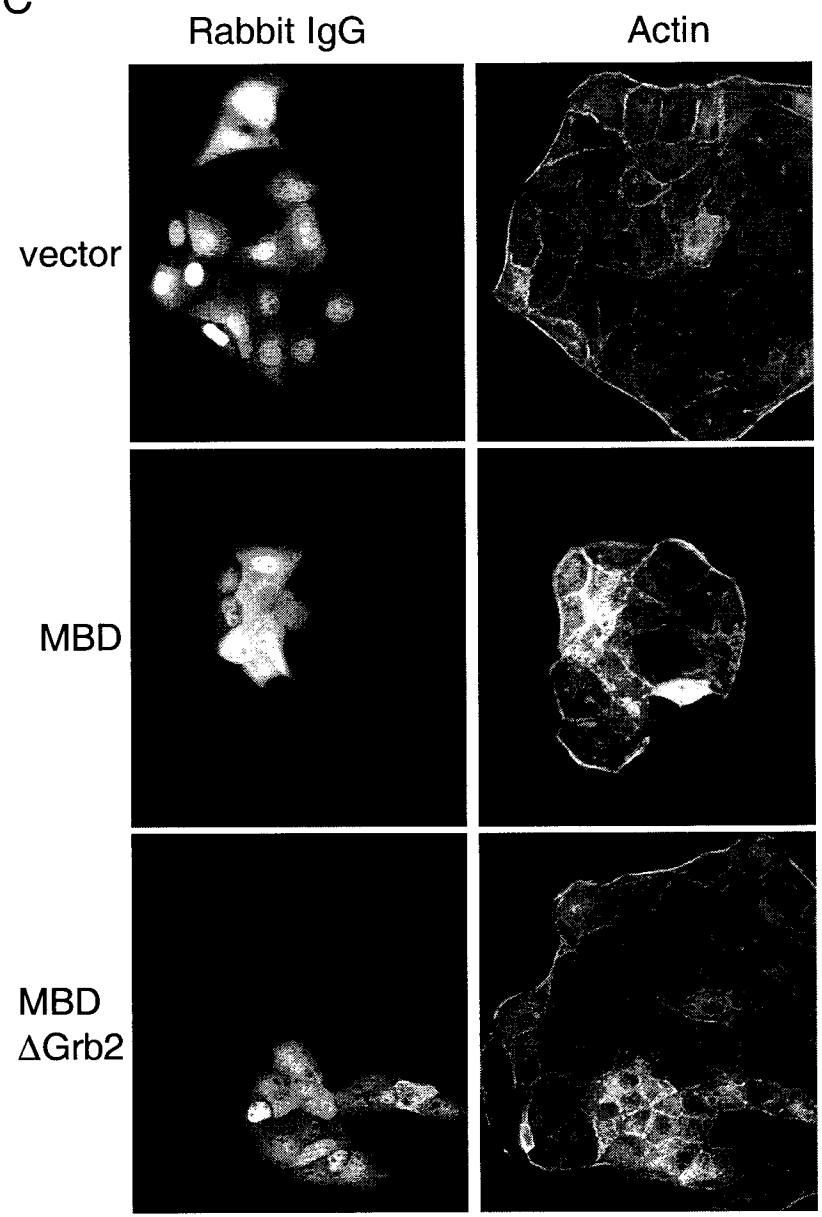
A


-
HGF
CSF
N1358H
N1358H
WT Gab1-3
N1358H
Gab1
ΔGrb2-4

B


% Branching tubules in response to CSF
90
80
70
60
50
40
30
20
10
0
WT-3
Vector
ΔGrb2-2
ΔGrb2-3
ΔGrb2-4
N1358H(17) MDCK cell lines
branching tubules
partial response

C


Rabbit IgG
Actin
vector
MBD
MBD
ΔGrb2

Chapter III: Distinct recruitment and function of Gab1 and Gab2 in Met-receptor mediated epithelial morphogenesis.

Preface

The previous chapter describes the identification of two highly conserved Grb2 C-terminal SH3 domain binding sites in Gab1, as well in the recently identified family member Gab2. Gab1 and Gab2 have highly homologous PH domains; contain tyrosine residues within a consensus for recruitment of various signaling proteins, and are both phosphorylated in response to similar growth factors and cytokines. Studies of knockout mice suggest that Gab1 and Gab2 have distinct functions during development; Gab1 -/- mice are embryonic lethal, whereas Gab2 -/- mice are deficient in the allergic response. However, 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 recruitment and function in epithelial morphogenesis downstream of the Met receptor tyrosine kinase.

Abstract

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 if 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 utilized chimeric 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; Fournier *et al.*, 1996; Bardelli *et al.*, 1997; Fixman *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).

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 and Daughter of Sevenless (DOS), belong to a group of docking proteins, including Insulin Receptor Substrates 1-4, Downstream of Kinases (Dok) 1-5, and FGF 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). These proteins lack enzymatic activities, but following 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). Following stimulation with HGF, Gab1 couples with the p85 subunit of 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 PLCγ1, 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 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 PI3K-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 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-2-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 recently identified (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 EGF, IL-6, IL-3, TPO, and EPO 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 has not been evaluated.

We show that both Gab1 and Gab2 are expressed in MDCK epithelial cells, show a similar localization to cell-cell junctions, and associate with similar signaling proteins following 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.

Experimental Procedures

Plasmids and Mutagenesis - WT Gab1-pCDNA 1.1-HA, Gab1ΔGrb2, GST-MBD (pGEX 2TK) and GST-MBDΔGrb2 were described previously (Lock *et al.*, 2000). All mutants were generated with the Quik Change mutagenesis kit (Stratagene) unless otherwise noted, and sequenced prior to use. All primers are written 5' to 3'. Gab2ΔGrb2-pEBB-HA was generated by making the mutations Δ348-355/P500A/R504A in Gab2-bluescriptKS+ using the primers: P518A/R504A-F, GAGATCCAGCCAGCCCCTGTCAACGCAAACCTCAAGCC; P518A/R504A-R, GGCTTGAG-GTTTGCGTTGACAGGGGCTGGCTGGATCTC; Δ348-355-F, CTGGAGATTCAGCGATCGCT-AGTCAGGCAGAAACATC; Δ348-355-R, GATGTTTCTGCCTGACTAGCGATCGCTGAATC-TCCAG. Gab2ΔGrb2 was then inserted back into the pEBB-HA vector. GST-Gab2MBDΔGrb2 was generated using primers P500A/R504A. The Δ3P mutation (deletes prolines 491-493) was generated in WT Gab1, Gab1ΔGrb2, GST-Gab1MBD and GST-Gab1MBDΔGrb2 using the primers: Δ491-493-F, CCTTTGGAATGCAAGTCGCTCACATGG-GCTTCAGGTCC and Δ491-493-R GGACCTGAAGCCCATGTGAGCGACTTGCATTCCAA-AGG. Alanine scanning mutagenesis on WT Gab1 pCDNA1.1 was performed with the Chameleon Double-Stranded mutagenesis kit (Stratagene), using primers: G487A, GGACCTTTGACTTTTCAAGCTTTGCAATGCGAGTCC-CTCCTCC; R489A, GGACCTTTGACTTTTCAAGCTTTGGAATGGCAGTCCCTCCTCCTG-CTC; P491A, CTTTTCTTCCTTTGGCATGCGAGTCGCTCCTCCTGCTCATATGG; P492A, CTTCCTTTGGAATGCGGGTACCTGCTCCTGCTCATATGGGCTTC; P493A, CTTCCTTTG-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 NheI site into Gab1MBD at nucleotide 1458 (aa 484 Ser to Ala). Gab2MBDXbaI-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 Gab1MBDNheI 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 to 496, replaces aa 476-478 of Gab2) was generated by creation of a 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 Gab2MBDNdeI. 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 MBD2NdeI 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, using primers: 484-502-F, CTCCTGCTCATATGGGCTTCCGATCGAGTCCACTTCCTATTCA-CAGAGGC; and 484-502-R, GCCTCTGTGAATAGGAAGTGGACTCGA-TCGGAAGCCCATATGAGCAGGAG. MBD2/1/2*-Δ3P was generated from MBD2/1/2* with the primers: 2/1/2Δ491-F, CTTCCTTTGGAATGCG-AGTCGCTCACATGGGCTTCCGATCGAGTCC; 2/1/2Δ491-R, GGACTCGATC-GGAAGCCCATGTGAGCGACTCGCATTCCAAAGGAAG. 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 $1x10^6$/ 100-mm petri dish and transfected 24h later by the calcium phosphate precipitation method (Wigler *et al.*, 1979) with 2 mg of DNA. Cos-1 cells were seeded at $8x10^5$/ 100-mm petri dish and transfected 24 h later with 6μg of plasmid DNA by a standard DEAE-dextran precipitation method as described previously (Rodrigues *et al.*, 1991). For co-transfections of Met receptor with Gab DNA's, 3 μg of Met receptor DNA and 1 μg of Gab DNA was used. Cos-1 and 293T cells were serum starved in 0.1% FBS for 24h, 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 mg aprotinin/ml, 10 mg leupeptin/ml, 1 mM Phenylmethylsulfonyl fluoride, 1 mM sodium fluoride, and 1 mM sodium vanadate). Following 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 colony-stimulating factor-1 (CSF-1) Met receptor and mutants thereof by retroviral infection have 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 per 100mm-dish. At 24h later, they were washed twice with DMEM and then starved for 24h in 10 ml of DMEM containing 0.02% FBS. HGF was added at 100U/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.

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 μg) immobilized on glutathione sepharose beads were incubated with either 300 mg of lysate from 293T cells transiently expressing the Met receptor, or 350 μg 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 hours 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 $1x10^4$ for 3 days in DMEM containing 10% serum on glass coverslips in a 24-well dish. For stimulations, $5x10^4$ cells were plated overnight in 10% serum-containing medium, washed two times and serum starved in 0.02% serum for 6 hr, and stimulated with 50U of HGF per ml for 15 min. Cells were fixed in 2% paraformaldehyde in 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-HA (1:300 in 0.2%Triton-

PBS) was added to the cells for 30 min, and after 3 washes, CY3-conjugated goat anti-mouse IgG (1:2000, Jackson ImmunoResearch Laboratories, West Point, DA, USA) 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) and visualized with a Zeiss (Thornwood, NY) Axiovert 135 incident-light fluorescence microscope.

Collagen assays. MDCK cells were resuspended in a collagen matrix as described previously (Maroun *et al.*, 1999a). HGF (15U/mL) or recombinant CSF rhCSF-1 (50 ng/mL) were added to the medium after 5 days. Quantitation of the morphogenic response was performed as described (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 Inc., Lake Placid, N.Y.). Anti-phosphotyrosine (4G10) and anti-Gab1 were purchased from Upstate Biotechnology Inc., Lake Placid, N.Y.; or RC20H from Transduction Laboratories; anti-HA (HA-11) from BABCO (Richmond, Calif.); and anti-GST from Santa Cruz Biotechnology (Santa Cruz, Calif.). Anti-phosphoMAPK, phosphoJNK, and 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, and Dr. John Blenis, Harvard Medical School, respectively. HGF and recombinant CSF were generously provided by Dr. George Van de Woude, Van Andel Research Institute, Grand Rapids, Michigan; and Genetics Institute, Boston, Mass., respectively. HA-tagged Gab2, Gab2MBD-pGEX 4T-1 and anti-Gab2 sera were gifts from Dr. Ben Neel (Beth Isreal-Deaconess Medical Centre, Boston). SH2 domain containing GST fusion proteins were generously provided by Dr. Bruce Mayer, University of Connecticut Health Center (GST-Crk II SH2); Dr. Tony Pawson, Samuel Lunenfeld Resarch Institute, Toronto (GST-PLCγ and GST-p85 SH2); and Dr. Gen-Sheng Feng, Burnham Institute, La Jolla, Calif. (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 minutes following HGF stimulation, and is sustained for at least 2 hours, similar to the time course of endogeneous Gab1 phosphorylation (Fig. 1A). As 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 have previously shown that Gab1 is localized to sites of cell-cell attachment in the presence of serum but localizes diffusely to the cytosol following serum starvation (Maroun *et al.*, 1999a). In a similar manner to Gab1, Gab2 is localized at sites of cell-cell contact (Fig. 1B). Under serum starved conditions, both Gab1 and Gab2 show a similar diffuse localization and are recruited to the membrane upon HGF stimulation (Fig. 1C), indicating that Gab1 and Gab2 share a similar subcellular distribution.

Gab2 is tyrosine phosphorylated at reduced levels when compared to 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 (Fig. 2A). To address whether Gab2 is capable of associating with similar signaling proteins as Gab1 following Met receptor activation, we utilized cell lines that express Gab2 to levels higher than Gab1 (highGab2, Fig. 2). Under these conditions, Gab1 and Gab2 show comparable levels of phosphorylation following stimulation of cells with HGF (Fig. 2B, top two panels and C). To investigate proteins that can associate with Gab1 and Gab2 in MDCK cells, we have performed either coimmunoprecipitation assays to detect endogenous proteins, or utilized GST fusion proteins encoding the SH2 domains of signaling proteins known to bind to Gab1 and Gab2 (Nguyen *et al.*, 1997; Maroun *et al.*, 1999a) (Garcia-Guzman *et al.*, 1999; Gual *et al.*, 2000; Lamorte *et al.*, 2000; Sakkab *et*

al., 2000) (Gu *et al.*, 1998; Pratt *et al.*, 2000; Crouin *et al.*, 2001). SHP-2 was identified as a major binding protein for Gab2 (Gu *et al.*, 1998) and by co-immunoprecipitation, both Gab1 and Gab2 associate with endogenous SHP-2 protein following stimulation of cells with HGF (Fig. 2B). GST fusion protein pulldown assays of cell lysates prepared from the MDCK cell lines overexpressing Gab1 or Gab2, showed that, following HGF stimulation, Gab2 is able to associate with various SH2 domain containing fusion proteins, including Crk II, p85, PLC-γ and SHP-2 (Fig. 2B). In addition, following 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 (Fig. 2D). Hence, although the level of Gab2 phosphorylation is lower than that of Gab1, when overepressed, 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 have 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 if Gab1 and Gab2 have similar functions in MDCK cells, we tested whether overexpression of Gab2 in the N1358H CSF-Met (MetΔGrb2) cell line could rescue the morphogenesis defect upon CSF stimulation. Multiple stable cell lines that overexpress Gab2 in the CSF-MetΔGrb2 cell line were isolated, and expression levels of representative lines are shown in Fig. 3A. When tested for their ability to rescue epithelial morphogenesis, cell lines expressing low levels of Gab2 (i.e. Gab2-B3 and Gab2-H1, Fig. 3A) were unable to rescue tubulogenesis upon stimulation, where cells remained as cysts, similar to the vector control (Fig. 3B and C). In cell lines expressing 5-7 times more Gab2 than Gab1 (i.e. Gab2-C1 and C9, Fig. 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 (Fig. 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 (Fig. 3B). A partial response is defined as any structure that is no longer a cyst, but whose length is less than 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 when compared with cells expressing Gab1, where branching tubule formation is approximately 80% (Fig. 3C and (Maroun *et al.*, 1999a)). Importantly, all cell lines elicited a morphogenic response following stimulation with HGF, indicating that the signals required for this response are intact (data not shown). 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 (MetΔGrb2) 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 (Fig. 3D, vector), possibly due to its ability to recruit and phosphorylate the Shc adapter protein and to promote Shc-Grb2 coupling. Noteably, CSF stimulation of WT MDCK cells does not induce MAPK phosphorylation (data not shown), indicating that activation of MAPK in the CSF-MetΔGrb2 cell lines occurs exclusively through signals generated from the CSF-MetΔGrb2 receptor. Importantly, overexpression of either Gab1 or Gab2 produced similar MAPK or JNK responses upon stimulation of the CSF-MetΔGrb2 mutant receptor (Fig. 3D). These results suggest that the inability of Gab2 to rescue the morphogenic phenotype of the mutant MetΔGrb2 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 if Gab2 is phosphorylated upon activation of the CSF-MetΔGrb2 mutant receptor. Whereas Gab1 is readily phosphorylated following stimulation of the CSF-MetΔGrb2 mutant, Gab2 is poorly phosphorylated, even when overexpressed (Fig. 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 (data not shown), as shown for Gab1 (Lock *et al.*, 2000), while mutation of both abrogates association with Grb2 as assessed by *in vitro* association assay (Fig. 4B). To define if Gab2 phosphorylation by the Met receptor was Grb2-dependent or -independent, the phosphorylation of a Gab2ΔGrb2 protein was assessed in stable MDCK cell lines following stimulation with HGF. Whereas WT Gab2 was tyrosine phosphorylated following stimulation of cells with HGF, a Gab2ΔGrb2 mutant was not detectably phosphorylated (Fig. 4C). In contrast, a Gab1ΔGrb2 mutant was efficiently phosphorylated (Fig. 4C). These data suggest that Gab2 lacks the ability to be recruited to the Met receptor in a Grb2-independent fashion.

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 expressed by transient transfection in 293T cells. When overexpressed, 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 when compared to MBD1, MBD2 binds less efficiently to the Met receptor, even though equal levels of fusion proteins were used (Fig. 4D). Elimination of the Grb2 binding site in the Gab2 MBD (MBD2ΔGrb2), abolishes this weak association, whereas the deletion of the Grb2 binding site in the Gab1 MBD1 (MBD1ΔGrb2), decreases but does not eliminate the association of the Gab1 MBD1 with the Met receptor (Fig. 4D) (Lock *et al.*, 2000). To establish, if as suggested by these

experiments that Gab2 is recruited to the Met receptor in a Grb2-dependent fashion, 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, (Gab1ΔGrb2 or Gab2ΔGrb2, Fig. 4E). Following immunoprecipitation, WT Gab1 or a Gab1ΔGrb2 mutant readily coimmunoprecipitate 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 (Fig. 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-dependent and 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 (Fig. 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, Fig. 5A), severely reduced the association of a GST-MBD1Δ3P fusion protein with the Met receptor in an *in vitro* association assay, when compared to GST-MBD1 WT and GST-MBD1ΔGrb2 fusion proteins (Fig 5B). Similarily, co-immunoprecipitation of the Met receptor with the full length Gab1Δ3P mutant protein is significantly reduced when compared to WT Gab1 or Gab1ΔGrb2 protein. Moreover, association with the Met receptor is abolished with either a GST-MBD1Δ3P/ΔGrb2 fusion protein (Fig. 5B) or the full length Gab1Δ3P/ΔGrb2 protein (Fig. 5C). The Gab1Δ3P mutant associates with a GST-Grb2 fusion protein *in vitro* to a similar level as WT Gab1 (Fig. 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 that 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, 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 asparagine 489 or proline 493 had no effect (Fig. 4E). Thus, the Δ3P mutant and more specifically a substitution of alanine for proline 491 or 492 abrogates the Grb2-independent recruitment of Gab1 to the Met receptor.

Insertion of the 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 (Fig. 6A), and tested these for their ability to bind to Met in an *in vitro* association assay (Fig. 6B). A chimeric protein containing the C-terminus of the Gab1MBD (MBD2/1484-end, Fig. 6B, lane 4), showed similar Met binding capacity to MBD1 (Fig. 6B, lane 1), whereas a chimeric protein, containing the C-terminus of the Gab1MBD (MBD2/1(495-end) Fig. 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), Fig. 6A), was insufficient to confer full binding (Fig. 6B, lane 6), whereas a Gab2MBD 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 (lane 7, Fig 6B). This is in agreement with data from Schaeper et al., 2000, who showed that insertion of 13 amino acids, GMQVPPPAHMGFR, into Gab2 was sufficient to mediate Met association in a yeast two hybrid assay (Schaeper *et al.*, 2000). The 6 additional amino acids identified here reflect our cloning strategy to generate these mutants, and from our mutational analysis 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*Δ3P, lane 8). As expected, a Gab2 MBD2/1/2*ΔGrb2 fusion protein (lane 9) retained the ability to associate with Met, but showed decreased association, similar to that of the Gab1 MBD1ΔGrb2 fusion protein (Fig. 5B). The difference between the MBD2/1/2 (lane 6) and the MBD2/1/2* (lane 7) proteins are 6 amino acids of Gab1 (GFRSSP, aa 497 to 502, Fig. 6C), which correspond

to amino acids 479-484 (GYPSTA) in Gab2 (Fig 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 (Fig. 6B, lane 10) has a severely decreased association with Met, while MBD2/1/2*P502A (Fig. 6B, lane 11) binds Met to the same level as MBD2/1/2* (lane 7). GST 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, SSFGMQVPPPAHMGFRSSP, where the first and second prolines and the last arginine are critical, and the first glycine is preferred (Fig. 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 (Fig. 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 (Fig. 6E). In accordance with these results, in stable MDCK cell lines, the Gab1Δ3P protein is significantly less phosphorylated than WT Gab1 (Fig. 6E). This indicates that not only are these amino acids from Gab1 required for Grb2-independent association with the Met receptor, but are also 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 the Gab1Δ3P mutant (Fig. 7A) was unable to rescue branching tubulogenesis in response to CSF-1 in five independent cell lines (Fig. 7C 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 (Fig. 7C-E). In a similar manner to WT Gab2 (Fig. 3), cell lines that express low levels of Gab2/1/2* (Fig. 7A) remain as cysts upon stimulation of the MetΔGrb2 receptor. Cell

lines that express high levels of Gab2/1/2* (Fig. 7A) are able to generate a partial response, with a higher percentage of long, unbranched tubules than Gab2 overexpressing cells (Fig.7C). However these cell lines are unable to generate a full branching morphogenic response comparable to that of the Gab1 expressing cell lines (Fig. 7A, and C-E; 4 representative cell lines are shown). Importantly, Gab2/1/2* protein becomes phosphorylated upon stimulation of the CSF-MetDGrb2 receptor, although consistently to lower levels than Gab1 (Fig. 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 utilized 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 (Fig. 1A) and that they show a similar subcellular localization and concentrate at cell-cell junctions in colonies of epithelial cells (Fig. 1B) (Maroun *et al.*, 1999a). Gab2 becomes phosphorylated upon HGF simulation (Fig. 1A), and can associate with similar signaling proteins as Gab1 (Fig. 2B 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 MetΔGrb2 receptor mutant (Fig. 3B 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 (MetΔGrb2) to induce branching morphogenesis (Maroun *et al.*, 1999a). In contrast, the overexpression of Gab2 fails to rescue the morphogenic defect (Fig. 3B and C). When compared to 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 (Fig. 2A, 4D and E). The reduced Gab2 association with the Met receptor when compared with Gab1, reflects different mechanisms of recruitment of these two related docking proteins. The recruitment of Gab2 to Met, and its subsequent phosphorylation, is dependent predominantly on a Grb2 binding site in Gab2 (Fig. 4C-E), whereas the recruitment of Gab1 to Met, is both Grb2-dependent and Grb2-independent (Fig. 5B 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 (Fig. 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*, Fig. 6A-D). The mutation of three proline residues within this region of Gab1 (Gab1Δ3P, Δ491-493), abolishes Grb2-independent recruitment of Gab1 (Fig. 5B and C), and decreases HGF-induced tyrosine phosphorylation of Gab1 (Fig. 6E). Noteably, the Gab1Δ3P mutant fails to rescue the branching morphogenic program downstream from the MetΔGrb2 receptor mutant (Fig. 7C 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 MetΔGrb2 mutant (Fig. 4A-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*, Fig. 7C-E), in spite of the increased tyrosine phosphorylation of the Gab2/1/2* protein (Fig. 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 following Met stimulation

(data not shown). Moreover, when overexpressed and phosphorylated following activation of Met, Gab2 can associate with the same signaling proteins as Gab1, suggesting that Gab2 can be extensively phosphorylated by Met (Fig. 2B). Two potentially phosphorylated tyrosines, (Y307 and Y373), are absent in Gab2. However, although these tyrosines contain a consensus binding site for Crk/PLCγ, Gab2 retains the ability to bind these signaling proteins (Fig.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 currenly 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 (Fig. 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 appears 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). While the MBD appears 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 EGFR, PDGF-Rβ v-Sea, TrkA, c-Ros, the insulin receptor, DDR, c-Ret, Sek-1, c-Kit, c-Abl, CSF-1R, and KGFR (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), while it interacts with the FGF receptor through a novel sequence in a non-phosphotyrosine dependent manner (Xu *et al.*, 1998; Ong *et al.*, 2000). In addition, the Grb7/Grb10/Grb14 family of adapter proteins contain 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 (KRLB) domain that interacts with the phosphorylated regulatory loop of the insulin receptor β-subunit (Sawka-Verhelle *et al.*, 1996; Sawka-Verhelle *et al.*, 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 if 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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Fig. 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 ($1x10^4$) stably expressing either WT HA-tagged Gab1 or WT HA-tagged Gab2 were grown for 72 hours on glass coverslips in DMEM containing 10% FBS. (C) MDCK cells ($5x10^4$) 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 hours, and stimulated with HGF (50U/mL) for 15 minutes. Cells were fixed in 2% paraformaldehyde and subjected to indirect immunofluorescence using anti-HA, followed by CY3-conjugated anti-mouse antibody. Photographs were taken at a magnification of 63X.

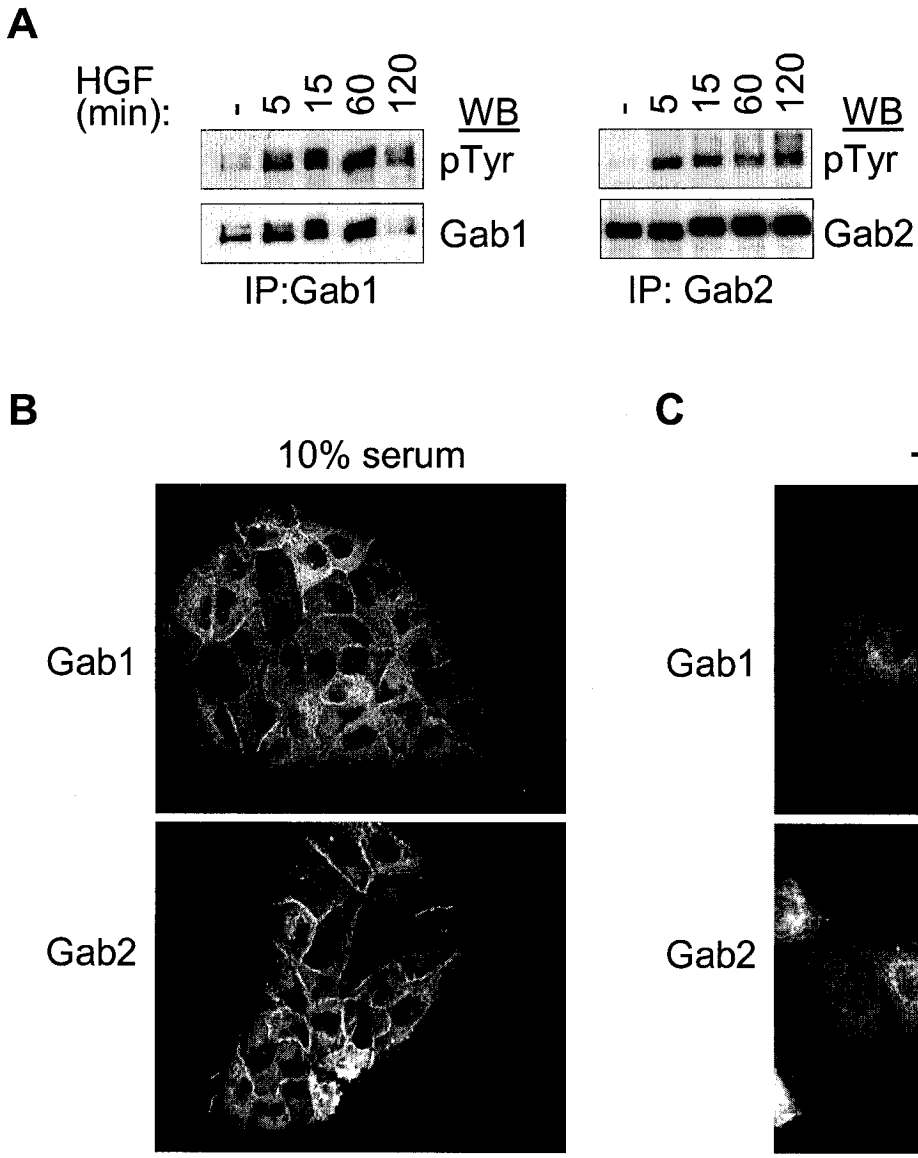

A
HGF (min):
- 5 15 60 120
WB
pTyr
Gab1
IP:Gab1
- 5 15 60 120
WB
pTyr
Gab2
IP: Gab2
B
10% serum
Gab1
Gab2
C
-
HGF (15 min)
Gab1
Gab2

Fig. 2. Gab2 is tyrosine phosphorylated at reduced levels in response to HGF when compared to 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 minutes. Gab proteins were immunoprecipitated with anti-HA, separated by SDS-PAGE and western blotted with anti-SHP-2 antibody. Membranes were subsequently stripped and re-probed with RC20H anti-phosphotyrosine and anti-HA. Total cell lysates (20 μg) 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 minutes and lysates were pooled together. 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 μg of total cell lysates were separated by SDS-PAGE and western blotted (WB) with pMAPK or pJNK antibodies. Membranes were stripped and subsequently blotted with MAPK or JNK antibodies.

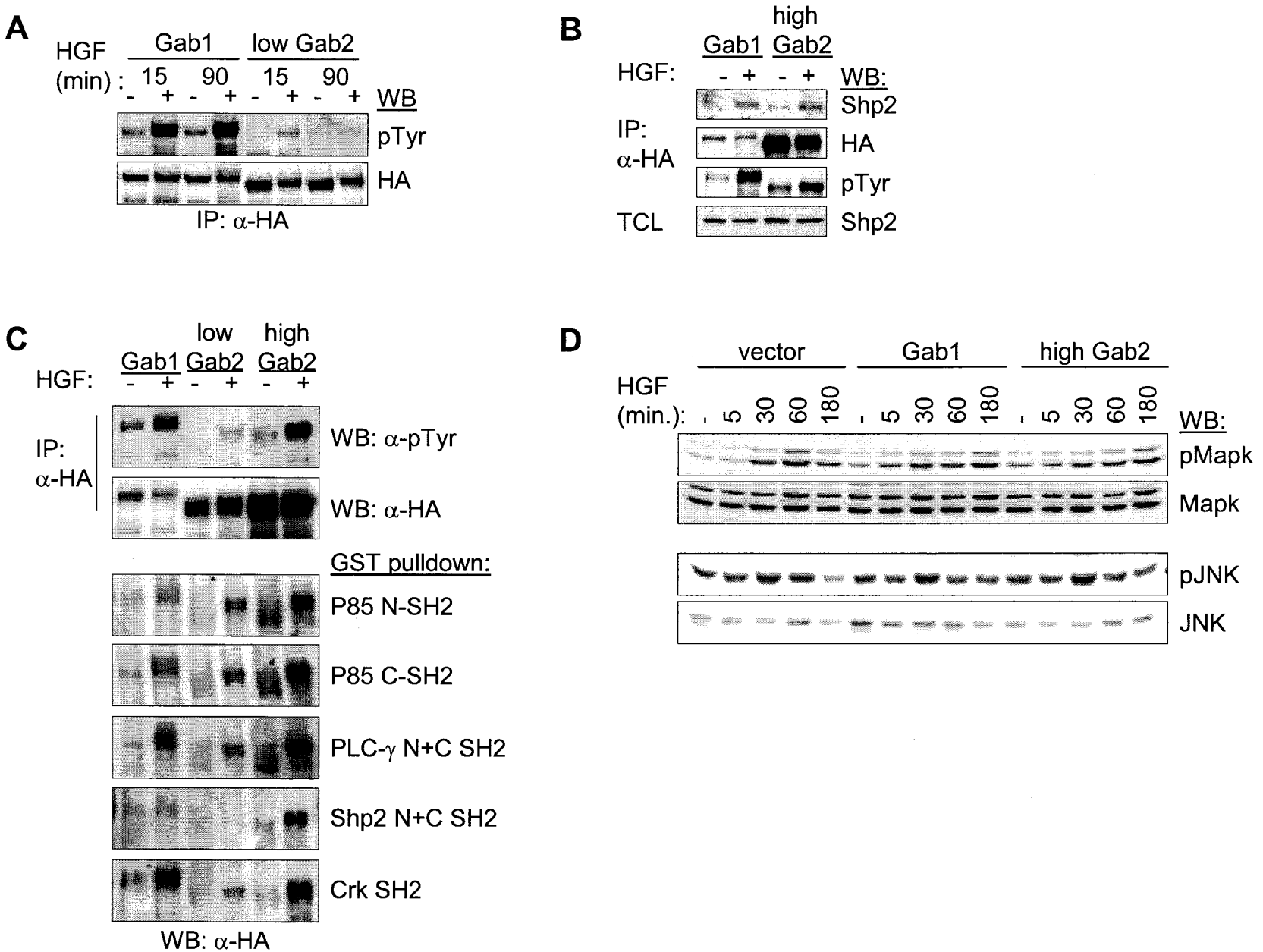

A
HGF
(min) :
Gab1
low Gab2
15
90
15
90
- + - + - + - +
WB
pTyr
HA
IP: α-HA
B
high
Gab1
Gab2
HGF:
- + - +
WB:
Shp2
IP:
α-HA
HA
pTyr
TCL
Shp2
C
low
high
Gab1
Gab2
Gab2
HGF:
- + - + - +
IP:
α-HA
WB: α-pTyr
WB: α-HA
GST pulldown:
P85 N-SH2
P85 C-SH2
PLC-γ N+C SH2
Shp2 N+C SH2
Crk SH2
WB: α-HA
D
vector
Gab1
high Gab2
HGF
(min.):
- 5 30 60 180 - 5 30 60 180 - 5 30 60 180
WB:
pMapk
Mapk
pJNK
JNK

Fig. 3. Gab2 is unable to rescue a Met-mediated morphogenic program.

MDCK cells expressing the CSF-MetΔGrb2 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) $5x10^3$ cells were grown in collagen for 5 days, during which time they formed cysts. RhCSF-1 or HGF was added, and 14 days later, branching tubules were visualized at a magnification of 10X. 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 5 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 less than 5 times its width and is unbranched. Structures referred to as partial response do not develop into branched tubules over time. (D) CSF-MetΔGrb2 (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 μg of total cell lysates were separated by SDS-PAGE and western blotted (WB) with pMAPK or pJNK antibodies. Membranes were stripped and subsequently blotted with MAPK or JNK antibodies.

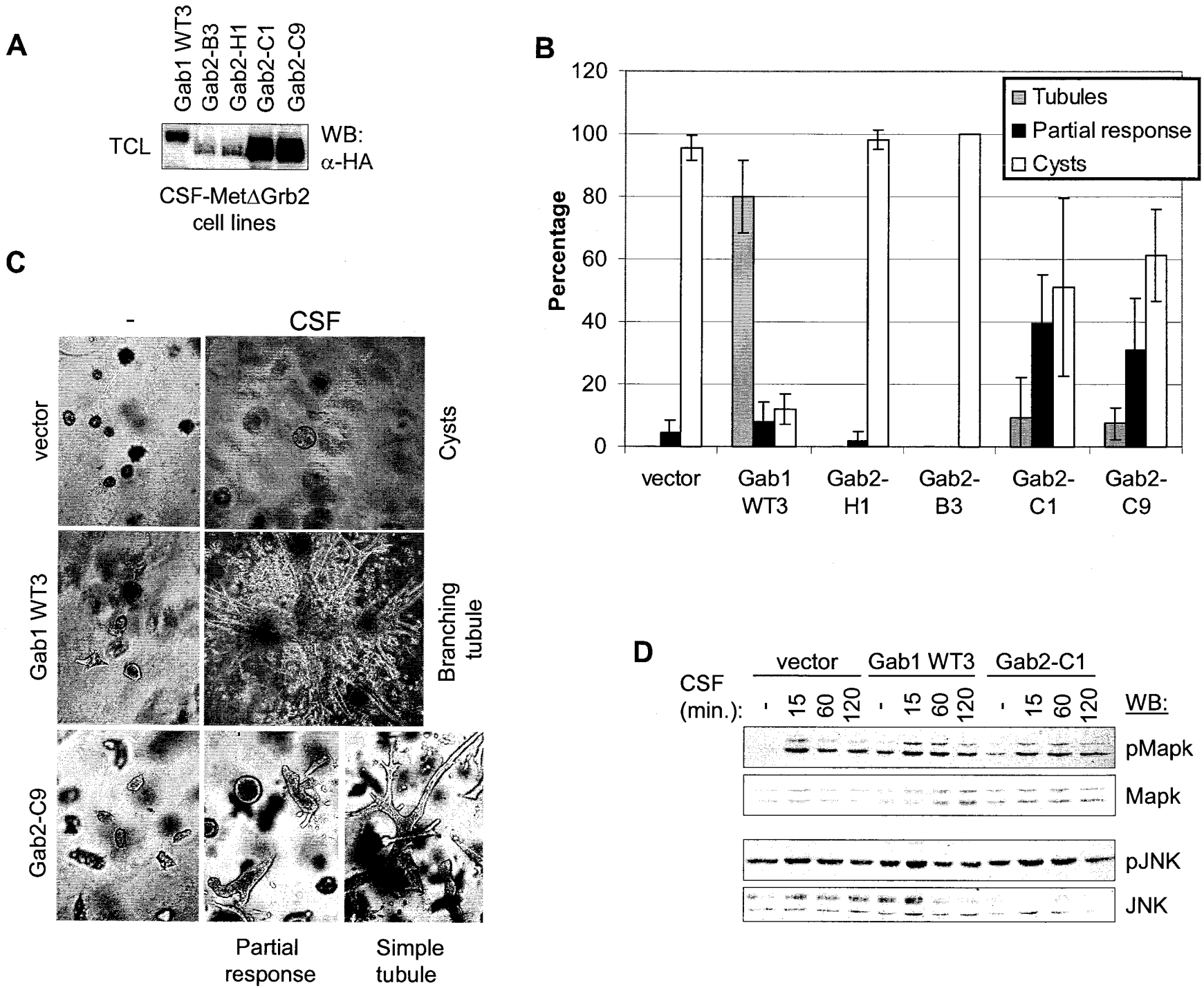

A
Gab1 WT3
Gab2-B3
Gab2-H1
Gab2-C1
Gab2-C9
TCL
WB:
α-HA
CSF-MetΔGrb2
cell lines
B
120
100
80
60
40
20
0
Percentage
Tubules
Partial response
Cysts
vector
Gab1 WT3
Gab2-H1
Gab2-B3
Gab2-C1
Gab2-C9
C
-
CSF
vector
Gab1 WT3
Gab2-C9
Cysts
Branching tubule
Partial response
Simple tubule
D
vector
Gab1 WT3
Gab2-C1
CSF (min.):
-
15
60
120
WB:
pMapk
Mapk
pJNK
JNK

Fig. 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 minutes. 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 western blotting (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 western blot (WB) analysis using anti-Met antibody. Levels of GST fusion proteins was determined by western blotting with anti-GST. (E) 293T cells were transiently co-transfected 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.

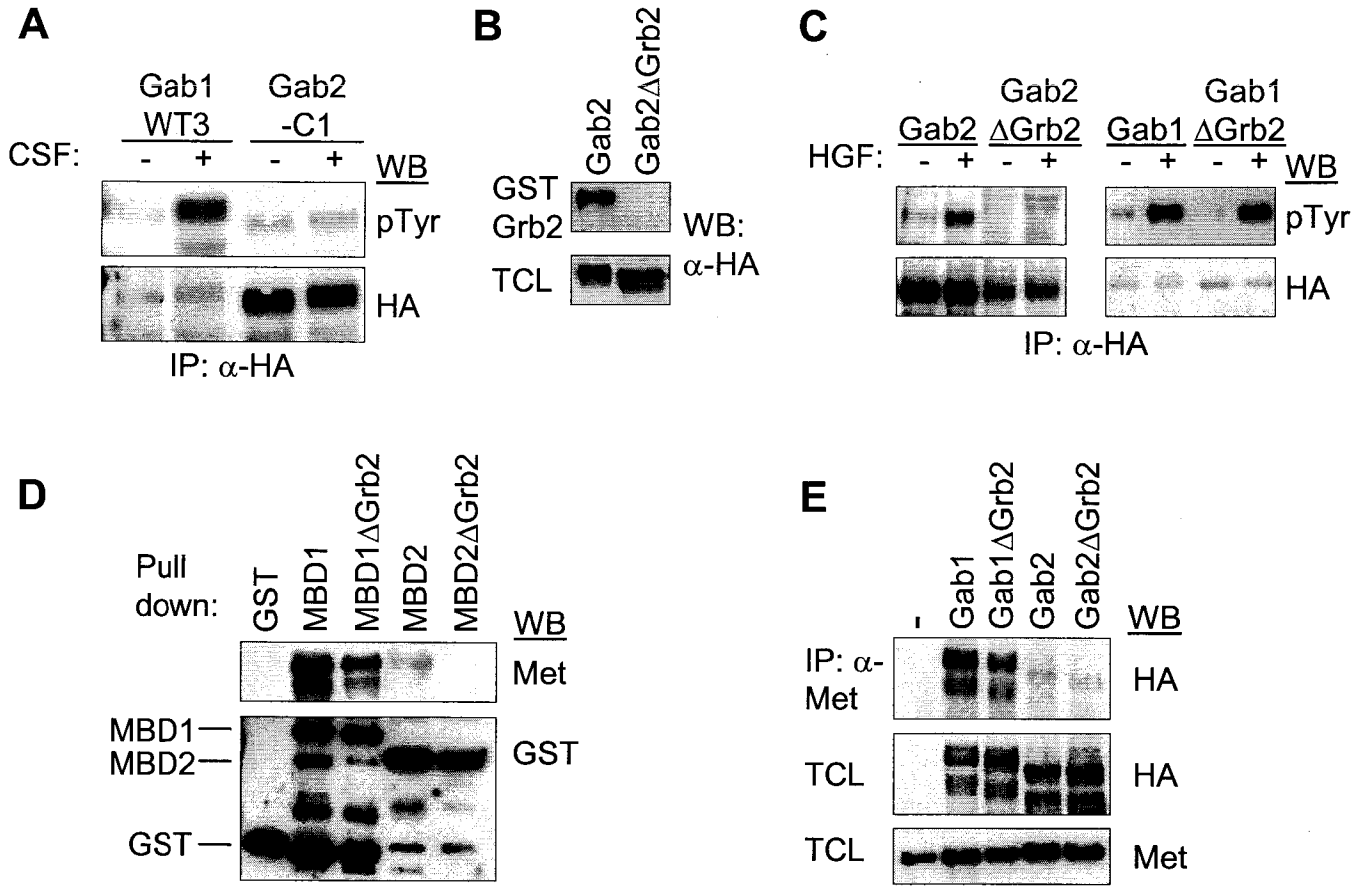

A
Gab1 WT3
Gab2 -C1
CSF:
- + - +
WB
pTyr
HA
IP: α-HA
B
Gab2
Gab2ΔGrb2
GST Grb2
TCL
WB: α-HA
C
Gab2
Gab2 ΔGrb2
Gab1
Gab1 ΔGrb2
HGF:
- + - + - + - +
WB
pTyr
HA
IP: α-HA
D
Pull down:
GST
MBD1
MBD1ΔGrb2
MBD2
MBD2ΔGrb2
WB
Met
MBD1
MBD2
GST
GST
E
-
Gab1
Gab1ΔGrb2
Gab2
Gab2ΔGrb2
WB
IP: α-Met
HA
TCL
HA
TCL
Met

Fig. 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 Δ3P deletion mutant are indicated. (B) Deletion of prolines 491-493 (Δ3P) significantly reduces association with Met via *in vitro* association assay. Gab1 MBD GST fusion proteins (MBD1, MBD1ΔGrb2, MBD1Δ3P, MBD1ΔGrb2/Δ3P) 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 co-transfected with the Met receptor and either full length WT HA-Gab1, HA-Gab1ΔGrb2, HA-Gab1Δ3P, or HA-Gab1ΔGrb2/Δ3P. Proteins from lysates were immunoprecipitated with anti-Met antibody, separated by SDS-PAGE, and western blotted (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) The Gab1Δ3P 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, R489, P491, P492 and P493 were substituted individually with alanine. Lysates prepared from Cos cells transiently co-transfected with Met and Gab1 mutants were immunoprecipitated with anti-HA antibody and western blotted with anti-Met.

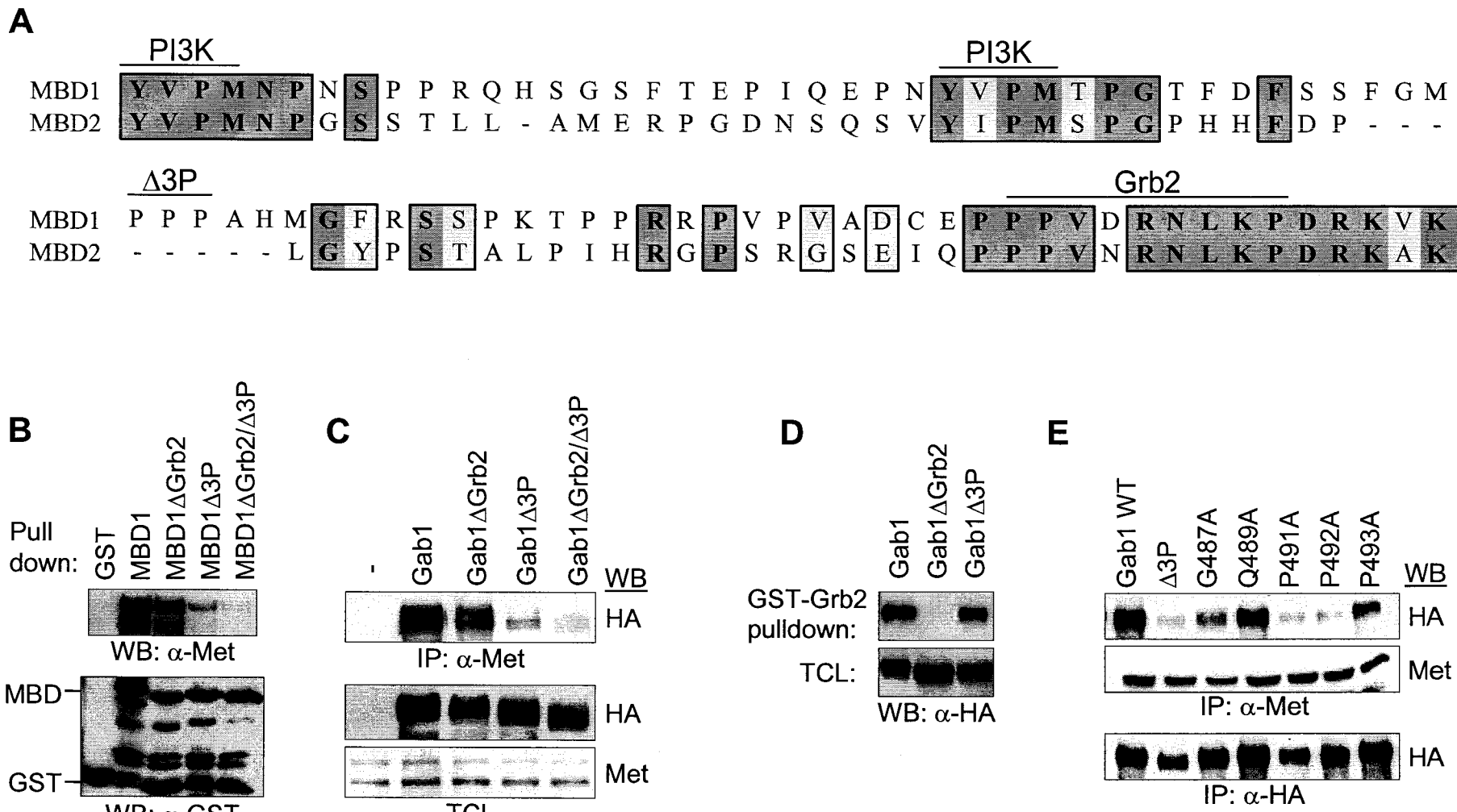

A
PI3K
PI3K
MBD1 Y V P M N P N S P P R Q H S G S F T E P I Q E P N Y V P M T P G T F D F S S F G M Q V
MBD2 Y V P M N P G S S T L L - A M E R P G D N S Q S V Y I P M S P G P H H F D P - - - - -
Δ3P
Grb2
MBD1 P P P A H M G F R S S P K T P P R R P V P V A D C E P P P V D R N L K P D R K V K P
MBD2 - - - - - - L G Y P S T A L P I H R G P S R G S E I Q P P P V N R N L K P D R K A K P
B
Pull down:
GST
MBD1
MBD1ΔGrb2
MBD1Δ3P
MBD1ΔGrb2/Δ3P
WB: α-Met
MBD
GST
WB: α-GST
C
-
Gab1
Gab1ΔGrb2
Gab1Δ3P
Gab1ΔGrb2/Δ3P
WB
HA
IP: α-Met
HA
Met
TCL
D
Gab1
Gab1ΔGrb2
Gab1Δ3P
GST-Grb2 pulldown:
TCL:
WB: α-HA
E
Gab1 WT
Δ3P
G487A
Q489A
P491A
P492A
P493A
WB
HA
Met
IP: α-Met
HA
IP: α-HA

Fig. 6. Insertion of the novel amino acid 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 Fig. 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 using 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 6B 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 western blotted (WB) with RC20H anti-phosphotyrosine. Blots were stripped and reprobed with anti-HA to show Gab protein levels in the immunoprecipitate.

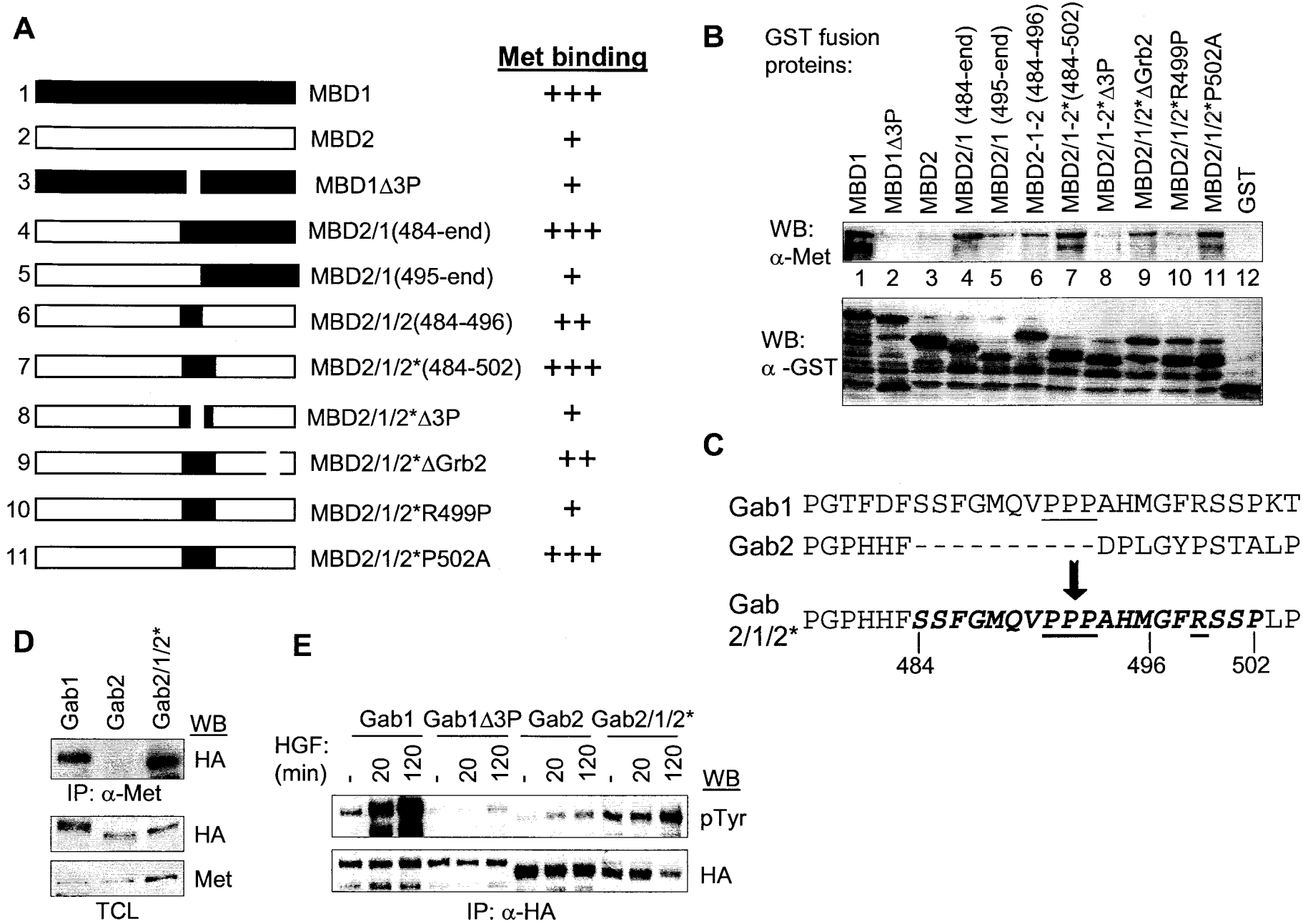
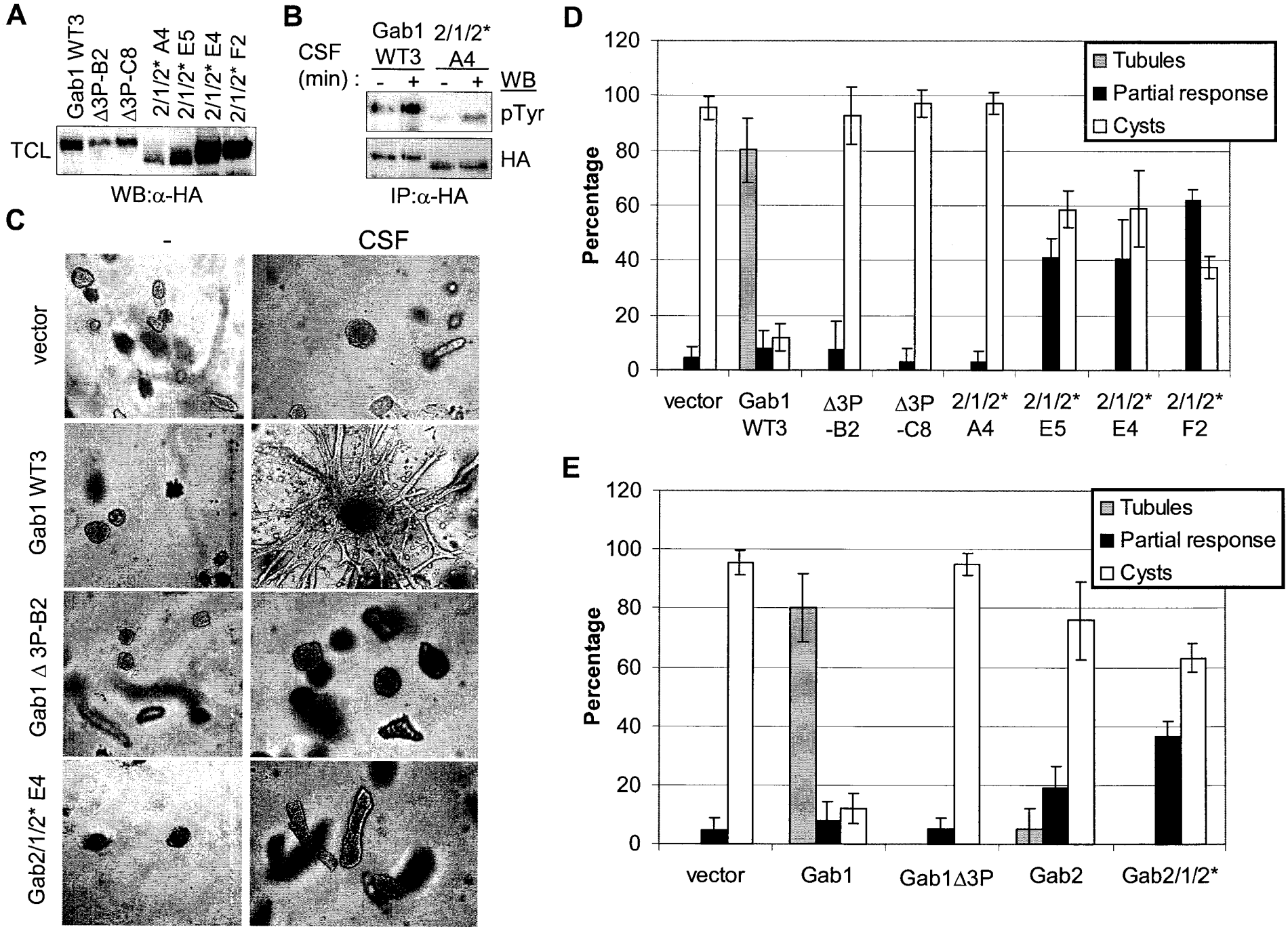

A
Met binding
1 MBD1 +++
2 MBD2 +
3 MBD1Δ3P +
4 MBD2/1(484-end) +++
5 MBD2/1(495-end) +
6 MBD2/1/2(484-496) ++
7 MBD2/1/2*(484-502) +++
8 MBD2/1/2*Δ3P +
9 MBD2/1/2*ΔGrb2 ++
10 MBD2/1/2*R499P +
11 MBD2/1/2*P502A +++
B
GST fusion proteins:
MBD1
MBD1Δ3P
MBD2
MBD2/1 (484-end)
MBD2/1 (495-end)
MBD2-1-2 (484-496)
MBD2/1-2*(484-502)
MBD2/1-2*Δ3P
MBD2/1/2*ΔGrb2
MBD2/1/2*R499P
MBD2/1/2*P502A
GST
WB: α-Met
1 2 3 4 5 6 7 8 9 10 11 12
WB: α -GST
C
Gab1 PGTFDFSSFGMQVPPPAHMGFRSSPKT
Gab2 PGPHHF----------DPLGYPSTALP
Gab 2/1/2* PGPHHFSSFGMQVPPPAHMGFRSSPLP
484
496
502
D
Gab1
Gab2
Gab2/1/2*
WB
HA
IP: α-Met
HA
Met
TCL
E
Gab1 Gab1Δ3P Gab2 Gab2/1/2*
HGF: (min) - 20 120 - 20 120 - 20 120 - 20 120
WB
pTyr
HA
IP: α-HA

Fig. 7. The 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 minutes. 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 Experimental Procedures. Results are derived from at least three independent experiments, with 5 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 2 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.


A
Gab1 WT3
Δ3P-B2
Δ3P-C8
2/1/2* A4
2/1/2* E5
2/1/2* E4
2/1/2* F2
TCL
WB:α-HA
B
Gab1 WT3
2/1/2* A4
CSF (min) : - + - +
WB
pTyr
HA
IP:α-HA
C
-
CSF
vector
Gab1 WT3
Gab1 Δ 3P-B2
Gab2/1/2* E4
D
Percentage
120
100
80
60
40
20
0
Tubules
Partial response
Cysts
vector
Gab1 WT3
Δ3P -B2
Δ3P -C8
2/1/2* A4
2/1/2* E5
2/1/2* E4
2/1/2* F2
E
Percentage
120
100
80
60
40
20
0
Tubules
Partial response
Cysts
vector
Gab1
Gab1Δ3P
Gab2
Gab2/1/2*

Chapter IV: Grb2-independent recruitment of Gab1 requires the C-terminal lobe and structural integrity of the Met receptor kinase domain.

Reproduced with permission from Lisa S. Lock, Melanie Frigault, Caroline Saucier, and Morag Park. 2002. *Grb2-independent recruitment of Gab1 requires the C-terminal lobe and structural integrity of the Met receptor kinase domain.* Submitted to Molecular and Cellular Biology, December 2002.

Preface

The previous chapters demonstrated that Gab1 can interact with the Met receptor in a Grb2-independent manner, and that this interaction is essential but not sufficient for the ability of Gab1 to rescue Met-mediated epithelial morphogenesis. Moreover, a 13 amino acid sequence within the Met binding domain (MBD), was shown to be the critical component of this interaction.

Although the MBD has been proposed to be a phosphotyrosine binding (PTB)-like domain that binds specifically to the Met receptor, neither the binding site on Met, nor the mechanism of their interaction had been delineated. This manuscript describes a novel type of interaction between the kinase domain of the Met receptor and an extended poorly structured motif in the MBD of Gab1.

Abstract

The Gab1 docking protein forms a platform for the assembly of a multiprotein signaling complex downstream from receptor tyrosine kinases (RTKs). In general, recruitment of Gab1 occurs indirectly, via the adapter 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 Y1349 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. In addition, the structural integrity of the Met receptor, and residues upstream of Y1349 located in the C-terminal lobe of the kinase domain, are required for Grb2-independent interaction with the Gab1 MBD, and the substitution of Y1349 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.

Introduction

The Gab family of docking proteins consists of Gab1, Gab2 and Gab3 in mammals, DOS (Daughter of Sevenless) in *Drosophila*, and SOC-1 (Suppressor of Clear-1) in *C. elegans*. Gab proteins are non-enzymatic and are characterized by a conserved amino-terminal pleckstrin homology (PH) domain that where investigated, binds phosphatidylinositol-3,4,5-P_3 (PIP_3) and mediates the PIP_3-dependent membrane association of Gab proteins (Maroun *et al.*, 1999b; Bausenwein *et al.*, 2000; Rodrigues *et al.*, 2000). 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 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 (Liu and Rohrschneider, 2002).

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 (Holgado-Madruga *et al.*, 1996). 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, as Gab1-deficient mice die in utero, displaying defects in the heart, placenta and skin, as well as reduced liver size. (Itoh *et al.*, 2000; Sachs *et al.*, 2000). *In vitro*, Gab1 promotes cell survival, neurite outgrowth, and DNA synthesis in neuronal cells downstream from the TrkA receptor (Holgado-Madruga *et al.*, 1997; Korhonen *et al.*, 1999) and regulates an invasive epithelial morphogenic program downstream from the HGF/Met receptor tyrosine kinase (Maroun *et al.*, 1999a; Maroun *et al.*, 1999b; Maroun *et al.*, 2000).

In epithelial cells, Gab1 is the major substrate for the Met receptor tyrosine kinase (Nguyen *et al.*, 1997), and upon tyrosine phosphorylation, Gab1 provides binding sites for proteins involved in signal transduction, including the tyrosine phosphatase, SHP-2, the p85 subunit of PI3'K, PLCγ, as well as the Crk adaptor protein (Garcia-Guzman *et*

al., 1999; Maroun *et al.*, 1999a; Gual *et al.*, 2000; Maroun *et al.*, 2000; Schaeper *et al.*, 2000). The association of Gab1 with several of these proteins, as well as an intact Gab1 PH domain, is required for the ability of Gab1 to promote the morphogenic program of Madin Darby canine kidney (MDCK) epithelial cells downstream from the Met receptor (Maroun *et al.*, 1999a; Maroun *et al.*, 1999b; Maroun *et al.*, 2000; Lamorte *et al.*, 2002). In contrast, the Gab2 protein fails to promote a morphogenic response, indicating that Gab1 and Gab2 are functionally distinct downstream from the Met receptor (Schaeper *et al.*, 2000; Lock *et al.*, 2002).

Gab1 and Gab2 contain highly conserved, but atypical binding sites for the C-terminal SH3 domain of the adapter protein Grb2, and are recruited indirectly via a Grb2-dependent interaction to the EGF receptor, FGF receptor-1, and the interleukin-3 receptor beta common chain (βc) (Gu *et al.*, 2000; Lock *et al.*, 2000; Rodrigues *et al.*, 2000; Ong *et al.*, 2001; Saxton *et al.*, 2001). Whereas the recruitment of Gab2 to the Met receptor is strictly Grb2-dependent, the interaction between Gab1 and the Met receptor is distinct, in that Gab1 is recruited by both a Grb2-dependent and Grb2-independent mechanism (Weidner *et al.*, 1996; Nguyen *et al.*, 1997). The Grb2-dependent recruitment requires tyrosine 1356 in the Met receptor C-terminus, which forms a Grb2 SH2 domain binding site (Nguyen *et al.*, 1997), and intact Grb2 SH3 domain binding sites on Gab1 (Lock *et al.*, 2000). The Grb2-independent recruitment of Gab1 requires an 83 amino acid, proline-rich region of Gab1, termed the Met binding domain (MBD), that was initially identified in a yeast-two hybrid interaction assay (Weidner *et al.*, 1996). This interaction required the kinase activity of the Met receptor, and phosphorylation of tyrosine 1349 in the C-terminus of Met (Weidner *et al.*, 1996).

Together, tyrosines 1349 and 1356 are required for the full recruitment of Gab1 to the Met receptor (Nguyen *et al.*, 1997). Tyrosine 1356 constitutes a docking site on the Met receptor required for the indirect recruitment of Gab1 through the Grb2 adapter protein, whereas tyrosine 1349 appears to be uniquely required for the direct recruitment of Gab1. Based on the observation that docking proteins of the IRS, Dok, and FRS2 families all contain phosphotyrosine binding (PTB)-like domains, the Gab1 MBD has been proposed to be a PTB-like domain that would bind directly to a phosphotyrosine-containing motif involving Y1349 in the Met receptor C-terminus (Weidner *et al.*, 1996).

However, the mechanism of interaction between the Gab1 MBD and Met has not been addressed.

Previous studies had identified a 13 amino acid sequence, GMQVPPPAHMGFR, within the MBD, as critical for the MBD-Met interaction (Schaeper *et al.*, 2000; Lock *et al.*, 2002). We show that this sequence is sufficient for interaction with the Met receptor, indicating that it is unlikely that the 83 aa Gab1 MBD forms a structured PTB-like domain. Moreover, we show that the structural integrity of the Met receptor, and residues upstream of tyrosine 1349 located in the C-terminal lobe of the kinase domain, are required for the Grb2-independent interaction with the Gab1 MBD. These results support the interpretation that the Gab1 MBD interacts with the Met receptor in a novel and previously unsuspected manner, where instead of the expected interaction of a phosphotyrosine binding domain in Gab1 with a phosphotyrosine-containing motif in the Met receptor, we propose that the activated kinase domain of Met and the negative charge of phosphotyrosine 1349 engage the Gab1 MBD as an extended peptide ligand.

Experimental Procedures

Plasmids and Mutagenesis

The generation of GST-MBD, GST-MBDΔGrb2 and MBDΔGrb2-myc was previously described (Lock et al., 2000). GST-MBD16 and GST-MBD16 R499A were generated by insertion of double-stranded oligos into BamHI/ EcoRI sites of pGEX 2TK; MBD16-F, GATCCTTTGGAATGCAAGTACCTCCTCCTGCTCATATGGGCTTTAGAAGTTG; MBD16-R, AATTCAACTTCTAAAGCCCATATGAGCAGGAGGAGGTACTTGCA-TTCCAAAG Alanine scanning mutations in Tpr Met-PXM were generated via the Quik Change mutagenesis protocol (Stratagene). Met C-terminal truncations mutants were generated by insertion of stop codons at amino acids 1348 and 1353 respectively. Tpr Met A and B mutants are p85 and Shc binding variants, respectively and are described in (Saucier et al., 2002). All mutants were sequenced prior to use.

Cell culture, DNA transfections and Total cell extracts - 293T cells were seeded at $1x10^6$/ 100-mm petri dish and transfected 24h later by the calcium phosphate precipitation method (Wigler et al., 1979) with 2 μg of DNA., while 293 cells were transfected with 10μg of DNA by Superfect method (Qiagen). Cells were serum starved in 0.1% FBS for 24h, 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 EGTA, 1.5 mM $MgCl_2$), or in RIPA buffer for GST-pulldown assays (0.05% SDS, 50 mM Tris pH 8, 150 mM NaCl, 1% NP-40, 0.5% sodium Desoxycholate). Inhibitors (10 mg aprotinin/ml, 10 mg leupeptin/ml, 1 mM PhenylMethylsulfonyl 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.

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 μg) were immobilized on glutathione sepharose beads for 30 minutes at room temperature and washed three times prior to a 1 hour incubation with the indicated lysates (lysed in RIPA buffer). For some experiments, the following peptides were added at the indicated concentrations: Gab1 MBD16 (FGMQVPPPAHMGFRSS), or Gab1 MBD16 R499A (FGMQVPPPAHMGFASS). The peptides were synthesized at the Sheldon Biotechnology Center (Montreal, Quebec, Canada). Immunoprecipitation and western blotting were performed as described in (Maroun et al., 1999a).

Far Western assay. - Lysates prepared from 293T cells transiently expressing Tpr Met mutants (300 μg) were immunoprecipitated with anti-Met 144 antibody, loaded on an 8% polyacrylamide gel, transferred, and blocked over night 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 minutes at room temperature. Washed membranes were then incubated with this reaction mixture in 10 ml of TBST for 30 minutes, prior to washing and ECL. Membranes were stripped and western blotted with anti-Met 144 antibody. Protocol is described in (Nollau and Mayer, 2001).

Antibodies and Reagents. Antibodies against the extreme C-terminus (144, (Rodrigues et al., 1991)) and the extracellular domain (DL-21, Upstate Biotechnology Inc., Lake Placid, N.Y.) of human Met protein were used. RC20H was purchased from Transduction Laboratories, anti-GST and anti-Grb2 were from Santa Cruz Biotechnology (Santa Cruz, Calif.), anti-pY1349Met from Cell Signaling (Mississauga, Ontario, Canada), and anti-Gab1 from Upstate Biotechnology Inc (Lake Placid, New York).

3-D modeling of Met. The catalytic domain of Met was modeled using Swiss Model Comparative Protein Modelling Server, and was visualized with Swiss-Pdb viewer (Peitsch, 1995, 1996; Guex and Peitsch, 1997). The Met kinase domain was modeled on available structures of the insulin and IGF-1 receptors (PDB ID codes, 1K3AA, 1IRK_, 1IR3A 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 (MBDΔGrb2) (Lock et al., 2000). Previous studies had identified 13 amino acids within the MBD as essential for Grb2-independent recruitment of Gab1 (Schaeper et al., 2000; Lock et al., 2002). To investigate the requirements for Met receptor binding, we first established whether a 16 amino acid peptide containing these 13 amino acids, (MBD16, FGMQVPPPAHMGFRSS, Fig. 1A) would act as a competitor in in vitro association studies. Addition of increasing levels of this peptide (10-250 μM) was able to compete the association of GST-MBDΔGrb2 from Met in an in vitro association assay (Fig. 1B). This was specific for the interaction of Met with the Gab1 MBDΔGrb2, as the peptide failed to compete the association of a GST-Grb2 fusion protein with Met (Fig. 1B), or the association of a WT Gab1 MBD fusion protein with Grb2 (Fig. 1C). Our previous studies had shown that Arg 499 within the Gab1MBD was required for the interaction of MBD with Met. Arg 499 is located within this 16 amino acid peptide, and a peptide where Arg 499 was converted to Ala (Gab1MBD16-R499A) failed to compete the association of the Gab1 MBDΔGrb2 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 (Lock et al., 2002). However, it was unclear whether this short sequence was sufficient on its own to interact with Met directly, or if surrounding amino acids in Gab2, that 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 MBDΔGrb2. 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 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. However, 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 since the interaction of Grb2 with Met requires only a short phosphotyrosine motif corresponding to Y1356 (YVNV) in the Met C-terminus, and the structured SH2 domain of Grb2 (Songyang et al., 1994; Fournier et al., 1996). In contrast, although the GST-MBDΔGrb2 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 assay. In contrast, a GST–Grb2 fusion protein efficiently binds Met both in boiled lysates and 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 MBM, 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-MBDΔGrb2 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 1356 with phenylalanine had little effect on MBDΔGrb2 binding by either assay, whereas, as expected, a mutant with both Y1349 and Y1356 substituted with phenylalanine residues, or a kinase inactive mutant (K1110A) were both unable to bind to a MBDΔGrb2 fusion protein (Fig. 3A and B). This identifies an important role for phosphotyrosine 1349 in the Grb2-independent interaction with the Gab MBD, and supports previous data obtained using a yeast 2-hybrid interaction assay (Weidner et al., 1996).

To determine whether amino acids upstream or downstream from Y1349 are required for Grb2 independent recruitment of the Gab1 MBD, we used existing mutants of the Met oncoprotein where amino acids 1353 to 1362 of Met were replaced with amino acids distinct from those in the Met receptor, but still capable of recruiting signaling proteins. Mutant A contains amino acids derived from the PDGFR, and mutant B contains amino acids derived from the TrkA receptor, with binding sites for the p85 subunit of PI3'K and the Shc adapter protein, respectively (Saucier et al., 2002). When subjected to an in vitro association assay, both the A and B variants were capable of associating with the MBDΔGrb2 fusion protein. As expected, substitution of Y1349 to F in either of these variants abolishes binding (Fig. 3C). Since the sequence of the A and B inserts are distinct from Met, and B adds additional amino acids, this indicates that residues 1353 to 1362 are not essential for association with the Gab1 MBDΔGrb2. To determine whether residues in Met C-terminal to 1362 are required, we constructed C-terminal truncation mutants in the Met receptor (Fig. 3E). A Met protein lacking the last 38 amino acids (Δ1353-1390) associates with MBDΔGrb2 to similar levels as WT, as revealed using an antibody that recognizes the extracellular domain of Met. As expected, a Met protein that lacks the last 43 amino acids, including Y1349, (Δ1348-1390) fails to bind (Fig. 3D). This indicates that residues in the C-terminus of Met, downstream of

Y1353 are not required for interaction with the Gab1 MBD, and suggests that residues upstream of Y1349 may be required.

Residues upstream of Y1349 are required for association with the Gab1 MBD.

To test this hypothesis, we performed alanine scanning mutagenesis on residues surrounding Y1349 and upstream of 1353. Amino acids 1340 to 1352 were individually substituted with alanines and tested for their ability to associate with a GST-MBDΔGrb2 fusion protein. Substitution of several residues upstream of Y1349 in Met was found to significantly decrease the association with the GST-MBDΔGrb2 fusion protein. Notably, substitution of F1341, F1344, I1345, G1346, and H1348 severely diminished the association, and as shown, the Y1349F Met mutant protein failed to associate with the GST-MBDΔGrb2 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 phospho-specific Y1349 antibody, all mutant proteins are tyrosine phosphorylated on Y1349 (Fig. 4C), indicating that the diminished association of GST-MBDΔGrb2 is not due to the inability of Met mutant proteins to phosphorylate Y1349. These data identify that residues upstream of Y1349 are required for binding to the Gab1 MBD.

A negative charge at residue 1349 is sufficient for MBD binding.

Phosphorylation of Y1349 is required for binding to the MBDΔGrb2 (Figure 3A and B). However, the role of this phosphorylation is unclear since 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 (Songyang et al., 1993; Waksman et al., 1993; Eck et al., 1996; Zhou et al., 1996). Since Y1349 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 Y1349 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 Y1349 and 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 (Wybenga-Groot et al., 2001). Whereas a Y1349F mutant protein was unable to associate with the MBDΔGrb2, the substitution of Y1349 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, Y1349/56E (Fig. 5A). This was specific for the MBDΔGrb2, as a Y1349/56E or a Y1356E mutant was unable to bind GST-Grb2 (Fig. 5B). Consistent with this, following transient transfection assays, a Y1349/56E mutant induced tyrosine phosphorylation of the endogenous Gab1 protein in 293 cells, whereas in cells expressing the Y1349/56F mutant Gab1 was only basally phosphorylated (Fig. 5B). This ability of the Y1349/56E mutant to bind and phosphorylate Gab1 in the absence of phosphotyrosines 1349/56 also correlated with the ability of this mutant to transform fibroblasts (Fig. 5C). Whereas the Y1349/56F mutant of Tpr-Met fails to phosphorylate Gab1, and is unable to transform fibroblasts (Fig. 5C) (Ponzetto et al., 1994; Fixman et al., 1995; Fixman et al., 1996; Fixman et al., 1997), the Y1349/56E mutant transformed fibroblasts to a low level (6 foci/ ug DNA). These results indicate that phosphorylation of tyrosine 1349 is not essential for binding of MBDΔGrb2, but instead indicates that a negative charge is sufficient.

Residues critical for MBD binding lie within the Met receptor kinase domain.

From sequence alignments, residues identified through alanine scanning mutagenesis as critical for MBD binding (F1341, F1344, and I1345, Fig. 4A), are located within the kinase domain of the Met receptor (Park et al., 1987). 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), while the larger C-terminal lobe contains at least two β strands (β7/β8) and 7-8 α helices (αD, αE, αEF, αF -αI/αJ) (Hanks et al., 1988). ATP is coordinated primarily by the N-terminal lobe, while substrate peptide binding and catalysis are performed by residues in the C-terminal lobe (reviewed in (Johnson et al., 1996)). Although the structure of the Met receptor kinase domain has not been solved, the 3-D 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, F1341 is located in the loop between α-helix I and α-helix J, while F1344 and I1345 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 (G1346, H1348 and pY1349) did not appear in the model as they are located C-terminal to the kinase domain. F1341, F1344 and I1345 are predicted to lie in close contact (less than 5 angstroms) to residues in α-helix E (K1179, I1182, L1186, and Q1187), as well as K1215 located in the loop between β-strand 7 and β-strand 8 (Fig. 6B). Since 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, placental defects, and are characterized by strongly reduced and delayed migration of myogenic precursor cells into the limbs (Itoh *et al.*, 2000; Sachs *et al.*, 2000), a phenotype similar to mice harboring mutations in Met or HGF genes (Schmidt *et al.*, 1995; Uehara *et al.*, 1995). In addition, our lab and others have previously demonstrated that Gab1 is required for the initiation of a Met receptor-mediated invasive morphogenic program in epithelial cells (Maroun *et al.*, 1999a). 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 (Lock *et al.*, 2002). The Grb2-independent interaction requires the integrity of 13 amino acids within a proline-rich region in Gab1 termed the Met binding domain (MBD) (Schaeper *et al.*, 2000; Lock *et al.*, 2002). The Gab1 MBD has been proposed to be a phosphotyrosine binding (PTB)-like domain (Weidner *et al.*, 1996), 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 classical 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, was sufficient to interact with Met. This interaction 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 (Feng *et al.*, 1995). 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 PXXP region in Nef (Lee *et al.*, 1995). Consistent with this, in a yeast 2 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 constructs containing amino acids 471-532, 450-511 and 471-511, retained only 15, 12 and 4% binding to Met, respectively (Weidner *et al.*, 1996). Moreover, only residues found within the MBD16 peptide (aa 487-499) were identified by random PCR mutagenesis as critical for Met binding (Schaeper *et al.*, 2000).

Together this supports a role for residues in Gab1 outside of the Met binding motif for efficient binding to Met. It is not clear which additional residues in Gab1 contribute to Met binding. Gab2 lacks the amino acids corresponding to the Met binding motif, and does not bind in a Grb2 independent manner to Met. The only conserved regions between Gab1 and Gab2 in the MBD are the binding sites for Grb2 and p85 (Fig. 1A) (Lock *et al.*, 2002). A Gab2 protein where the Gab1 Met binding motif was inserted, was able to bind Met as efficiently as Gab1 (Schaeper *et al.*, 2000; Lock *et al.*, 2002), suggesting that the p85 or Grb2 binding sites may confer some additional affinity. Although as shown here, an MBDΔGrb2 fusion protein can still bind to Gab1, indicating that the Grb2 binding site is not essential for this interaction.

Consistent with the Met binding motif in Gab1 being sufficient to bind Met, using multiple structure prediction methods including PHD (Rost and Sander, 1993, 1994; Rost, 1996), and PSIPRED (Jones, 1999; McGuffin *et al.*, 2000) the Gab1 MBD is highly predicted not to form any secondary structure and from this 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 (Chou and Fasman, 1974, 1978; Williams and Deber, 1991; Hurley *et al.*, 1992). 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 (Rocchi *et al.*, 1998; Roshan *et al.*, 1999; Lock *et al.*, 2000; Schaeper *et al.*, 2000). 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 rather than as a domain, led us to speculate that the Met receptor may contain a 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. 2A and B). This is in contrast to the interaction of Met with Grb2, which requires only a short YVNV phosphotyrosine-containing motif involving Y1356 in the C-terminus of Met, and the structured SH2 domain of Grb2 (Fig. 2A and B). In addition, we have shown that the interaction between the MBD and Met requires residues upstream of Y1349, as well as the presence of a phosphotyrosine or a negatively charged residue at 1349 (Figs. 3, 4 and 5). Several of these residues (F1341, F1344, and I1345) 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 (F1341) and comprise α-helix J (F1344 and I1345) (Fig. 6). The remainder of the residues critical for MBD binding (G1346, H1348 and pY1349) did not appear in the model as they are located C-terminal to the kinase domain. 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 (Shewchuk *et al.*, 2000). Therefore, it is unclear how the 47 amino acid C-terminus of Met, including G1346, H1348 and pY1349 fit into the model. The crystal structure of the Tie2 kinase domain and C-terminus 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 (Shewchuk *et al.*, 2000). The Met C-terminus may adopt a similar conformation, since 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, leading to inhibition of downstream signaling events, and biological function (Bardelli *et al.*, 1999).

We propose that residues identified through alanine scanning mutagenesis in the C-terminal lobe of the kinase domain function to create a binding surface for the MBM peptide and that phosphorylation of Y1349 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 Y1349 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 F1341, F1344 and I1345 on Met are aromatic and/or hydrophilic, and are predicted to lie in close contact with side chains of residues in α-helix E (K1179, I1182, G1183, L1186, and Q1187), as well as K1215 located in the loop between β-7 and β-8 (Fig. 6B). A potential binding surface for the MBD ligand is present in the region comprising helices E, I and J (Fig. 6B). However, the presence of two basic residues (K1179 in α-helix E and K1215 in the loop between β-7 and β-8) could be a deterrent to MBM binding as the positive charges of these side groups may repel the positive charge of the critical arginine 499 present at the C-terminus of the MBM. Rotation of the binding site for the MBM away from these basic residues may be required for MBM binding. Activation of the kinase and phosphorylation of tyrosines with the activation loop as well as phosphorylation of Y1349 could lead to a conformational change that would position the MBM binding region away from these basic residues, or may be required to expose the binding site for the MBM (Fig. 7, model).

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, which is critical for interaction with Met (Schaeper *et al.*, 2000; Lock *et al.*, 2002)(Fig. 1D and E). However, the MBD binding region on Met contains only one acidic residue, E1347, which was not essential (Fig. 4B). Alternatively, since phosphorylation of Y1349 or the creation of a negative charge is critical (Fig. 2A and B), the positively charged Arg 499 in the MBM may interact with the negatively charged phosphate group on Y1349, 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. These results suggest that the Met receptor kinase domain can now be added to the list of protein-protein interaction modules involved in signal transduction.

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/IPS/PIR) that allow them to interact differentially with receptors (He *et al.*, 1998; Kasus-Jacobi *et al.*, 1998). Specifically, interaction of Grb10 with the insulin and IGF-1 receptors predominantly requires its BPS domain (Stein *et al.*, 2001), while its interaction with the PDGF (Frantz *et al.*, 1997; Wang *et al.*, 1999) and EGF (Ooi *et al.*, 1995; He *et al.*, 1998) receptors requires the SH2 domain. 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 (Van Obberghen *et al.*, 2001). Intriguingly, the MBD, BPS and KRLB all bind to kinase domains, and all three interactions require receptor kinase activity (Sawka-Verhelle *et al.*, 1997; He *et al.*, 1998). 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.

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Figure 1. 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 bold. (B) Competition of Met from GST-MBDΔGrb2 or GST-Grb2 fusion proteins using the Gab1 MBD16 peptide (FGMQVPPPAHMGFRSS). The indicated concentrations of peptides were incubated for 1 hour 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 144 Met antibody. (C) Competition of Met or Grb2 from GST-MBD using the Gab1 MBD16 peptide. The indicated concentrations of peptides were incubated for 1 hour with 100 μg of lysates prepared from 293T cells transiently expressing Tpr Met or Grb2, 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 144 Met or Grb2 antibodies, respectively. (D) Competition of Met from GST-MBDΔGrb2 fusion protein using the Gab1 MBD16 peptide with arginine 499 substituted for an alanine residue. (MBD16-R499A, FGMQVPPPAHMGFASS), 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 hour. Bound Met was detected by western blot with the 144 Met antibody.

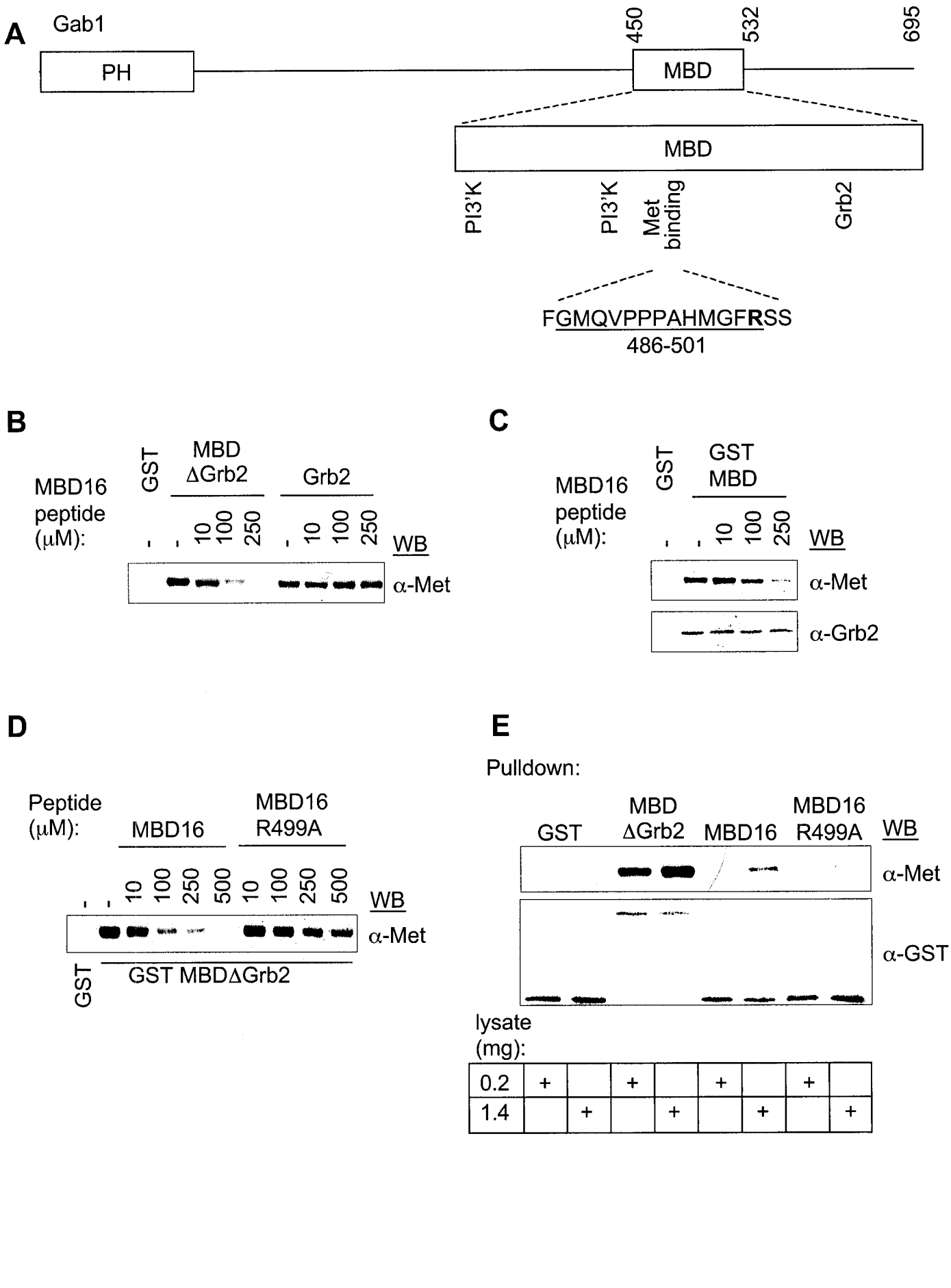
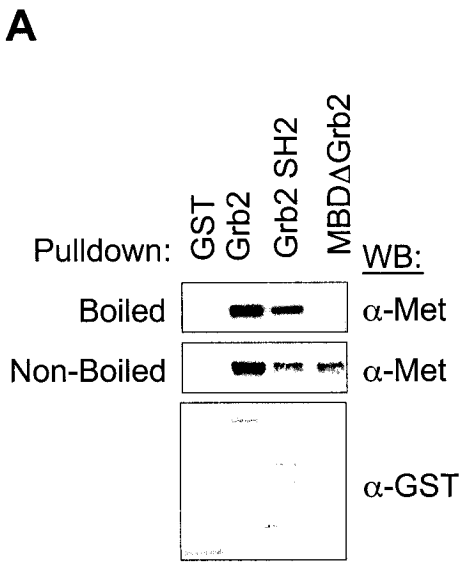

A
Gab1
PH
450
532
695
MBD
MBD
PI3'K
PI3'K
Met binding
Grb2
FGMQVPPPAHMGFRSS
486-501
B
MBD16 peptide (μM):
GST
MBD ΔGrb2
Grb2
- - 10 100 250 - 10 100 250
WB
α-Met
C
MBD16 peptide (μM):
GST
GST MBD
- - 10 100 250
WB
α-Met
α-Grb2
D
Peptide (μM):
MBD16
MBD16 R499A
- - 10 100 250 500 10 100 250 500
WB
α-Met
GST
GST MBDΔGrb2
E
Pulldown:
GST
MBD ΔGrb2
MBD16
MBD16 R499A
WB
α-Met
α-GST
lysate (mg):
0.2 + + + + + + + +
1.4 + + + + + + + +

Figure 2. The structural integrity of Met is required for the interaction of Met with the Gab1 MBD.

(A). Association with denatured versus non-denatured Met. For the boiled reaction, 1 mg of lysate prepared in RIPA lysis buffer from 293T cells transiently expressing Tpr Met was immunoprecipitated with 1 μl of 144 Met antibody for 1 hour, and incubated with protein A sepharose for 1 additional hour. After three washings, the beads were resuspended in 40 μl of 1% SDS, 50 mM Tris pH 6.8, and boiled for 5 minutes. The supernatant was frozen immediately on dry ice for 10 minutes before addition to 400 μl of RIPA lysis buffer containing the indicated GST fusion proteins bound to glutathione sepharose beads for one hour. For the non-boiled reaction, 400 μg of lysate was added to 400 μl of RIPA lysis buffer containing the indicated GST fusion proteins bound to glutathione sepharose beads for one hour. Membranes were western blotted with either anti-Met 144 or anti-GST antibodies. (B) Far western with GST-Grb2 and GST-MBD fusion proteins. Lysates prepared from 293T cells transiently expressing Tpr Met mutants (300 μg) were immunoprecipitated with anti-Met 144 antibody, loaded on an 8% polyacrylamide gel, transferred, and blocked over night in 10% milk in TBST containing 1 mM sodium vanadate. GST-Grb2 and GST-MBD fusion proteins were eluted from glutathione sepharose beads with reduced glutathione, and 20 μg of purified fusion protein was incubated with 2 μl of glutathione-conjugated horseradish peroxidase for 30 minutes at room temperature. Washed membranes were then incubated with this reaction mixture in 10 ml of TBST for 30 minutes, prior to washing and ECL. Membranes were stripped and western blotted with anti-Met 144 antibody.


A
Pulldown:
GST
Grb2
Grb2 SH2
MBDΔGrb2
WB:
Boiled
α-Met
Non-Boiled
α-Met
α-GST

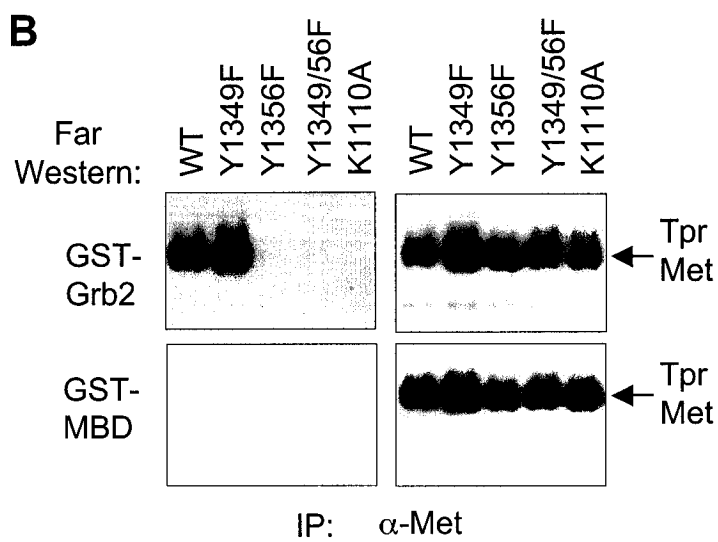

B
Far Western:
WT
Y1349F
Y1356F
Y1349/56F
K1110A
WT
Y1349F
Y1356F
Y1349/56F
K1110A
GST-Grb2
Tpr Met
GST-MBD
Tpr Met
IP: α-Met

Figure 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, as indicated. 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. The amino acid sequence of mutant A (p85 binding mutant) and B (Shc binding mutant) in comparison to WT Tpr Met are indicated (Saucier *et al.*, 2002). (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 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 (50 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).

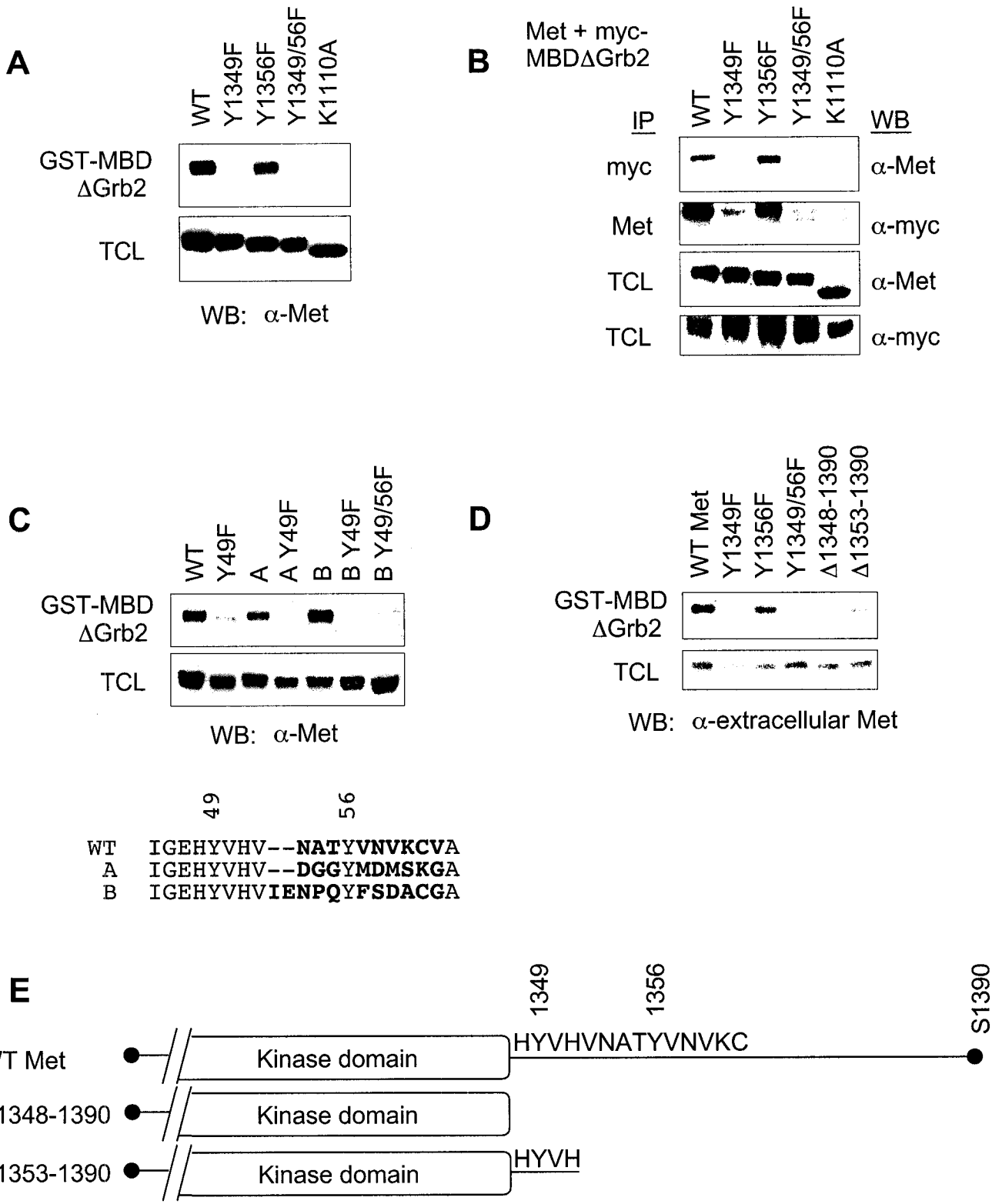

A
WT
Y1349F
Y1356F
Y1349/56F
K1110A
GST-MBD ΔGrb2
TCL
WB: α-Met
B
Met + myc-MBDΔGrb2
IP
WB
myc
α-Met
Met
α-myc
TCL
α-Met
TCL
α-myc
C
WT
Y49F
A
A Y49F
B
B Y49F
B Y49/56F
GST-MBD ΔGrb2
TCL
WB: α-Met
49
56
WT IGEHYVHV--NATYVNVKCVA
A IGEHYVHV--DGGYMDMSKGA
B IGEHYVHVIENPQYFSDACGA
D
WT Met
Y1349F
Y1356F
Y1349/56F
Δ1348-1390
Δ1353-1390
GST-MBD ΔGrb2
TCL
WB: α-extracellular Met
E
1349
1356
S1390
WT Met
Kinase domain
HYVHVNATYVNVKC
Δ1348-1390
Kinase domain
Δ1353-1390
Kinase domain
HYVH

Figure 4. Residues upstream of Y1349 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 western blotted with anti-pY1349Met, pTyr (RC20H), or 144 Met, as indicated.

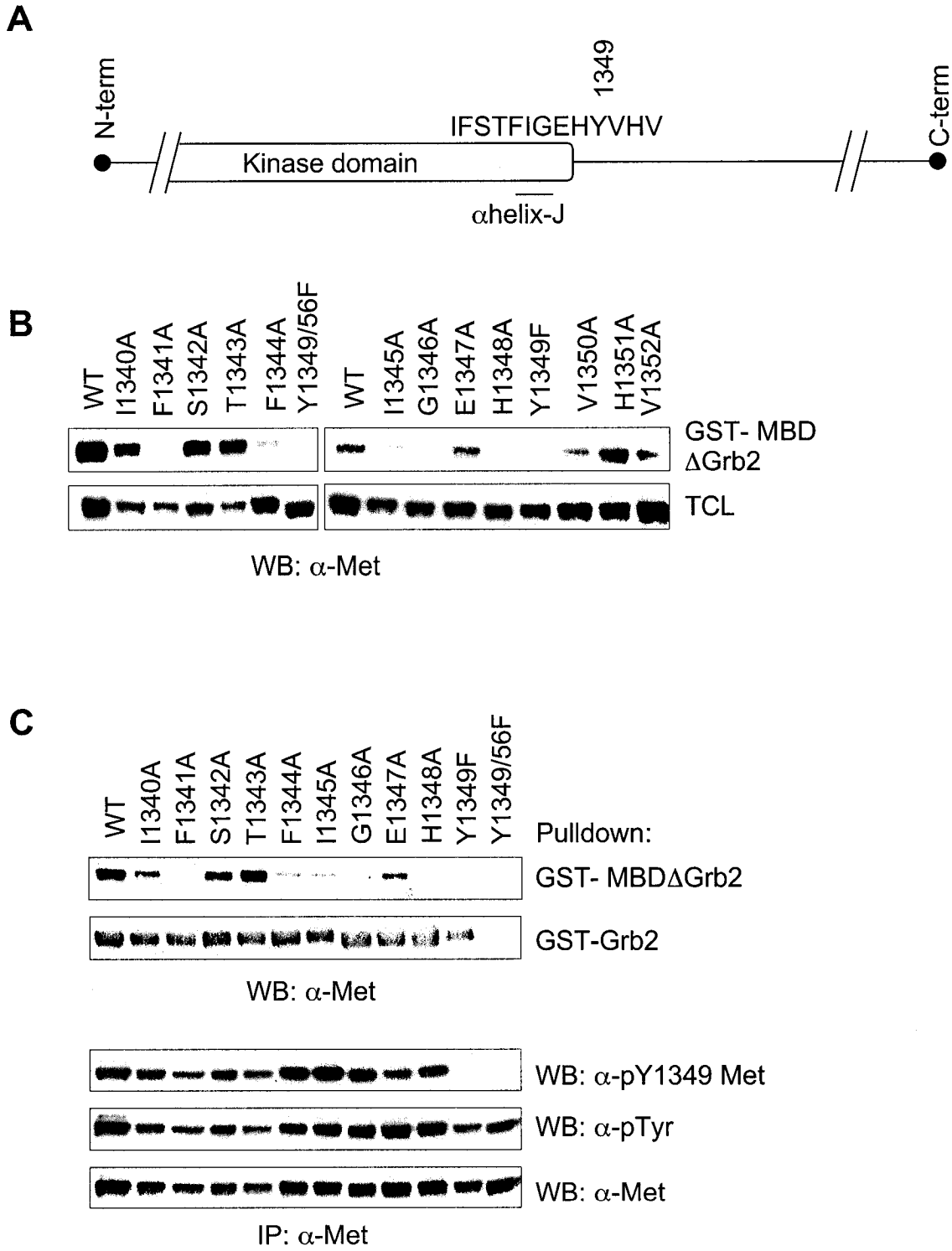

A
N-term
1349
IFSTFIGEHYVHV
C-term
Kinase domain
αhelix-J
B
WT
I1340A
F1341A
S1342A
T1343A
F1344A
Y1349/56F
WT
I1345A
G1346A
E1347A
H1348A
Y1349F
V1350A
H1351A
V1352A
GST- MBD ΔGrb2
TCL
WB: α-Met
C
WT
I1340A
F1341A
S1342A
T1343A
F1344A
I1345A
G1346A
E1347A
H1348A
Y1349F
Y1349/56F
Pulldown:
GST- MBDΔGrb2
GST-Grb2
WB: α-Met
WB: α-pY1349 Met
WB: α-pTyr
WB: α-Met
IP: α-Met

Figure 5. A negative charge at residue 1349 is sufficient for MBD binding.

(A) 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 Y to E 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. (B) 293 cells were transiently transfected with expression vectors for Tpr Met WT or mutants, as indicated. Lysates were either immunoprecipitated with anti-Gab1 and western blotted for Gab1 ptyr, or incubated with GST-Grb2 bound to glutathione sepharose beads, and 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 endogenous Gab1 and Tpr Met mutants. (C) Rat1 fibroblast cells were transfected with equal amounts of expression plasmids encoding Tpr Met mutants, and the number of foci from three experiments, performed in duplicate were assessed and the dishes are represented here.

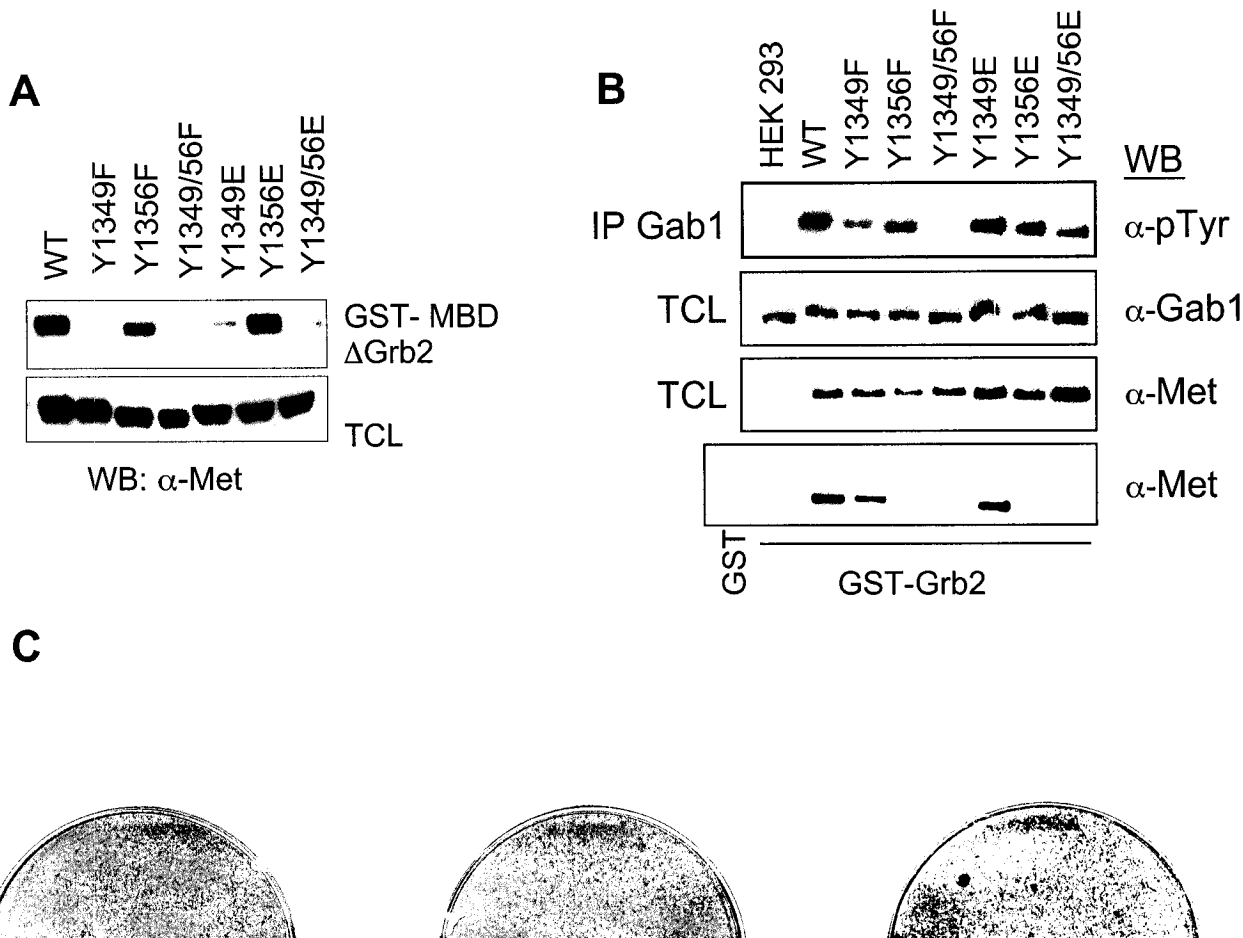

A
WT
Y1349F
Y1356F
Y1349/56F
Y1349E
Y1356E
Y1349/56E
GST- MBD ΔGrb2
TCL
WB: α-Met
B
HEK 293
WT
Y1349F
Y1356F
Y1349/56F
Y1349E
Y1356E
Y1349/56E
WB
IP Gab1
α-pTyr
TCL
α-Gab1
TCL
α-Met
α-Met
GST
GST-Grb2
C
Rat1monolayer
Y1349/56F Met
Y1349/56E Met

Figure 6. Residues critical for MBD binding lie within the Met receptor kinase domain.

(A) Ribbon diagram of the modeled Met receptor kinase domain. The α-helices are shown in red, β-strands in blue, the nucleotide binding loop in yellow, the catalytic loop in orange, and the activation loop in green. The termini are denoted by N and C. Residues required for MBD binding are located within the loop between α-I and α-J, and within α-J. (B) Critical residues for MBD binding are in close proximity to residues in α-helix E and β-7 and β-8. Residues identified through alanine scanning mutagenesis (F1341, F1344 and I1345) are shown in yellow, while residues less than 5 angstroms away are shown in aqua. The figures were prepared with Swiss PDB viewer (Peitsch, 1995, 1996; Guex and Peitsch, 1997).

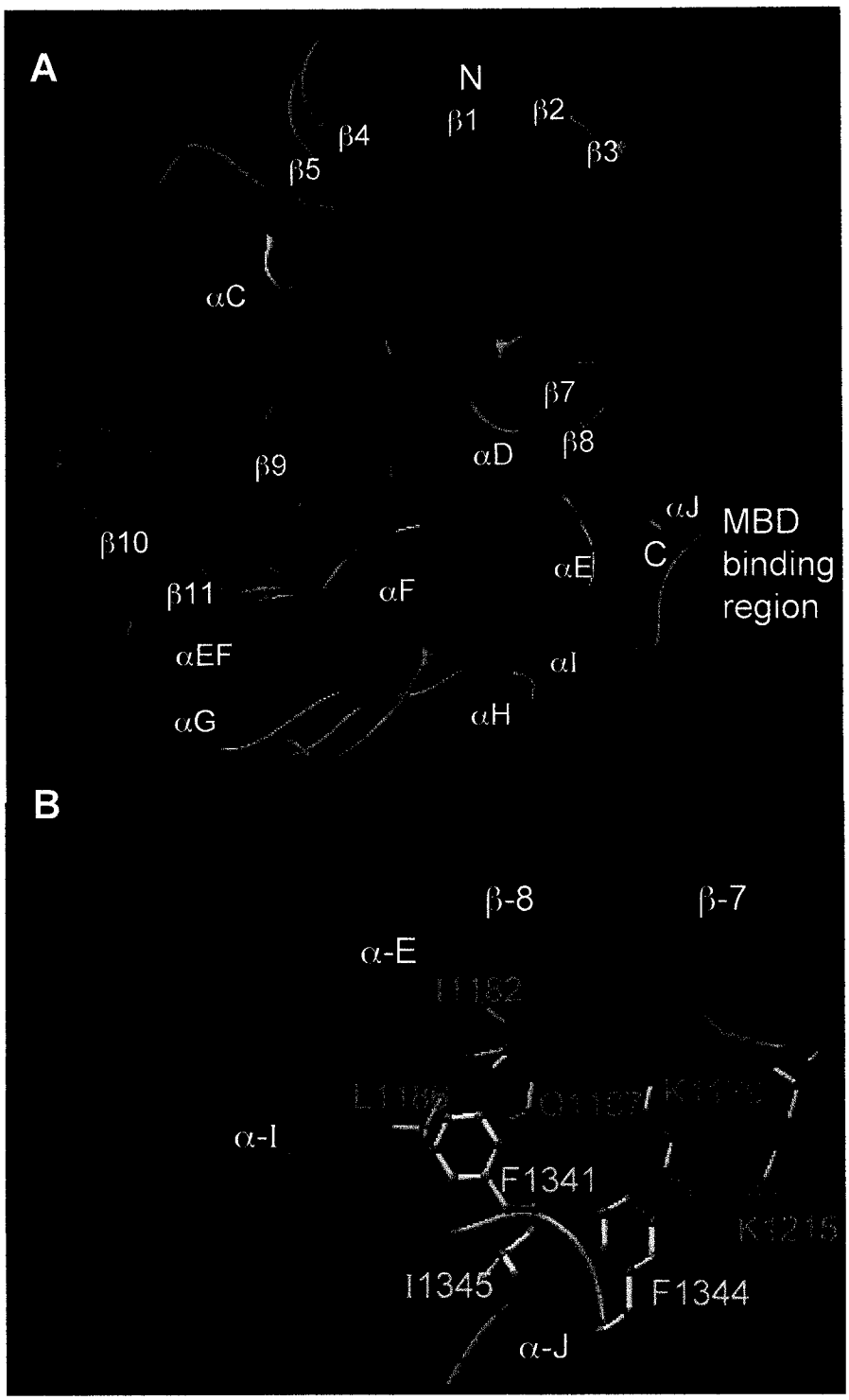
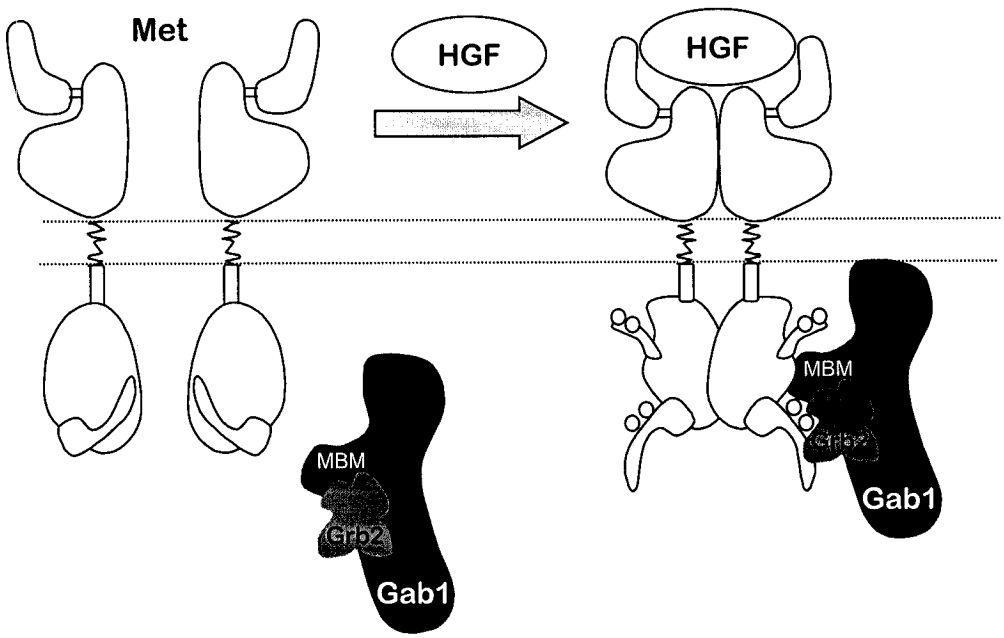

A
N
β1
β2
β3
β4
β5
αC
β7
β8
αD
β9
β10
β11
αF
αJ
MBD
binding
region
C
αE
αEF
αI
αG
αH
B
β-8
β-7
α-E
α-I
F1341
I1345
F1344
α-J

Figure 7. Model for Met -Gab1 MBM interaction.

In the absence of ligand, the Met receptor is in an inactive conformation. Binding of HGF to the Met receptor leads to dimerization, and phosphorylation of tyrosines in the activation loop, stabilizing it in an open and extended conformation that is permissive for substrate binding. Phosphorylation of Y1349 located at the junction between the kinase domain and the C-terminus may be required for a conformational shift in Met that exposes a binding surface for Gab1 Met binding motif (MBM) within the C-terminal lobe of the kinase domain.


Met
HGF
HGF
MBM
Grb2
Gab1
MBM
Gab1

Chapter V: Discussion

The Met/HGF receptor tyrosine kinase mediates pleiotropic effects including cell proliferation, survival, cell dispersal, and morphogenesis. These effects are fundamental for the diverse biological functions of HGF observed *in vivo*. During embryogenesis, as well as in the adult, HGF plays roles in axonal outgrowth, angiogenesis, tissue regeneration, and wound healing (reviewed in (Rosen *et al.*, 1994; Jeffers *et al.*, 1996; Birchmeier and Gherardi, 1998)). HGF and Met are deregulated in many human cancers, through mechanisms that include increased Met expression, increased Met receptor catalytic activity resulting from point mutations, and the formation of autocrine stimulatory loops (reviewed in (Danilkovitch-Miagkova and Zbar, 2002)), implicating both Met and HGF in tumor formation and progression.

Gab1 is the major phosphorylated protein downstream of the Met receptor in epithelial cells, and functions to couple a Met signal to various signaling proteins including PI3K, PLC-γ, SHP-2, and Crk. Importantly, Gab1 is a critical mediator of Met-receptor mediated epithelial remodeling required for the morphogenic response. Determination of the mechanisms involved in recruitment of Gab1 to the Met receptor, and its subsequent tyrosine phosphorylation, is crucial to permit a clearer understanding of the role of Gab1 in Met receptor-mediated biological processes. These studies could provide an approach to interfere specifically with the Gab1-dependent biological effects triggered by the Met receptor.

1. Mechanism of recruitment to receptors:

The recruitment of Gab1 to activated receptor complexes, and its subsequent phosphorylation was poorly understood. Gab1 was shown to associates constitutively with the C-terminal SH3 domain of the Grb2 adapter protein (Nguyen *et al.*, 1997), suggesting a role for Grb2 in the indirect recruitment of Gab1 to receptor tyrosine kinases. By identification and subsequent mutation of the Grb2 binding sites in Gab1, I established that the association of Gab1 with the EGF receptor is dependent on Grb2 (Lock *et al.*, 2000). In agreement, mutation of the Grb2 SH2 domain binding sites on the

EGF receptor abolished the association between Gab1 and the EGF receptor (Rodrigues *et al.*, 2000), while an absence of Gab1 tyrosine phosphorylation in response to EGF is found in fibroblast cells isolated from mice expressing a null/hypomorphic mutant of Grb2 (Saxton *et al.*, 2001). These results provide both biochemical and genetic evidence for a Grb2-dependent mechanism of recruitment of Gab1 to the EGF receptor.

The Grb2 binding sites are the most highly conserved regions in all members of the Gab family, including DOS and SOC-1. Moreover, many receptors contain binding sites for the SH2 domain of Grb2, suggesting that this may be a common mechanism of recruitment to activated receptors. However, recruitment of Gab proteins to receptors that do not contain binding sites for the SH2 domain of Grb2 may require an adapter protein such as Shc or FRS2, that is able to bind simultaneously to receptors and the SH2 domain of Grb2. Accordingly, Gab2 is recruited to the interleukin-3 receptor beta common chain (βc) through a Shc-Grb2-Gab2 mechanism (Gu *et al.*, 2000), while an FRS2-Grb2-Gab1 interaction is utilized for the recruitment of Gab1 to the FGFR1 receptor (Ong *et al.*, 2001) (Fig. 1).

2. Identification of an atypical Grb2 C-SH3 binding motif in Gab1

Gab1 had originally been identified as a Grb2-binding protein (Holgado-Madruga *et al.*, 1996) that associated constitutively with the C-terminal SH3 domain of Grb2 (Nguyen *et al.*, 1997). However, the Grb2 binding sites had not been determined, and were originally predicted to correspond to two PXXPXR sites present in Gab1 (Holgado-Madruga *et al.*, 1996). PXXPXR motifs are class II, polyproline (PP)-II left-handed helical sequences known to bind to SH3 domains, including the amino-terminal SH3 domain of Grb2 (Goudreau *et al.*, 1994; Lim and Richards, 1994; Terasawa *et al.*, 1994). However, mutation of both of these sites had no effect on the binding of Gab1 to Grb2 (Lock *et al.*, 2000).

By combining multiple approaches including deletion analyses, alanine scanning mutagenesis, and peptide competition, I identified two Grb2 carboxyl-terminal SH3 domain binding sites in Gab1. One site contains several typical P*XX*P motifs, which favor the formation of the PP-II helix, and are requirements for SH3 domain interactions.

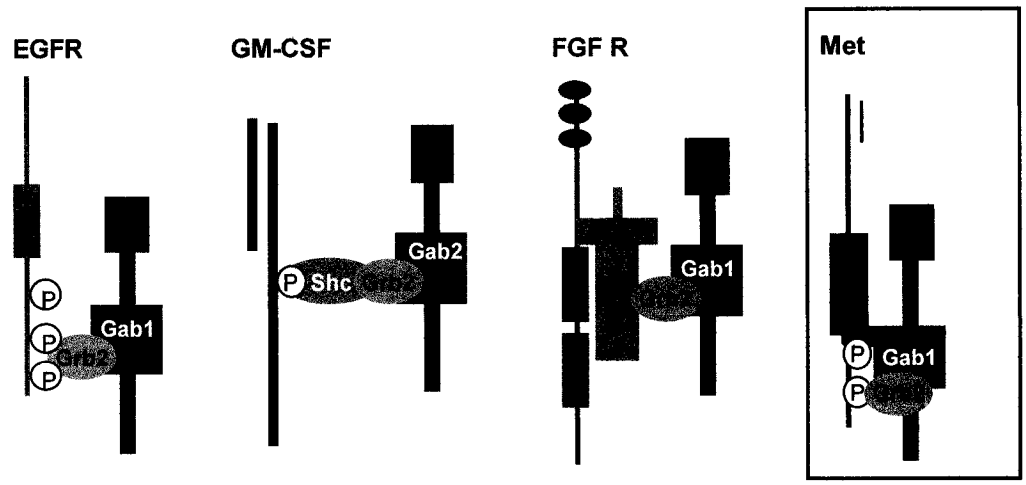


Figure 1: Critical role for Grb2 in the recruitment of Gab proteins to receptors

Importantly, the second Grb2 binding site contains an atypical non-P*XX*P motif, where a proline and an arginine separated by three amino acids (PXXXR) are critical. These two sites are also present in Gab2, and are required for its association with Grb2. Moreover, the PXXXR site is also present in the unrelated Grb2-binding protein Slp-76, and alignment of Gab1,Gab2 and Slp-76 further identified an elongated motif of PX(3)RX(2)KPX(7)PLD. This motif is also required for binding to the C-SH3 domain of the Grb2-related protein Gads/Mona (Lock *et al.*, 2000).

Other groups have further defined the consensus as PXXXRXXKP (Kato *et al.*, 2000; Schaeper *et al.*, 2000; Lewitzky *et al.*, 2001), while recently, Berry and colleagues found that the interaction of Slp-76 with the Grb2 and Gads/Mona C-SH3 domains required an RXXK motif, within the previously defined PXXX**RXXK**P motif. Although the flanking prolines significantly enhanced this interaction, they were not absolutely essential (Berry *et al.*, 2002). The P-X-X-X-R-X-X-K-P motif is found in all Gab family

members, as well as Gab-unrelated proteins, including the B-cell linker family (BLNK, and Slp-76), AMSH (associated molecule with the SH3 domain of STAM), and UBPY (member of ubiquitin-specific protease family). The motifs in these proteins bind to specific SH3 domains, thus far including the C-terminal SH3 domains of Grb2 and Gads/Mona, STAM and Hbp (STAM2/Hrs-binding protein). Accordingly, phylogenetic analysis of the complete set of 211 nonredundant human SH3 domains indicates that these SH3 domains cluster as a group comprised of 16 SH3 domains that separate from the bulk of the SH3 domains at the first branch point (Berry *et al.*, 2002).

2.1. SH3 domains recognize more diverse motifs than PxxP

SH3 domains have long been recognized to bind to proline-rich peptides containing the core PXXP, which adopts a pseudo-symmetrical polyproline class II (PP-II) helix. However, recent studies have shown that SH3 domains utilize their hydrophobic surface with considerable versatility, and the repertoire of SH3 recognition has been extended beyond the classical proline-rich PXXP. The crystal structures of Fyn SH3 with HIV-1 Nef (Lee *et al.*, 1996), $p67^{phox}$ SH3 with $p47^{phox}$ (Kami *et al.*, 2002), and that of the 53BP2 SH3 with the core domain of the p53 protein (Gorina and Pavletich, 1996), have shown that other regions in the protein bear the major responsibility for accomplishing the high affinity binding, in conjunction with the PPII helix-binding site. Moreover, sequences lacking a typical PXXP motif have been reported to bind a variety of SH3 domains. In addition to the PXXXRXXKP motif identified above, sequences with the consensus PXXDY derived from the e3b1/Abi-1 and RN-tre proteins, interact with the Eps8 SH3 domain (Mongiovi *et al.*, 1999), while sequences with the consensus RKXXYXXY, from SKAP55, bind to the SH3 domains in the Fyb, Fyn and Lck proteins (Kang *et al.*, 2000), and a WXXXFXXLE pattern from Pex5p interacts with the Pex13p SH3 domain (Barnett *et al.*, 2000; Urquhart *et al.*, 2000).

The C-terminal SH3 domain of Grb2 is extremely versatile, and can bind at least three diverse types of ligands: canonical PXXP-containing ligands such as Sos (Lim *et al.*, 1994), PXXXRXXKP-containing ligands such as Gab1, and the SH3 domain of Vav (with which it dimerizes) (Nishida *et al.*, 2001). Noteably, although the binding sites for all three ligands are distinct, they all overlap at a small portion of the Grb2 C-SH3

domain, including F165, N208, and Y209 (Lim *et al.*, 1994; Nishida *et al.*, 2001; Kami *et al.*, 2002).

3. Grb2-independent association of Gab1 with the Met receptor

In contrast to the association of Gab1 with the EGF receptor, mutation of the Grb2 binding sites on Gab1 only slightly diminishes the association of Gab1 with the Met receptor, by co-immunoprecipitation or *in vitro* association assay (Lock *et al.*, 2000). Moreover, a Gab1 mutant that lacks Grb2 SH3 domain binding sites is able to rescue the morphogenic phenotype of MetΔGrb2-expressing MDCK cells as efficiently as WT Gab1 (Lock *et al.*, 2000; Lock *et al.*, 2002). These results indicate that Gab1 can interact with the Met receptor in the absence of a Grb2-dependent interaction (Fig. 1), and suggests that another mechanism of interaction exists. Indeed, Gab1 had been identified previously as a direct binding partner for the Met receptor in a yeast two-hybrid assay. This interaction required the kinase activity of the receptor, phosphorylation of tyrosines 1349 (major) and 1356 (minor) in the Met receptor, and an 83 amino acid, proline-rich region in Gab1 termed the Met binding domain (MBD) (Weidner *et al.*, 1996). Although the MBD has been proposed to be a phosphotyrosine binding (PTB)-like domain (Weidner *et al.*, 1996), the mechanism of interaction between the Gab1 MBD and Met was unclear.

I have shown that mutation of the Grb2 binding sites in Gab2 abolishes the association of Gab2 with the Met receptor, indicating that their interaction is strictly dependent on Grb2 (Lock *et al.*, 2002). Therefore, to define the region in the Gab1 MBD required for its Grb2-independent interaction with Met, I generated chimeric fusion proteins of Gab1 and Gab2 MBD regions. Insertion of a 19 amino acid sequence, SSFGMQV**PP**PAHMGF**R**SSP, into the Gab2 MBD region was sufficient to confer Met binding capability on Gab2, thereby identifying this region as the critical component of the MBD-Met interaction. The first and second prolines, in addition to the arginine are essential for this interaction (Lock *et al.*, 2002). Another group also identified the same sequence in Gab1, consisting of 13 amino acids (underlined) (Schaeper *et al.*, 2000). I have shown that these 19 amino acids are sufficient for interaction with the Met receptor (Chapter IV, Fig. 1). This suggests that the MBD functions not as a PTB-like domain,

but rather, as an extended peptide motif, with surrounding amino acids in the MBD conferring higher affinity binding to the Met receptor. In accordance, the MBD is highly predicted not to form any secondary structure using multiple computer-based structure prediction methods. However, secondary structure analysis of purified MBD by circular dichroism is needed to verify these results. We have therefore changed the name from Met binding domain, to Met binding motif. (MBM).

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 rather than as a domain, led us to speculate that the Met receptor may contain a domain that interacts with the MBM peptide. In support of this, we have shown that the structural integrity of the Met receptor is required for its interaction with the MBM and that this interaction requires phosphorylation of tyrosine 1349, and residues upstream, located in the C-terminal lobe of the kinase domain, after α-helix I (Chapter IV, Fig. 2-5). We propose that residues identified through alanine scanning mutagenesis in the C-terminal lobe of the kinase domain may function to create a binding surface for the MBD peptide. However, we cannot rule out the possibility that mutation of these residues may affect the binding of the MBD to another location in the Met kinase domain. Indeed, it is feasible that phosphorylation of the tyrosines in the activation loop of the kinase domain, and/or the C-terminal tyrosines could lead to a conformational change that would expose the binding site for the MBD. NMR analysis is required to verify that these residues in the Met receptor kinase domain are involved in binding the MBD. A recently developed NMR method, cross-saturation, which can identify residues involved in the molecular interface between two proteins, based on the saturation transfer from a non-labeled to a labeled protein, could be used (Wuthrich, 2000).

These results support the interpretation that the Gab1 MBM interacts with the Met receptor in a novel and previously unsuspected manner, where instead of the expected interaction of a phosphotyrosine binding domain in Gab1 with a phosphotyrosine-containing motif in the Met receptor, we propose that the activated kinase domain of Met and the negative charge of phosphotyrosine 1349 engage the Gab1 MBM as an extended peptide ligand. Solving the three-dimensional structure of Met, in conjunction with an MBD peptide, will permit a clearer understanding of this novel interaction, and could

provide an approach to interfere specifically with the biological effects triggered by the Met receptor.

3.1 Unique interaction with specific receptors: A mechanism to modulate distinct biological responses?

To date, the Gab1 MBD interacts in a Grb2-independent manner with only the Met receptor and its close family member Ron, (L.S. Lock and M. Park, unpublished results), and not with other receptors tested, including TrkA, c-Ros, DDR, c-Ret, v-Sea, Sek-1, c-Kit, c-Abl, and the insulin, EGF, PDGF, CSF-1, and keratinocyte growth factor receptors (Weidner *et al.*, 1996; Lock *et al.*, 2000). 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 contains novel receptor-specific interaction domains (BPS/IPS/PIR) (He *et al.*, 1998; Kasus-Jacobi *et al.*, 1998), in addition to their SH2 domains that allow them to interact differentially with receptors. Specifically, interaction of Grb10 with the insulin and IGF-1 receptors predominantly requires its BPS domain (Stein *et al.*, 2001), while interaction with the PDGF (Frantz *et al.*, 1997; Wang *et al.*, 1999) and EGF (Ooi *et al.*, 1995; He *et al.*, 1998) receptors requires the SH2 domain. Moreover, the kinase regulatory loop-binding (KRLB) domain of IRS-2 interacts specifically with the insulin receptor but not the highly related IGF-1 receptor (Van Obberghen *et al.*, 2001). 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 distinct biological responses downstream from receptor tyrosine kinases.

3.2 Novel interactions with kinase domains of receptors

I have shown in chapter four, that the MBD region of Gab1 interacts with the kinase domain of the Met receptor. Intriguingly, the BPS of Grb10 and the KRLB of IRS-2 also bind to kinase domains, and all three interactions require receptor kinase activity (Sawka-Verhelle *et al.*, 1997; He *et al.*, 1998). Furthermore, all three proteins have alternate modes of interacting with receptors; SH2, PTB/PH and Grb2-dependent,

for Grb10, KRLB and MBD, respectively. Binding of the BPS domain of Grb10 to the insulin or IGF-1 receptor kinase domains negatively regulates both kinase activity and substrate phosphorylation (Stein *et al.*, 2001). Although this requires phosphorylation of tyrosines in the activation loop, the BPS domain does not appear to bind directly to the phosphotyrosines in the activation loop (Stein *et al.*, 2001). In accordance, overexpression of Grb10 negatively regulates insulin-dependent phosphorylation of IRS-1 and activation of PI3K in Chinese hamster ovary cells (Liu and Roth, 1995).

The KRLB of IRS-2 appears to bind the phosphorylated activation loop of the insulin receptor, as shown using peptide competition studies (Sawka-Verhelle *et al.*, 1997). However, the precise molecular nature of this interaction, in addition to a biological role for the KRLB, is still undefined. 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. Interestingly, alignment of the Gab1 MBS with amino acids 698-714 from KRLB shows 47% identity within a 17 amino acid sequence, with two gaps added to enhance alignment (Fig. 2). Although it is not clear whether this is significant, it is intriguing to speculate that these two proteins may interact with the kinase domains of their respective receptors through similar mechanisms.

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Gab1 MBM     GMQVP-PPAHMGFRSSP
             :  :: ::. .: :. :
IRS-2 KRLB   GAAVPAPPSGVG-RTFP
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Figure 2: Alignment of the Gab1 MBM with the IRS-2 KRLB.

Amino acids 487-502 from the Gab1 MBM were aligned with amino acids 698-714 from the IRS-2 KRLB.Residues in bold are those determined by (Schaeper *et al.*, 2000) to be critical for Met binding.

3.3 The role of Grb2-independent association of Gab1 with the Met receptor in tyrosine phosphorylation and epithelial morphogenesis

My studies have established that the Grb2-independent interaction of Gab1 with Met is required for efficient tyrosine phosphorylation of Gab1 and for rescue of epithelial morphogenesis in MetΔGrb2-expressing MDCK cells. A Gab1 mutant with a disrupted MBM (Gab1Δ3P) is both less phosphorylated and unable to mediate morphogenesis (Lock *et al.*, 2002). It is not clear whether this lower level of phosphorylation is due to fewer Gab1Δ3P molecules being phosphorylated relative to WT Gab1, or to phosphorylation on fewer tyrosines per molecule of Gab1Δ3P. Therefore, a possible mechanism for the inability of Gab1Δ3P to mediate morphogenesis could be due to a lack of binding to unidentified signaling proteins, or to the inability to reach a threshold of phosphorylated Gab1 proteins required for the morphogenic response. In support of the latter possibility, a Met receptor mutant lacking the Grb2 binding site shows a reduced tyrosine phosphorylation of endogenous Gab1, and is unable to induce the morphogenic phenotype. Only upon overexpression of Gab1, such that a threshold of Gab1 tyrosine phosphorylation is reached, is morphogenesis achieved (Maroun *et al.*, 1999a). A dual mode of contact (Grb2-dependent and independent) between Gab1 and Met may promote the formation of a more stable complex, producing increased tyrosine phosphorylation of Gab1 and subsequent increases in the docking of other signaling proteins, thereby leading to enhanced signaling downstream of the Met receptor.

To clearly define the biological implications of this interaction, cell-permeable peptides containing an antennapedia signal linked to an MBM peptide could be tested for their ability to disrupt Met-mediated processes such as cell scatter and morphogenesis. The ultimate test would be to "knock-in" a Gab1 mutant with a disrupted MBM into Gab1-deficient mice and assess the biological impact resulting from disruption of a direct Gab1-Met interaction *in vivo*.

3.4 Possible effects of MBD binding on Met receptor action

The binding of the MBD to the kinase domain of Met may affect the Met receptor itself. For example, the phosphatase PTP-1B binds to phosphotyrosines in the activation loop of the Met receptor, leading to dephosphorylation of the receptor (V. Sangwan and M. park, unpublished results). The MBD may function to inhibit binding to and dephosphorylation of tyrosines by PTP-1B, thereby preventing or delaying inactivation of the Met receptor. Alternatively, interaction of the MBD with the kinase domain of the Met receptor may function to positively modulate the kinase activity or substrate phosphorylation by Met. The effects of the MBD on Met receptor kinase activity, receptor phosphorylation, or even receptor stability and degradation, have not been examined thus far, and remain as key experiments to perform.

4. Gab1 and Gab2 are not functionally redundant in MDCK cells

In vivo, Gab1 is critical for embryonic development, as Gab1-deficient mice die in utero, displaying defects in the heart, placenta and skin, as well as reduced liver size. (Itoh *et al.*, 2000; Sachs *et al.*, 2000). In contrast to Gab1, mice lacking Gab2 are viable and generally healthy, but have a defect in FcεRI-mediated allergic responses, including passive cutaneous and systemic anaphylaxis, cytokine gene expression and mast cell degranulation, as well as mast cell development (Gu *et al.*, 2001; Nishida *et al.*, 2002). Whether these differences are mainly attributed to distinct tissue expression during development or to a distinct function of Gab proteins had not been directly evaluated. To address this issue, we used the MDCK epithelial cell system to study the roles of Gab1 and Gab2 in epithelial morphogenesis downstream of the Met receptor tyrosine kinase. In contrast to Gab1, Gab2 is unable to rescue the epithelial morphogenesis defect of the MetΔGrb2 receptor mutant (Lock *et al.*, 2002), providing evidence for the first time for a distinct role of Gab1 and Gab2 downstream from the Met receptor, and suggesting that possible differences in expression during development are not solely responsible for the distinct phenotypes of the Gab null mice. Gab3 has not been as extensively studied as Gab1 or Gab2, however preliminary studies in our lab indicate that Gab3 is unable to rescue the morphogenic phenotype (V. Sangwan and M. Park, unpublished).

4.1 Grb2-independent recruitment to Met is essential but not sufficient for rescue of epithelial morphogenesis

I have shown that Gab2 is phosphorylated to lower levels than Gab1 upon HGF stimulation. Moreover, Gab2 does not contain a functional Met binding domain, and hence its association with the Met receptor is strictly dependent on Grb2 (Lock *et al.*, 2002). We had therefore hypothesized that the inability of Gab2 to rescue morphogenesis could be attributed to the absence of the Gab1 Met binding sequence, and hence the low levels of Gab2 phosphorylation. To test this, I inserted the Met binding motif (MBM) into Gab2, to generate a Gab2/1/2* protein, that was capable of binding Met to similar levels as WT Gab1. Moreover, in stable MDCK cell lines, Gab2/1/2* was phosphorylated to higher levels than Gab2, although it was not as highly phosphorylated as Gab1. Importantly, the Gab2/1/2* protein was unable to rescue epithelial morphogenesis, even when expressed at levels much higher than WT Gab1 (Lock *et al.*, 2002). These results indicate that although the Grb2-independent recruitment of Gab proteins to the Met receptor is essential (as discussed in section 3.3), it is not sufficient.

4.2 Possible differences between Gab1 and Gab2

We hypothesized that in addition to its lack of direct association with Met, the inability of Gab2 to rescue morphogenesis could be due to two reasons. One, Gab2 lacks a region found in Gab1 that is required for morphogenic capability; or two, Gab2 contains a region not found in Gab1, that inhibits the morphogenic capability. To investigate these possibilities, we have created Gab1/Gab2 chimeric proteins (Fig. 3), and are currently testing their ability to rescue morphogenesis. These chimeric Gab1/Gab2 proteins will allow us to identify the region(s) required for the morphogenic capability of Gab1, and to further delineate specific interactions or mechanisms required for the complex process of morphogenesis. The differences in biological function of Gab1 and Gab2 could be attributed to multiple possibilities, some of which will be discussed in the subsequent sections. Although my studies have allowed us to rule out several possibilities, the

difference(s) between Gab1 and Gab2 are still unclear and are currently under investigation.

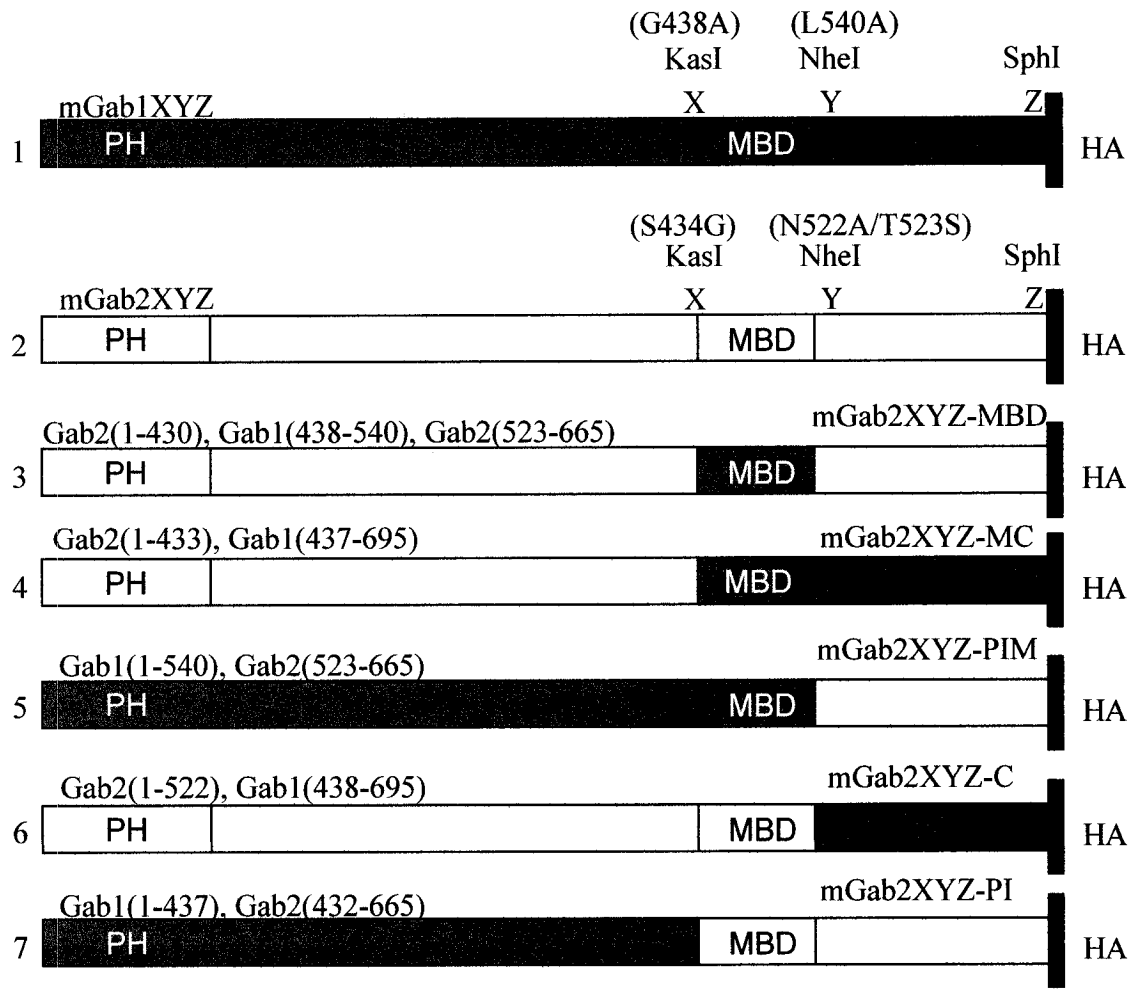

Figure 3: Gab1/Gab2 chimeras.

4.3.1 The potential role of tyrosine phosphorylation

Both Gab2 and Gab2/1/2* are less phosphorylated than Gab1 upon HGF stimulation, suggesting that a high level of tyrosine phosphorylation may be required for rescue of epithelial morphogenesis. However, I have shown that when overexpressed and phosphorylated after activation of Met, Gab2 can associate to the same extent as Gab1 with known signaling proteins (Shp-2, p85, Crk and PLC-γ) (Lock *et al.*, 2002), indicating that the tyrosines involved in binding to these proteins are efficiently phosphorylated, and that the inability of Gab2 to rescue morphogenesis is not due to an inability to associate with known signaling molecules. Gab2 does however lack two potentially phosphorylated tyrosines (Y307 and Y373 in Gab1), which could contribute to the lower level of tyrosine phosphorylation, and/or be required for association with an unidentified protein that is critical for the morphogenic response. However, although these two tyrosines contain a consensus binding site for Crk/PLCγ, Gab2 retains the ability to bind these signaling proteins (Gual *et al.*, 2000; Lamorte *et al.*, 2000; Sakkab *et al.*, 2000; Schaeper *et al.*, 2000; Lock *et al.*, 2002).

It is also possible that other kinases activated downstream of Met, such as Src family kinases, 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 (L.S. Lock and M. Park, unpublished data). Moreover, PKB/Akt negatively regulates heregulin-mediated tyrosine phosphorylation of Gab2 through serine phosphorylation in epithelial cells (Lynch and Daly, 2002), providing a possible mechanism for the lower level of Gab2 phosphorylation in MDCK cells upon HGF stimulation (see section 4.3.3).

4.3.2 The potential role of subcellular localization

The Gab1 PH domain binds specifically to the PI3K product, phosphatidylinositol-3,4,5-P_3 (PIP_3) (Maroun *et al.*, 1999b; Rodrigues *et al.*, 2000), and is necessary and sufficient for the localization of Gab1 at sites of cell-cell contact in epithelial cells, and for Gab1-dependent morphogenic responses (Maroun *et al.*, 1999a;

Maroun *et al.*, 1999b). Importantly, whereas deletion of the Gab1 PH domain abolishes epithelial morphogenesis, replacement of the Gab1 PH domain with a myristoylation tag was sufficient to allow Gab1 to mediate epithelial morphogenesis, indicating that the PH domain of Gab1 functions predominantly as a localization module, rather than having additional roles in regulating intracellular signaling (Maroun, C.R., et al, in press).

Although the phosphoinositide binding specificity of Gab2 has not been determined, it also localizes to cell-cell junctions in MDCK epithelial cells, both in response to HGF, and in steady-state conditions (Lock *et al.*, 2002). Moreover, the PH domains of Gab1 and Gab2 are 87% similar, suggesting that the Gab2 PH domain would likely also bind the PI3K product, PIP_3. However, preliminary results indicate that substitution of the Gab2/1/2* PH domain with a myristoylation tag to is sufficient to rescue morphogenesis (M. Frigault and M. Park, unpublished), suggesting that constitutive membrane localization, plus the ability to associate directly with the Met receptor, is sufficient to mediate epithelial morphogenesis. It is not clear yet whether a myristoylated WT Gab2 protein can also rescue morphogenesis. Further studies will be required to verify these results, including evaluation of the morphogenic capacity of a chimeric Gab2 or Gab2/1/2 protein containing the Gab1 PH domain, as well as the determination of the phosphoinositide binding specificity of the Gab2 PH domain.

4.3.3 The potential role of serine/threonine phosphorylation

It has been postulated that Gab proteins are extensively serine/threonine (S/T) phosphorylated, due to their delayed migration in SDS-PAGE gels (Holgado-Madruga *et al.*, 1996). Moreover, activation of serine/threonine kinases by okadaic acid, a specific inhibitor of the serine/threonine phosphatases, PP1 and PP2A, leads to hyper-S/T phosphorylation of Gab1 (Gual *et al.*, 2001). Interestingly, Gab1 and Gab2 have a distinctly different array of potential S/T phosphorylation sites (Table 1), possibly leading to a distinct regulation of the two proteins. Potential effects of S/T phosphorylation include increased or decreased tyrosine phosphorylation, leading to increased or decreased association with signaling proteins; creation of a new binding site (eg. FHA domains or 14-3-3 proteins), altered conformation, or altered localization. Only a few studies thus far have examined the role of S/T phosphorylation of Gab proteins.

Table 1: Potential Ser/Thr phosphorylation sites in Gab1 and Gab2.
Casein kinase II (S/T-X-X-E/D),cAMP-dependent kinase (R/K-R/K-X-S/T), Cdc2 kinase (S/T-P-X-K/R), MAP kinase (P-X-S/T-P), and protein kinase C (S/T-X-R/K).

	Gab1	*Gab2*	*Conserved*
Casein kinase II	24	13	7
Protein kinase C	9	6	2
MAP kinase	5	2	2
Cdc2 kinase	5	1	1
cAMP protein kinase	0	4	0
Total	43	26	12

It was recently shown that Gab2, but not Gab1, is negatively regulated by its constitutive association with PKB/Akt, leading to phosphorylation of Gab2 on serine 159. An S159A mutant abolishes this negative regulation and leads to increased tyrosine phosphorylation of Gab2 and other cellular proteins, increased Mapk and Akt activation, and growth of NIH 3T3 cells in soft agar, revealing a proto-oncogenic role for Gab2 (Lynch and Daly, 2002). To address the role of PKB-mediated negative regulation of Gab2, a Gab2 S159A mutant, as well as a Gab2/1/2*-S159A mutant are currently being expressed in MetΔGrb2-expressing epithelial cells, to determine if PKB/Akt-mediated negative regulation prevents Gab2 from rescuing morphogenesis (M. Frigault and M. Park, in progress).

In contrast, Gab1, but not Gab2, binds to and is a substrate of Erk1/2 Mapk (Roshan *et al.*, 1999). Phosphorylation of a threonine within the MBD of Gab1 upon growth factor stimulation, leads to either increased (HGF) or decreased (EGF) association of Gab1 with the p85 subunit of PI3K (Yu *et al.*, 2001; Yu *et al.*, 2002). However, a

Gab2 mutant containing a Mapk binding site from Gab1 was unable to rescue morphogenesis (L.S. Lock and M.Park, unpublished results).

4.3.4 Expression levels

It is clear that a threshold of Gab1 expression is required for rescue of epithelial morphogenesis, since MetΔGrb2-expressing epithelial cells normally express endogenous Gab1, and only upon overexpression of exogenous Gab1 can the morphogenic phenotype be rescued (Maroun *et al.*, 1999a). To rule out the possibility that exogenous Gab2 could not rescue morphogenesis due to its level of expression, we tested cell lines that express levels of Gab2 that were both lower and much higher than that of exogenous Gab1 cell lines. Although some of the lines expressing high levels of Gab2 did give a small partial morphogenic response upon growth factor stimulation, no cell lines were able to recapitulate branching morphogenesis (Lock *et al.*, 2002).

4.3.5 Other Possibilities

Other possibilities for the distinct biological functions of Gab1 and Gab2 include association with distinct binding partners in a non-phosphorylation dependent manner, a distinct manner and time course of degradation/ endocytosis/ recycling, or potential association with phosphatases. These mechanisms of regulation have yet to be studied.

4.4 Docking proteins within a family function as distinct molecular platforms

Similar to mice deficient in Gab proteins, the phenotypes of IRS deficient mice are also distinctly different. The major role of IRS-1 appears to be in embryonic and post-natal somatic cell growth, as mice lacking IRS-1 have a reduced body size and are mildly insulin-resistant (Araki *et al.*, 1994). IRS-2 on the other hand, appears to play a key role in the regulation of carbohydrate metabolism and reproduction. Deletion of IRS-2 leads to insulin resistance associated with a defect in pancreatic β-cell development resulting in diabetes (Withers *et al.*, 1998), as well as female infertility (Burks *et al.*, 2000). IRS-3 and IRS-4 deficient mice have no apparent phenotype (Liu *et al.*, 1999; Fantin *et al.*, 2000). Importantly, IRS-2 expressed in IRS-1-deficient fibroblasts does not

reconstitute normal insulin-IGF-1 signaling (Bruning *et al.*, 1997). Moreover, expression of IRS-1, IRS-2, or IRS-4 in 32D myeloid progenitor cells, which lack endogenous IRS proteins, has identified several key differences in their signal transmission (Uchida *et al.*, 2000). Similarily, the Dok family of docking proteins, all appear to interact with a slightly different array of signaling proteins. While Dok1-3 play a negative role in the regulation of MAP kinase signaling (Jones and Dumont, 1999; Tamir *et al.*, 2000; Yamanashi *et al.*, 2000), Dok4 and 5 play a positive role (Grimm *et al.*, 2001). Thus, together, all these studies suggest that each docking protein within a family is a distinct molecular platform, coupling receptors to a unique cohort of regulatory signals.

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Chapter VI: Contributions to original research

1. Identified two binding sites for the C-terminal SH3 domain of Grb2 in the Gab family of proteins.
2. Identified an atypical, non-PXXP type motif for binding to the C-terminal SH3 domain of Grb2 and Gads.
3. Demonstrated that the recruitment of Gab1 to the EGF receptor was indirect, and dependent on the adapter protein, Grb2.
4. Demonstrated that the recruitment of Gab1 to the Met receptor was both Grb2-dependent and independent.
5. Demonstrated that the recruitment of Gab2 to the Met receptor occurred in a Grb2-dependent manner.
6. Established that Gab1 and Gab2 are not functionally redundant in MDCK cells, as Gab2 is unable to rescue a morphogenic phenotype.
7. Demonstrated that the Grb2-independent interaction of Gab1 with the Met receptor is essential but not sufficient for rescue of morphogenesis in MDCK epithelial cells.
8. Identified the Met binding motif as the critical component of the Grb2-independent interaction of Gab1 with the Met receptor.
9. Determined that the Met binding motif of Gab1 is sufficient for interaction with the Met receptor, suggesting that the Met binding domain actually binds to Met as an extended, peptide ligand.
10. Demonstrated that the structural integrity of the Met receptor and residues in the C-terminal lobe of the kinase domain are essential for interaction with the Met binding motif of Gab1, suggesting a novel and previously unsuspected mechanism of interaction between Gab1 and Met.