Distinct Involvement of the Gab1 and Grb2 Adaptor Proteins in Signal Transduction by the Related Receptor Tyrosine Kinases RON and MET

Received for publication, March 12, 2011, and in revised form, July 21, 2011. Published, JBC Papers in Press, July 22, 2011, DOI 10.1074/jbc.M111.239384

Amitabha Chaudhuri, Ming-Hong Xie, Becky Yang, Kaushiki Mahapatra, Jinfeng Liu, Scot Marsters, Sweta Bodepudi, and Avi Ashkenazi

From the Department of Molecular Oncology, Genentech, Inc., South San Francisco, California 94080

Although the signal transduction mechanisms of the receptor tyrosine kinase MET are well defined, less is known about its close relative RON. MET initiates intracellular signaling by autophosphorylation on specific cytoplasmic tyrosines that form docking sites for the adaptor proteins Grb2 and Gab1. Grb2 binds directly and is essential for all of the biological activities of MET. Gab1 docks either directly or indirectly via Grb2 and controls only a subset of MET functions. Because MET and RON possess similar adaptor binding sites, it was anticipated that their adaptor interactions would be conserved. Here we show that in contrast to MET, RON relies primarily on Gab1 for signal transmission. Surprisingly, disruption of the Grb2 docking site of RON or Grb2 depletion augments activity, whereas enhancement of Grb2 binding attenuates Gab1 recruitment and signaling. Hence, RON and MET differ in their adaptor interactions; furthermore, Grb2 performs a novel antagonistic role in the context of RON signaling.

Receptor tyrosine kinases (RTKs) form a large family of transmembrane, cell surface proteins that transmit signals into the cell from a variety of ligands (1, 2). RTK ligands, which include growth factors, hormones, cytokines, and matrix proteins, regulate a variety of cellular characteristics, such as proliferation, differentiation, survival, shape, size, and motility (1, 2). Several RTKs are abnormally hyperactivated in cancer, through mechanisms that involve mutation, chromosomal translocation, gene amplification, or mRNA overexpression (3, 4). Indeed, aberrant RTK signaling often plays an important role in malignant cell transformation. Dysregulation of specific RTKs has been implicated also in a number of other illnesses, including insulin-resistant diabetes, skeletal and craniofacial disorders, and autoimmune disease, to name a few (5). Therefore, better understanding of the basic signaling mechanisms employed by RTKs may help elucidate their roles in physiology and disease and facilitate the development of therapeutic strategies to regulate these receptors.

Upon engagement by their cognate ligands, most RTKs initiate intracellular signaling by autophosphorylating on specific cytoplasmic tyrosines, thereby augmenting their own kinase activity and creating docking sites for primary adaptor proteins that mediate further signal transmission (6). The adaptor proteins attach to these sites via unique structures, such as phosphotyrosine-binding and Src homology 2 domains (6–8). Subsequently, the RTK phosphorylates the bound adaptors on multiple tyrosines, creating further binding sites for additional signal-transmitting molecules, such as FRS2 among others (8, 9). Additional tyrosines are subsequently phosphorylated to create binding sites for downstream signaling effectors or modulators.

The hepatocyte growth factor receptor MET and the macrophage-stimulating protein (MSP) receptor RON are structurally related RTKs (human MET and RON share 63% amino acid sequence homology) (10). Like MET, RON is frequently overexpressed in various human cancers. Both receptors activate the wound healing response by promoting epithelial cell migration, proliferation, and survival at the wound site (11). However, whereas the biological activities of MET are primarily restricted to the epithelial compartment, RON signaling also controls the innate immune response by regulating the migration and phagocytic activity of macrophages (12–14). These additional RON functions are critical to resolution of acute inflammatory responses and prevention of tissue damage. Accordingly, mice deficient in RON kinase activity show a hyperactive immune phenotype, characterized by sensitivity to sublethal doses of lipopolysaccharide (LPS) (15). Further, these mice show delayed tumor growth and reduced metastasis upon induction of mammary tumorigenesis by expression of polyoma middle-T antigen under control of the mouse mammary tumor virus (16). A similar delay in papilloma growth was observed in an H-Ras-induced skin carcinogenesis model (17), suggesting that RON signaling can promote tumor growth.

Both MET and RON initiate signaling by autophosphorylating specific cytoplasmic tyrosines that form docking sites for the adaptor proteins growth factor receptor bound protein-2 (Grb2) and Gab2-associated binder (Gab1) (18). Phosphorylation of tyrosines 1349 and 1356 on MET is sufficient to drive its oncogenic activity (19, 20). Grb2 binds to phosphorylated Tyr-1356 through Src homology 2 domain interactions. Gab1 can...
bind to MET directly via interaction with phosphorylated Tyr-1349 or indirectly through Grb2 (21-23). Whereas Grb2 mediates most of the oncogenic transforming activity of MET, Gab1 specifically directs MET signals to regulate cell shape and motility (19, 24, 25). Disruption of the Grb2-binding site of MET by mutation results in complete loss of biological activity, including Gab1-dependent functions (25). These and other observations indicate that Grb2 is a pivotal adaptor for MET, acting directly by engaging the RAS-MAPK pathway as well as indirectly, through Gab1-mediated signals.

RON has two adaptor docking-site tyrosines, Tyr-1353 and Tyr-1360, which are homologous to the docking site tyrosines Tyr-1349 and Tyr-1356, respectively, on MET (18). The sequence similarity between these sites suggested that the mode of interaction of the two RTKs with adaptor proteins might be conserved. Our present results show unexpectedly that RON relies primarily on Gab1, rather than Grb2, for signal transduction. Moreover, our studies uncover a unique antagonistic activity of Grb2, exerted in the context of RON signaling.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Cell Culture**—Cells from human cancer cell lines A2780, ZR75.1, HCT15, U2OS, and A549 and human embryonic kidney cell lines HEK293, HEK293T, and NIH3T3 were from the American Type Culture Collection (Manassas, VA). A2780, A549, HCT15, and U2OS cells were cultured in RPMI 1640 medium. ZR75.1, HEK293, and HEK293T cells were cultured in F-12/DMEM (50:50) medium. Culture media were supplemented with 10% (v/v) fetal bovine serum (FBS) (Thermo Fischer Scientific, Rockford, IL) and 2 mM L-glutamine. NIH3T3 were cultured in DMEM supplemented with 10% (v/v) bovine serum albumin (Thermo Fischer Scientific) and 2 mM L-glutamine. Recombinant human MSP was from R&D Systems (Minneapolis, MN). Antibodies against phosphorylated and total p42/44 ERK, AKT, S6, the pan-phosphotyrosine antibody Tyr(P)-100, Gab1, Grb2, and c-MET (clone L41G3) were from Cell Signaling Technology (Rockford, IL) and 2 mM L-glutamine. NIH3T3 were cultured in DMEM supplemented with 10% (v/v) bovine serum albumin (Thermo Fischer Scientific) and 2 mM L-glutamine. Recombinant human MSP was from R&D Systems (Minneapolis, MN). Antibodies against phosphorylated and total p42/44 ERK, AKT, S6, the pan-phosphotyrosine antibody Tyr(P)-100, Gab1, Grb2, and c-MET (clone L41G3) were from Cell Signaling Technology (Beverly, MA); the PathScan ELISA kit for total and phosphorylated p42/44 ERK and AKT were from Cell Signaling Technology. RON (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), β-actin, and anti-FLAG (M2) (Sigma), anti-c-Myc tag (clone 9E10) (Upstate Biotechnology, Inc., Lake Placid, NY), and anti-HA tag (Roche Applied Science) were used in this study. All secondary antibodies were from Rockland Immunochemicals (Gilbertsville, PA).

**Expression Constructs and Transfection**—RON, MET, FLAG-tagged Gab1 and Myc, and HA-tagged-Grb2 were generated using standard PCR and cloning strategies. Docking site mutants of RON and MET were generated by site-directed mutagenesis using the QuickChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA). All of the constructs were subcloned into the expression plasmid pRK5 (Genentech). For transient expression of proteins, 293T cells were transfected with expression constructs using Fugene-HD (Roche Applied Science) according to the manufacturer’s protocol. Stable cell lines used in this study were generated by transfecting cells with Lipofectamine-2000 (Invitrogen) according to the manufacturer’s protocol. Stable clones were selected in the presence of Geneticin (Invitrogen). The siRNAs against Gab1 and Grb2 were from Dharmacon (Lafayette, CO), and siRNA against RON was from Qiagen: siGab1 (A), 5’-CAUCAAGCUAGACCUAU-3’; siGab1 (B), 5’-GAUGCUGGUAUGACAAU-3’; siGrb2 (A), 5’-UAGAUGACGUGGUGAUAU-3’; siGrb2 (B), 5’-CGAAGAAUGUGAUCAGAC-3’; siRON, 5’-GGAGUAACUAUGUGUCAAA.

Cells were transfected using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s protocol.

**Generation of RON-expressing Stable Cell Lines**—Stable cell lines expressing RON or RON mutants were generated by transfecting cells with Lipofectamine-2000 (Invitrogen) along with RON expression plasmids (pRK5, Genentech) for 72 h. Subsequently, cells were selected in Geneticin (G418) to obtain stable cell lines. Single clones expressing a similar level of RON protein were used in biochemical and biological assays.

**Immunoblot (IB) Analysis and Immunoprecipitation (IP) Experiments**—RON signaling was analyzed in lysates of MSP-stimulated cells. Lysates were prepared after washing cells once with cold PBS followed by incubation in ice in lysis buffer (10 mM Tris/HCl, pH 7.5, containing 150 mM NaCl, 10% glycerol, 0.1% Triton X-100, 5 mM β-glycerophosphate, 2 mM NaF, 1 mM sodium orthovanadate, and protease and phosphatase inhibitors (Sigma)). Lysates were cleared by centrifugation at 14,000 × g. Clarified lysates were resolved on SDS-polyacrylamide gels and transferred to nitrocellulose. Membranes were blocked and probed with appropriate antibodies. Proteins were detected by fluorescence-labeled antibodies using the LI-COR scanner (LI-COR Biosciences, Lincoln, NE). Binding experiments were done by transient transfection of receptor and adaptor proteins in 293T cells. RON was immunoprecipitated from cell lysates using anti-RON antibody 1A2.2 (Genentech). Gab1 and Grb2 proteins were immunoprecipitated using α-FLAG or α-HA antibodies. Immune complexes were washed three times in cold lysis buffer and resuspended in sample buffer (Invitrogen) for analysis by Western blotting. The fluorescent intensity of protein bands was determined separately for the red and the green channels using the LI-COR program.

**In Vitro Kinase Assay**—RON immunoprecipitates were washed with cell lysis buffer followed by three washes with kinase assay buffer (Cell Signaling Technology). Protein phosphorylation was initiated by adding 200 μM ATP (Cell Signaling Technology) to the reaction. The kinase assay was stopped by adding 1× sample buffer and heating the samples immediately for 2 min at 95 °C.

**Cell Migration Assay**—The cell migration assay was carried out in 24-well Fluoroblok plates (BD Biosciences). Briefly, 30,000 serum-starved cells were added to the top well of the plate in a total volume of 100 μl of medium containing serum-free RPMI medium. In the bottom well was 300 μl of 0.5% serum-containing medium supplemented without or with 50 ng/ml MSP. Plates were incubated for 16 h. Media from the top wells were aspirated, and cells were fixed in cold methanol at 4 °C for 30 min. The wells were air-dried, and cells were stained with the fluorescent dye 10 μM YO-PRO-1 iodide (Invitrogen) in PBS at room temperature for 30 min. Wells were washed twice with PBS, and cells migrating to the bottom layer of the plate were quantitated in a fluorescent plate reader (485-nm
siRNA-mediated Knockdown—Small interfering RNA (siRNA)-mediated knockdown of genes was carried out by transfecting 50,000 cells with 40 pmol of siRNA mixed with 2 μl of RNAi-MAX (Invitrogen) in a total volume of 200 μl. Lipid-siRNA complex was added to the cells growing in complete medium and incubated for 24 h. Medium from each well was replaced with serum-free medium, and the cells were serum-starved for an additional 16 h. RON signaling was determined after stimulating the cells with MSP. A non-targeted control siRNA was used as a negative control.

Quantitative Analysis of Adaptor Binding to RON—293-T cells were co-transfected with wild-type or mutant RON proteins and different amounts of FLAG-tagged Gab1 or Grb2 plasmids. After 48 h, Gab1 or Grb2 adaptor protein was immunoprecipitated, and the level of wild-type or mutant RON protein in the immunoprecipitates was quantitated using LI-COR.

Microarray Analyses—Messenger RNA expression profiling of A2780-RON and HCT15 cells treated with MSP was carried out on an Affymetrix GeneChip Human Genome U133 Plus 2.0 Array as per the manufacturer’s protocol. Expression summary values for all probe sets were calculated using the RMA algorithm as implemented in the RMA package from Bioconductor (26). Statistical analyses of differentially expressed genes were performed using linear models and empirical Bayes moderated statistics as implemented in the limma package from Bioconductor.

RESULTS

RON Signaling Requires the Docking Site Tyrosine Tyr-1353 but Not Tyr-1360—To interrogate the molecular signal transduction mechanisms of RON, we examined human ZR75.1 breast cancer cells. Analysis of cell lysates by IP and IB with a RON-specific antibody revealed the full-length, unprocessed (U) and 130 kDa (processed (P)), as indicated. RON phosphorylation was analyzed by immunoprecipitating RON from lysates using anti-RON antibody coupled to agarose beads and immunoblotting with anti-phosphotyrosine antibody Tyr(P)-100. Migration assays were performed as described under “Experimental Procedures.” Cell migration was analyzed by microscopy (C) and quantified (D). Data in D are depicted as mean ± S.D. (error bars) of two separate experiments performed in triplicate.
cells stably transfected to express human RON (Fig. 1B). Kinetic analysis of RON signaling in HCT15 colon cancer cells and ZR75.1 breast cancer cells revealed rapid phosphorylation of AKT and MAPK by 15 min, followed by a decrease in MAPK phosphorylation (supplemental Fig. S1A). Although AKT phosphorylation returned to baseline within 4 h in HCT15 cells, it persisted longer in ZR75.1 cells, which are deficient in PTEN (phosphatase and tensin homolog) (supplemental Fig. S1A). Gene expression profiling of MSP-stimulated HCT15 and A2780-RON cells indicated up-regulation of several MAPK target genes (50% of all up-regulated genes) by 1 h. Among the stimulated genes were transcription factors (EGR2–4, ATF3, and FOSB), three different ligands of the epidermal growth factor (EGF) family, and the phosphatase DUSP6, previously shown to down-modulate MAPK responses as part of a feedback mechanism (28) (supplemental Fig. S1B and Table S1). MSP stimulation for 8 h revealed persistent expression of MAPK target genes (20% of the up-regulated genes), including genes that participate in cell growth and differentiation (supplemental Fig. S1B and Table S1). Taken together, the biochemical and gene expression analyses indicate that RON activation results in sustained MAPK stimulation, a feature that is shared with MET (29, 30). Ectopic RON expression in A2780 cells as well as human 293 kidney and U2OS sarcoma cells promoted cell motility, as measured by a trans-well migration assay, and this effect was substantially enhanced by MSP (Fig. 1, C and D, and supplemental Fig. S1C). Transfection of RON-expressing A2780 cells with siRNA against RON attenuated both basal and MSP-induced cell migration (supplemental Fig. S1, D and E), confirming RON-dependent activation of this biologic response.

The two docking site tyrosines of RON are tandemly arranged and reside within amino acid sequence regions that share homology with corresponding MET sequences (Fig. 2A). To assess the importance of Tyr-1353 and Tyr-1360 for RON function, we substituted these tyrosines either simultaneously or individually with phenylalanine and stably expressed the corresponding RON mutants in A2780 cells. Cell lines with similar surface expression of wild-type and mutant RON protein were selected for further characterization (Fig. 2B). A2780 cells expressing wild-type RON or the Y1360F mutant displayed an elongated, spindle-like morphology, whereas those expressing Y1353F or the double mutant exhibited a round morphology, similar to the parental cells (Fig. 2C). This marked change in morphology suggested that the two docking site tyrosines fulfill different signaling functions. As compared with wild-type RON, the Y1353F/Y1360F double mutant supported little or no phosphorylation of AKT, MAPK, or S6 in response to MSP (Fig. 2, D and E). The Y1353F mutant also mediated much less MSP-induced phosphorylation of these downstream targets than did wild-type RON, whereas Y1360F further increased the phosphorylation of AKT, MAPK, and S6 relative to wild-type RON (Fig. 2, D and E). The level of autophosphorylated RON (pRON) in relation to total RON protein was lower by ~20% for Y1353F and Y1360F single mutant and ~60% for the double mutant (supplemental Fig. S2). Whereas Y1353F and Y1353F/Y1360F were incapable of mediating MSP induction of cell migration, Y1360F surprisingly promoted an even stronger cell migration activity than did wild-type RON (Fig. 2, F and G). These results suggest that the Tyr-1353 docking site is critical for RON function, whereas Tyr-1360 may negatively regulate signal transduction by RON.

Distinct Docking Site Tyrosines Mediate RON Binding to Gab1 and Grb2—These latter observations prompted us to examine the interactions between RON and the RTK adaptors Gab1 or Grb2, both of which are implicated in signaling by MET (31). We first examined whether endogenous Gab1 or Grb2 interact with RON. Co-immunoprecipitation of RON in A2780-RON cells indicated a low level of tyrosine-phosphorylated RON in unstimulated cells, which was increased by MSP stimulation (Fig. 3A). Ligand stimulation induced recruitment and phosphorylation of Gab1 as observed by an increase in tyrosine-phosphorylated Gab1 and total Gab1 in RON immunoprecipitates (Fig. 3A, IP-RON). By contrast, there was no detectable increase in Grb2 binding or Grb2 phosphorylation (Fig. 3A). This observation suggested that RON activation promotes Gab1 recruitment and phosphorylation preferentially over Grb2. To assess whether the binding preference of RON to Gab1 was due to a non-functional Grb2-binding site on the receptor, we interrogated the interaction between RON and each adaptor protein by ectopic co-expression in 293-T cells.

Upon co-transfection with FLAG epitope-tagged Gab1 or Grb2, wild-type RON displayed detectable autophosphorylation (Fig. 3B, IP-RON). In contrast, a “kinase-dead” mutant RON variant (RON-KD) (Δexon-14) did not undergo autophosphorylation (Fig. 3B, IP-RON). IP of Gab1 or Grb2 with anti-FLAG antibody followed by IB analysis of RON, Gab1, or Grb2 revealed association of each adaptor with wild-type RON but not the RON-KD mutant (Fig. 3B, IP-Flag). These results verified that adaptor protein docking requires autophosphorylation of RON. We therefore examined which of the two docking sites was important for recruitment of Gab1 or Grb2. Whereas the Y1353F and Y1353F/Y1360F mutants showed little binding to Gab1, Y1360F retained partial Gab1 association as compared with wild-type RON (Fig. 3C, IP-Flag). Analysis of Gab1 immunoprecipitates by IB with anti-phosphotyrosine antibody indicated that both wild-type RON and the Y1360F mutant were phosphorylated, whereas the other RON mutants were not. Of note, the phosphorylated Y1360F mutant migrated slower than wild-type RON on SDS-polyacrylamide gels. On the other hand, whereas Y1353F retained intact Grb2 interaction as compared with wild-type RON, neither Y1360F nor Y1353F/Y1360F showed significant Grb2 binding (Fig. 3D, IP-Flag). Gab1 appeared to have a preference for binding to the mature, processed wild-type RON polypeptide (Fig. 3C), whereas Grb2 bound both processed and unprocessed RON (Fig. 3D). We detected only weak phosphorylation of wild-type RON in Grb2 immunoprecipitates as compared with Gab1 immunoprecipitates (Fig. 3D). A weaker band, migrating at the same position as phosphorylated RON, was detected in association with Y1360F and Y1353F/Y1360F in the absence of any Grb2 binding (Fig. 3D), suggesting that a nonspecific band may be present at that position. Together, these results suggest that RON autophosphorylation on Tyr-1353 is required for Gab1 recruitment, whereas similar modification of Tyr-1360 is important for Grb2 binding.
Grb2 Antagonizes RON Signaling

A

MET: GEHY\textsubscript{1349}VHV\textsubscript{1356}NATY\textsubscript{1356}VNVK
RON: GDHY\textsubscript{1353}VQLP\textsubscript{1360}ATY\textsubscript{1360}MNLG

B

C

RON
Y1353F
Y1360F
Y1353F/Y1360F
X200

D

Docking site mutants

E

\[ \text{Fold change in phosphoproteins (+MSP/−MSP)} \]

F

- MSP
+ MSP

G

Cell migration (RFU)

RT
Y1353F
Y1360F Y1353F/Y1360F

X10
Grb2 Antagonizes RON Signaling

To further validate our experimental strategy, we examined the binding of MET to Gab1 and Grb2 using a similar mutational approach. Consistent with previous reports (32), both Tyr-1349 and Tyr-1356 contributed to MET interaction with Gab1, whereas only Tyr-1356 was required for association with Grb2 (Fig. 3E). pMET was easily detected both in Gab1 and Grb2 immunoprecipitates, in contrast to pRON, which was readily detected only in Gab1 immunoprecipitates. Thus, the docking site requirements for recruitment of Gab1 and Grb2 to RON and MET are not identical, particularly with regard to Gab1.

**RON Signaling Requires Gab1 but Not Grb2**—To evaluate the relative importance of Gab1 and Grb2 for RON function, we suppressed the expression of each adaptor individually or jointly by siRNA transfection of RON-expressing A2780 cells. We achieved significant knockdown (~90%) of each adaptor (Fig. 4A, top, and supplemental Fig. S3). Gab1 knockdown attenuated MSP-induced phosphorylation of AKT and MAPK by >75% and of S6 by ~50%, whereas Grb2 silencing increased phosphorylation of these targets (Fig. 4, A and B). As compared with control, knockdown of Grb2 induced a further shift in mobility of the Gab1 band (Fig. 4A, compare lanes 1 and 2 with lanes 5 and 6), suggesting increased Gab1 phosphorylation. Simultaneous knockdown of both adaptors inhibited these phosphorylation events to a similar extent as the knockdown of Gab1 by itself (Fig. 4, A and B). By contrast, suppression of Gab1 and Grb2 adaptor proteins singly in A549 cells attenuated hepatoctye growth factor-induced phosphorylation of MAPK and AKT by 70% and of S6 by 50%. Combined knockdown of both adaptors did not result in further inhibition of MAPK and AKT phosphorylation but reduced phosphorylation of S6 by 75%. These results suggest that although Gab1 may play a more prominent role in mediating RON signaling, both Gab1 and Grb2 are required to support the signaling activity of MET. Whereas Grb2 may perform an antagonistic role in RON signaling, it plays an agonistic role in MET signaling (32). Furthermore, ligands of more distantly related RTKs, namely EGF, IGF1, and FGFI, relied on Grb2 rather than Gab1 to activate MAPK phosphorylation (supplemental Fig. S4).

**Gab1 and Grb2 Differentially Regulate RON Autophosphorylation**—To further define the interaction between RON and its cognate adaptors, we investigated the extent of RON phosphorylation in association with Gab1 or Grb2. Surprisingly, the level of pRON was lower in RON immunoprecipitates from cells co-transfected with Grb2 as compared with Gab1 or vector control (Fig. 5A and supplemental Fig. S5A). This suggested that Grb2 binding might limit the extent of RON autophosphorylation. Consistent with our earlier observation (Fig. 3C), we detected less pRON in association with Grb2 as compared with Gab1, evident by weaker intensity and faster migration of the pRON band (Fig. 5B, top panels). Moreover, the ratio between pRON and total RON was 2–3-fold lower in Grb2 versus Gab1 immunoprecipitates (supplemental Fig. S5B). This latter difference was not due to changes in total RON levels, which were comparable in cells co-transfected with Gab1, Grb2, or vector control (Fig. 5B, Lysates). Treatment of Gab1 immunoprecipitates from Gab1 co-transfected cells with calf intestinal phosphatase led to depletion of both pRON and phospho-Gab1 and decreased the amount of the slower migrating RON species (Fig. 5C, top). By contrast to RON, MET autophosphorylation was not reduced upon MET co-transfection with Grb2 as compared with Gab1 or vector control (Fig. 5D). Indeed, phospho-MET detected in Grb2 immunoprecipitates as compared with Gab1 immunoprecipitates showed higher intensity and migrated more slowly (Fig. 5E, top panels). This observation strongly suggested that Grb2 may perform a distinct function in association with RON versus MET; although Grb2 association may prevent RON from reaching its full autophosphorylation potential, it enhances the autophosphorylation of MET.

Given that RON kinase activity (and, as a corollary, its autophosphorylation) was critical for adaptor recruitment (Fig. 3A), we reasoned that if Grb2 prevents RON from reaching a fully autophosphorylated state, then Grb2 binding should diminish the ability of RON to recruit Gab1. To test this prediction, we co-expressed RON with Gab1 alone or Gab1 plus Grb2 and compared the level of RON in Gab1 immunoprecipitates. Grb2 co-transfection significantly decreased the amount of pRON associated with Gab1 as well as the amount of phospho-Gab1 present (Fig. 6A). The migration of the RON and pRON bands was faster in the presence of Grb2 (Fig. 6A), consistent with less phosphorylation. The total amount of RON was slightly lower in the presence of Grb2 (Fig. 6A, IP-RON). The significant decrease in Gab1 binding to RON in the presence of Grb2 supports the possibility that Grb2 modulates the extent of RON phosphorylation to influence Gab1 binding. Grb2 was detected in Gab1 immunoprecipitates, potentially due to its independent ability to bind to Gab1 (32–34). To further assess whether Grb2 association affects the autophosphorylation capacity of RON, we immunoprecipitated RON, incubated the immunoprecipitates with ATP, and examined tyrosine phosphorylation by blotting with a-Tyr(P)-100 antibody. RON autophosphorylation increased in the presence of ATP (Fig. 6B). Both the processed and the unprocessed RON were phosphorylated following incubation with ATP (Fig. 6B), suggesting that processed RON has the ability to transphosphorylate unprocessed RON. Next we examined in vitro whether autophosphorylation of RON was affected by co-expression with Gab1 or Grb2. In the absence of ATP, RON autophosphorylation was diminished in the context of Grb2 as compared with Gab1 co-expression or

**FIGURE 2. Docking site tyrosine Tyr-1353 mediates RON-induced signaling and cell migration.** A, sequence homology surrounding the two docking site tyrosines of MET and RON. B, surface expression of wild-type or mutant RON variants by stably transfected A2780 cells was analyzed by FACS. C, morphology of transfected A2780 cells stably expressing RON variants. D, pathway activation in response to 30 min of MSP treatment in A2780 cells stably expressing wild type or the indicated docking-site mutants of RON. E, phosphorylation of p44/42 MAPK (Thr-202/Tyr-204), AKT (Ser-473), and S6 (Ser-235/236) was quantified using LI-COR based on the fluorescent intensity of the protein bands and normalized to the intensity of either total MAPK, AKT, and S6 proteins (from two independent experiments) or actin (from two additional independent experiments). Data represent mean ± S.D. (error bars) of four independent experiments. F and G, migration of A2780 cells stably expressing wild-type or mutant RON variants in response to MSP. Data in G are depicted as mean ± S.D. of two independent experiments performed in triplicates.

**SEPTEMBER 16, 2011 • VOLUME 286 • NUMBER 37**
vector control (Fig. 6C). The addition of ATP increased RON autophosphorylation with time, although minimal increase was observed upon RON co-expression with Grb2 as compared with Gab1 or vector control (Fig. 6C). We also detected phosphorylation of Gab1 in cells transfected with RON plus Gab1, suggesting that Gab1 was readily phosphorylated when bound by the receptor (Fig. 6C). In contrast, we did not observe phosphorylation of Grb2 in cells transfected with RON plus Grb2.

The inhibitory effect of Grb2 on RON autophosphorylation both in vivo and in vitro prompted us to examine whether this adaptor could also exert a similar effect on MET. Co-expression of MET with Gab1, Grb2, or both adaptors did not alter the amount of pMET associated with Gab1 (Fig. 6D, top). Furthermore, Grb2 co-transfection with MET did not affect the extent of Gab1 phosphorylation (Fig. 6B, top). In fact, the amount of pMET and total MET associated with Gab1 was greater in the
presence of Grb2 than in its absence. Moreover, Grb2 appeared to undergo phosphorylation in association with MET (Fig. 6D).

Thus, the inhibitory effect of Grb2 on receptor autophosphorylation is specific to RON versus MET.

Grb2 Negatively Regulates RON Signaling in a Gab1-dependent Manner—If Grb2 association limits the ability of RON to catalyze optimal autophosphorylation, then Grb2 should act as a negative regulator of RON signaling. Accordingly, disruption of the docking site for Grb2 on RON should augment signaling, whereas enhanced Grb2 binding should decrease RON activity. Indeed, as compared with wild-type RON, the Y1360F mutant, which was deficient in binding to Grb2 but not Gab1, mediated markedly greater phosphorylation of AKT, MAPK, and S6 as well as stronger cell migration activity in response to MSP (Fig. 2, B–D, and supplemental Fig. S6A). Further, despite the lack of Grb2 binding by Y1360F (Fig. 3C), siRNA silencing of Grb2 increased the induction of AKT and MAPK phosphorylation by Y1360F in response to MSP (Fig. 7A and supplemental Fig. S6B). To confirm

FIGURE 4. Differential requirement of Gab1 and Grb2 adaptor proteins in RON and MET signaling. Gab1 and Grb2 were depleted by siRNA transfection of A2780-RON cells (A and B) or A549 cells (C and D), as described under “Experimental Procedures.” Activation of intracellular signaling pathways in response to MSP (A) or hepatocyte growth factor (HGF) (C) was quantified using LI-COR, and the phosphorylated p42/44 MAPK, AKT, and S6 proteins were normalized to the expression of total MAPK, AKT, and S6 proteins, respectively. -Fold change shown in B for MSP and D for hepatocyte growth factor indicates the response of cells depleted of Gab1 or Grb2 individually or together relative to undepleted control cells. Data represent mean ± S.D. (error bars) from three independent experiments.
that this enhanced signaling by Y1360F was mediated exclusively through Gab1, we depleted Gab1 by siRNA transfection. Gab1 knockdown significantly decreased Y1360F-mediated phosphorylation of AKT and MAPK as compared with the non-targeted siRNA control (Fig. 7A and supplemental Fig. S6B). As expected, simultaneous knockdown of Gab1 and Grb2 inhibited phosphorylation of AKT and MAPK similarly to Gab1 depletion by itself (Fig. 7A and supplemental Fig. S6B). The enhanced signaling by the Y1360F in the absence of Grb2 suggested that Grb2 knockdown may release Gab1 from Gab1-Grb2 complexes, making it available for recruitment by the mutant RON receptor. To test this prediction, we co-transfected 293-T cells with RON-Y1360F, which is deficient in Grb2 binding, along with Grb2 siRNA. A control, we expressed wild-type RON in conjunction with Grb2 siRNA. Grb2 levels were decreased in cells transfected with Grb2 siRNA compared with the control siRNA (Fig. 7B, Lysates). Silencing of Grb2 increased auto-phosphorylation of Y1360F by ~1.5-fold and augmented binding of Gab1 to the receptor as well as Gab1 phosphorylation by ~2-fold (Fig. 7B, IP-RON, and supplemental Fig. S7). However, silencing of Grb2 did not affect the autophosphorylation of wild-type RON, although binding of Gab1 and Gab1 phosphorylation were slightly increased as compared with the control siRNA (Fig. 7B, IP-RON, and supplemental Fig. S7). Together these data support the hypothesis that Grb2 binding limits RON signaling via Gab1.

To assess this further, we asked whether tighter Grb2 binding by RON would result in stronger Grb2-mediated antagonism of RON activity. To augment Grb2 interaction, we replaced the methionine residue at the P+1 position of the RON Grb2 docking site with a valine residue (M1361V), rendering the sequence more akin to the Grb2 docking site on MET. The M1361V mutant displayed slightly less Gab1 association than did wild-type RON (supplemental Fig. S8A). Conversely, M1361V showed stronger binding to Grb2 than did wild-type RON (supplemental Fig. S8B). M1361V mediated less efficient phosphorylation of AKT and MAPK in response to MSP, as compared with wild-type RON (Fig. 7C). Further mutation of the Gab1 docking site (Y1353F/M1361V) abolished MSP-induced phosphorylation of AKT similar to Y1353F and significantly reduced

FIGURE 5. Phosphorylated RON is enriched in Gab1 immunoprecipitates compared with Grb2 immunoprecipitates. A and B, RON was transfected into 293-T cells singly or with FLAG-tagged Gab1 or FLAG-tagged Grb2. The amount of phosphorylated and non-phosphorylated RON in anti-RON immunoprecipitates (A) and anti-FLAG IPs (B) is shown, with the input of RON, Gab1, and Grb2 proteins in B (bottom). C, RON was co-transfected with FLAG-tagged Gab1. The FLAG (M2) immunoprecipitate was treated with buffer or calf intestinal phosphatase (CIP) for 1 h at 37 °C. Phosphorylated proteins were detected by IB as indicated. D, MET was transfected singly or with FLAG-tagged Gab1 or FLAG-tagged Grb2. The amount of phosphorylated and non-phosphorylated MET in anti-MET immunoprecipitates (D) and anti-FLAG immunoprecipitates (E) is shown.
the phosphorylation of MAPK (Fig. 7C). The reduced Gab1 binding of M1361V decreased MSP-dependent cell migration, which was further reduced with the Y1353F/M1361V mutant deficient in Gab1 binding (Fig. 7D and supplemental Fig. S8C). These data suggest that the residual signaling and induction of cell migration activity displayed by M1361V is mediated by Gab1. Thus, blocking Grb2 binding to RON by disruption of the Grb2 docking site or depletion of Grb2 augments whereas enhancement of Grb2 binding attenuates RON signaling, supporting the conclusion that Grb2 negatively regulates RON activity.

**DISCUSSION**

RTK signaling is spatially and temporally regulated by a wide array of signaling adaptors that are recruited to specific phosphotyrosines on the receptors (7). The Gab1 and Grb2 adaptor proteins are employed by many RTKs to transduce downstream signals. MET and RON are closely related RTKs that share similar docking site sequences for Gab1 and Grb2, suggesting that their molecular signaling mechanisms may be conserved. Contrary to this expectation, our findings suggest that Gab1 and Grb2 serve opposing functions in modulating RON signaling, unlike their concerted action in supporting MET activity (31, 32, 34). Whereas Gab1 regulates a subset of signals downstream of MET, our data suggest that it acts as a key adaptor downstream of RON. On the other hand, although Grb2 plays a pivotal role in transmitting MET signals, it fulfills a negative regulatory function in the context of RON activation. The antagonistic effect of Grb2 on RON signaling appears to be
mediated by the stabilization of a hypophosphorylated state of the receptor, whereas Gab1 reinforces the autophosphorylation of RON to promote efficient signal transmission.

Many RTKs recruit Grb2 directly, by forming a phosphotyrosine docking site for this adaptor’s Src homology 2 domain. Most RTKs lack a Gab1 binding site and recruit the latter adaptor indirectly, via a primary adaptor such as Grb2 (for EGF receptor) or FRS2 (for FGF receptor) (6). MET and RON employ two separate docking site tyrosines to bind Gab1 and Grb2 (18, 35–37). A third member of the so-called hepatocyte growth factor receptor family, Sea, has two binding sites for Grb2, similar to other RTKs, such as the EGF and PDGF receptors (38, 39). Why some RTKs possess a direct binding site for Gab1 remains unclear. In vitro, MET recruits Gab1 more efficiently via Grb2 than through direct binding, as do other RTKs, including EGF, PDGF, and insulin receptors (32, 39). In contrast, RON bound to Gab1 directly, and this appeared to be its preferred mode of interaction with this adaptor. Although disruption of the Grb2 binding site significantly reduced Gab1 binding to MET, it had a minimal effect on Gab1 recruitment by RON.
Research from several laboratories has shed critical light on the contribution of Gab1 and Grb2 to MET signaling. During development, Gab1 serves as an important adaptor protein for MET. Gab1 and MET gene knock-out mice have very similar developmental defects (40). Furthermore, Gab1 mediates a wide range of cellular effects downstream of MET, including epithelial morphogenesis, cell transformation, and tumor progression (21, 31, 41, 42). Early studies using the TPR-MET oncoprotein suggested that the cellular effects of MET are dependent on the Grb2 docking site tyrosine Tyr-1356, rather than the Gab1-binding tyrosine Tyr-1349 (19, 20, 23, 32, 34, 43). This was further elucidated by the observation that MET recruits Gab1 primarily by indirect binding through Grb2 (32). However, the mechanism of Gab1 recruitment by MET is context-dependent. In vitro, both wild-type MET (this study) and TPR-MET (32, 34, 44) rely more on Grb2 to recruit Gab1. In contrast, liver growth and placental development in mice require both Grb2-dependent and Grb2-independent mechanisms for Gab1 recruitment to MET (45). Taken together, these findings suggest that MET depends on both Grb2 and Gab1 for its full spectrum of biological activity.

Our studies suggest that RON has a distinct mode of interaction with Gab1 and Grb2. Perturbation of Gab1 binding abrogated signal transduction and induction of cell migration downstream of RON, whereas disruption of Grb2 binding augmented these activities. Similarly, knockdown of Gab1 significantly reduced RON signaling, whereas Grb2 depletion augmented RON activity. These findings indicate that, contrary to its established role as a positive and often a pivotal mediator of RTK signaling (41, 46), Grb2 acts as a negative regulator in the context of RON signaling.

The extent of RON phosphorylation was substantially diminished in association with Grb2 as compared with Gab1. In contrast, Grb2 interaction with MET did not affect the levels of receptor phosphorylation, indicating that the inhibitory effect of Grb2 is specific to RON. The two different phosphorylation states of RON may represent temporally distinct phases of receptor activation. Indeed, recent studies have demonstrated that FGFR1 undergoes three sequential phases of tyrosine phosphorylation before reaching maximal activation (47, 48). We suspect that RON may similarly undergo multiple rounds of phosphorylation following initial activation of the kinase domain. The presence of phosphorylated RON in Gab1 and Grb2 immunoprecipitates suggested that the receptor undergoes at least two phases of tyrosine phosphorylation: phosphorylation of the kinase-domain tyrosines and, consequently, phosphorylation of the docking site tyrosines. It is likely that the receptor then undergoes additional rounds of autophosphorylation, depending on whether it is bound to Gab1 or Grb2.

On the basis of these new findings, we propose a model for RON signaling as depicted in Fig. 7E. The two docking site tyrosines, Tyr-1353 and Tyr-1360, bind to Gab1 and Grb2, respectively. Recruitment of Gab1 to RON results in Gab1 phosphorylation, reinforces RON phosphorylation, and generates a strong signal. In contrast, binding of Grb2 stabilizes RON in a hypophosphorylated state and hence effectively attenuates signal transmission. Moreover, Tyr-1353 may be inefficiently phosphorylated when Grb2 is bound to phospho-Tyr-1360, preventing Gab1 binding and thereby further attenuating RON signaling. The inhibitory effect of Grb2 on RON signaling requires the presence of Gab1, perhaps because Grb2 association with Gab1, which has previously been shown (32–34), sequesters the latter adaptor away from binding to RON.

In support of our model, Grb2 overexpression decreased Gab1 binding to RON and suppressed Gab1 phosphorylation. In contrast, binding of Gab1 to MET and Gab1 phosphorylation were not affected by Grb2 overexpression. Conversely, Grb2 knockdown increased Gab1 binding to RON and its phosphorylation. The Grb2 binding site of MET (Y1356VNV) differs from that of RON (Y1360VNL). The sequence YVNV is present in many proteins that bind to Grb2, including both receptor and non-receptor tyrosine kinases; this sequence is conserved as a binding motif for type 1 Src homology 2 domains such as the one possessed by Grb2 (49). Mutation of the Grb2 binding site on RON to Y1360VNL favored binding to Grb2 over Gab1, perturbing efficient signaling. The residual activity of the mutant was further attenuated upon mutation of the Gab1 binding tyrosine Tyr-1353, to phenylalanine. These observations strongly suggest that the Grb2 docking site on RON has lower affinity for Grb2 as compared with the corresponding site on other RTKs. In turn, weaker Grb2 binding to RON facilitates direct binding of Gab1 to its own site. In contrast to RON, MET has a high affinity interaction with Grb2, which favors efficient, albeit indirect, recruitment of Gab1.

Gab1 and Grb2 have overlapping yet distinct roles in RTK signaling. For example, the two adaptors differentially regulate the duration of p42/44 MAPK phosphorylation, by coupling to different downstream signaling mediators. Gab1 supports sustained MAPK phosphorylation, by recruiting the tyrosine phosphatase SHP2, which dephosphorylates negative regulators of the Ras-MAPK pathway (50–52). Grb2, on the other hand, mediates transient MAPK phosphorylation, through coupling to Ras (53). Furthermore, Gab1 can directly recruit the p85 regulatory subunit of PI3K to activate PI3K and promote AKT phosphorylation, unlike Grb2, which relies on other adaptors (including Gab1 itself) to activate the PI3K pathway (54). Gab1-mediated PI3K signaling is further sustained, likely via translocation of Gab1 to the membrane after the adaptor protein is recruited to RTKs (55, 56). Therefore, by engaging Gab1, RON is likely to deliver extended MAPK and AKT activation. Gene expression analysis further confirmed sustained up-regulation of MAPK target genes in two different cell lines. Prolonged activation of these pathways promotes cell differentiation, migration, and survival, functions that are characteristic to RON signaling (10, 57). Unlike mouse RON, human RON fails to support growth of NIH3T3 cells under anchorage-independent conditions in vitro or as grafted tumors in vivo (58). We propose that this lack of transforming capacity of human RON may be linked to its inability to transduce Grb2-dependent signals. We have observed that mouse RON bound Gab1 and Grb2. Further, Grb2 did not attenuate Gab1 binding to mouse RON upon co-transfection, similar to its effect on MET and distinct from its effect on human RON regarding interaction.

4. A. Chaudhuri, M.-H. Xie, B. Yang, K. Mahapatra, J. Liu, S. Marsters, S. Bode-pudi, and A. Ashkenazi, unpublished data.
Grb2 Antagonizes RON Signaling

with Gab1. In keeping with this notion, the oncogenic activity of MET and Sea is dependent on their docking site tyrosine for Grb2 (34, 38, 59).

In conclusion, our findings demonstrate that the adaptor interactions of RON are surprisingly distinct from those of its relative MET. Furthermore, we have uncovered a novel antagonistic function of Grb2 in the context of RON signaling. To our knowledge, this is the first characterization of Grb2 as modulating the phosphorylation state of a cognate RTK to antagonize Gab1 binding.

REFERENCES

1. Matsumura, L., Mizuki, M., and Kanakura, Y. (2008) Cancer Sci. 99, 479–485
2. Haber, D. A., and Settleman, J. (2007) Nature 446, 145–146
3. Harris, T. J., and McCormick, F. (2010) Nat. Rev. Clin. Oncol. 7, 251–265
4. Sharma, S. V., Bell, D. W., Settleman, J., and Haber, D. A. (2007) Nat. Rev. Cancer 7, 169–181
5. Lahiry, P., Torkamani, A., Schork, N. J., and Hegele, R. A. (2010) Nat. Rev. Genet. 11, 60–74
6. Lemmon, M. A., and Schlessinger, J. (2010) Adv. Exp. Med. Biol. 611B, 181–194
7. Scott, J. D., and Pawson, T. (2009) Science 326, 1220–1224
8. Pawson, T., and Kofler, M. (2009) Curr. Opin. Cell Biol. 21, 147–153
9. Pawson, T. (2004) Cell 116, 191–203
10. Trusolino, L., and Comoglio, P. M. (2002) Nat. Rev. Cancer 2, 289–300
11. Cowin, A. J., Kallinicos, N., Hatzisodos, N., Robertson, J. G., Pickering, K. J., Couper, J., and Belford, D. A. (2001) Cell Tissue Res. 306, 239–250
12. Leonard, E. J., and Skeel, A. H. (1979) J. Biol. Chem. 254, 13116–13122
13. Maroun, C. R., Naujokas, M. A., Holgado-Madruga, M., Wong, A. J., and Park, M. (2002) Hum. Mutat. 21, 6140–6144
14. Morrison, A. C., and Correll, P. H. (2002) Proc. Natl. Acad. Sci. U.S.A. 109, 10854–10859
15. Scheicher, M., Audero, E., Maina, F., Bardelli, A., Basile, M. L., Giordano, S., Naujokas, M. A., Comoglio, P. M. (2006) J. Biol. Chem. 281, 30083–30090
16. Zhu, H., Naujokas, M. A., Fixman, E. D., Torossian, K., and Park, M. (1994) J. Biol. Chem. 269, 29943–29948
17. Gentelman, R. C., Carey, V. J., Bates, D. M., Bolstad, B., Dettling, M., Dudoit, S., Ellis, B., Gautier, L., Ge, Y., Gentry, J., Hornik, K., Hothorn, T., Huber, W., Iacus, S., Irizarry, R., Leisch, F., Li, C., Maechler, M., Rossini, A. J., Sawitzki, G., Smith, C., Smyth, G., Tierney, L., Yang, J. Y., and Zhang, J. (2004) Genome Biol. 5, R80
18. Gaudino, G., Follenzi, A., Naldini, L., Collesi, C., Santoro, M., Gallo, K. A., Godowski, P. J., and Comoglio, P. M. (1994) EMBO J. 13, 3524–3532
19. Owens, D. M., and Keyse, S. M. (2007) Oncogene 26, 3203–3213
20. Rosário, M., and Birchmeier, W. (2003) Trends Cell Biol. 13, 328–335
21. Maroun, C. R., Naujokas, M. A., Holgado-Madruga, M., Wong, A. J., and Park, M. (2000) Mol. Cell. Biol. 20, 8513–8525
22. Schaeper, U., Gehring, N. H., Fuchs, K. P., Sachs, M., Kempkes, B., and Birchmeier, W. (2000) J. Cell Biol. 149, 1419–1432
23. Paro, R., and Schernthaner, G. (2003) Cell 114, 241–252
24. Darnell, J. E., Green, B. H., and Levine, A. S. (2006) Cell 124, 267–275
25. Palade, G. E. (1975) J. Cell Biol. 67, 441–458
26. Moriyama, Y., and Ogasawara, M. (1999) J. Biol. Chem. 274, 19925–19938
27. Nishizuka, Y. (2002) Sci. Signal. 5, ra6
28. Songyang, Z., Shoelson, S. E., Glomme, G. S., Orlowski, P., Pawson, T., Bustelo, X. R., Barbacid, M., Sabe, H., Hanafusa, H., and Yi, T. (1994) Mol. Cell. Biol. 14, 2777–2785
29. Kiyokita, K., Asahina, M., Akamatsu, T., Nakamura, N., Kitamura, Y., and Hanafusa, H. (1997) Mol. Cell. Biol. 17, 7021–7029
30. Zang, J. X., and Kornberg, A. D. (2001) Cell. Mol. Life Sci. 58, 1961–1974
31. Maroun, C. R., Naujokas, M. A., and Park, M. (2003) Mol. Biol. Cell 14, 1691–1708
32. Eulenfeld, R., and Schaper, F. (2009) J. Cell Sci. 122, 55–64
33. Kretschmann, K. L., Eyob, H., Buys, S. S., and Welm, A. L. (2010) Curr. Drug Targets 11, 1157–1168
34. Zhou, Y. Q., He, C., Chen, Y. Q., Wang, D., and Wang, M. H. (2003) Oncogene 22, 186–197
35. Giordano, S., Bardelli, A., Zhen, Z., Menard, S., Ponzetto, C., and Comoglio, P. M. (1997) Proc. Natl. Acad. Sci. U.S.A. 94, 13868–13872