Coupling of Grb2 to Gab1 Mediates Hepatocyte Growth Factor-induced High Intensity ERK Signal Required for Inhibition of HepG2 Hepatoma Cell Proliferation*

Asuka Kondo, Naoki Hirayama, Yasuko Sugito, Michihiro Shono, Toshiaki Tanaka, and Naomi Kitamura

From the Department of Biological Sciences, Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, Nagatsuta, Midori-ku, Yokohama 226-8501, Japan

Activation of the extracellular signal-regulated kinase (ERK) pathway is a key factor in the regulation of cell proliferation by growth factors. Hepatocyte growth factor (HGF)-induced cell cycle arrest in the human hepatocellular carcinoma cell line HepG2 requires strong activation of the ERK pathway. In this study, we investigated the molecular mechanism of the activation. We constructed a chimeric receptor composed of the extracellular domain of the NGF receptor and the cytoplasmic domain of the HGF receptor (c-Met) and introduced a point mutation (N1358H) into the chimeric receptor, which specifically abrogates the direct binding of Grb2 to c-Met. The mutant chimeric receptor failed to mediate the strong activation of ERK, up-regulation of the expression of a Cdk inhibitor p16INK4a and inhibition of HepG2 cell proliferation by ligand stimulation. Moreover, the mutant receptor did not induce tyrosine phosphorylation of the docking protein Gab1. Knockdown of Gab1 using siRNA suppressed the HGF-induced strong activation of ERK and inhibition of HepG2 cell proliferation. These results suggest that coupling of Grb2 to Gab1 mediates the HGF-induced strong activation of the ERK pathway, which is required for the inhibition of HepG2 cell proliferation.

Transforming growth factor-β inhibits cell proliferation through a transient activation of ERK1/2 (3). The G protein-coupled sst2 somatostatin receptor inhibits cell proliferation through the activation of ERK2 (4). Thus, the level of ERK activity may determine the proliferative response of cells to extracellular signals.

Hepatocyte growth factor (HGF) inhibits serum-dependent proliferation of the human hepatocellular carcinoma cell line HepG2. We have previously shown that HGF induces strong activation of ERK in HepG2 cells, and a reduction of this strong activation to a weak activation by a low concentration of MEK inhibitors restores the proliferation of HepG2 cells inhibited by HGF (5). These results indicate that the activation of ERK is required for both the stimulation and inhibition of HepG2 cell proliferation, and the level of ERK activation determines the opposing proliferation responses: the strong activation is required for the inhibition of proliferation, whereas the weak activation leads to the stimulation of proliferation. The strong activation of ERK by HGF changes the expression of regulators of the cell cycle including cyclin-dependent kinase (Cdk) inhibitors, p16INK4a, p21CIP1, and p27KIP1, cyclin A, and E2F1 (6, 7). Detailed analysis of the expression of p16 revealed that the activated ERK pathway induces the activation of a member of the Ets family of transcription factors, which up-regulates the expression of p16. The p16 protein forms a complex with Cdk4, leading to the redistribution of p21 and p27 from Cdk4 to Cdk2. The association of p21 and p27 with Cdk2 represses Cdk2 activity, resulting in hypophosphorylation of pRb. The hypophosphorylated pRb eventually causes cell cycle arrest at G1 (6, 7). Thus, one crucial molecular pathway linking the strong activation of the ERK pathway to the HGF-induced cell cycle arrest in HepG2 cells is the up-regulation of p16 expression.

The biological effects of HGF on the proliferation of cells are transduced through the activation of its high affinity receptor, the c-Met proto-oncogene product (c-Met) (8–11). The association of HGF with c-Met elicits recruitment of the Grb2/Sos complex to the activated c-Met (12). The translocation of Sos to the plasma membrane occurs close to Ras and can stimulate the exchange of GTP for GDP. The binding of GTP to Ras initiates a protein cascade, which leads to ERK activation through the intervening protein kinases Raf and MEK (13). Thus, the HGF-induced strong activation of ERK in HepG2 cells is a consequence of the up-regulation of one of these reactions. However, the mechanism of the up-regulation remains to be elucidated.

The abbreviations used are: ERK, extracellular signal-regulated kinase; NGF, nerve growth factor; HGF, hepatocyte growth factor; Cdk, cyclin-dependent kinase; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; GST, glutathione S-transferase; SEAP, secreted alkaline phosphatase; RRE, Ras-responsive element; SRE, serum response element; siRNA, small interfering RNA.

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† To whom correspondence should be addressed: Dept. of Biological Sciences, Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, 4259 Nagatsuta, Midori-ku, Yokohama 226-8501, Japan. Tel.: 81-45-924-5701; Fax: 81-45-924-5771; E-mail: nkitamura@bio.titech.ac.jp.

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Two mechanisms have been described for the association of Grb2 with c-Met, that is the initial event of the Ras/ERK pathway. One is direct binding of Grb2 to c-Met. The association of HGF with c-Met triggers phosphorylation of two C-terminal tyrosines (Tyr1349 and Tyr1356) essential for the c-Met signaling (12). The phosphorylation of Tyr1356 is required for Grb2 to directly bind to c-Met. In addition, the asparagine residue (Asn1358) at position +2 of Tyr1356 is also essential for the binding of Grb2 to c-Met, because replacement of the asparagine with histidine impairs the binding (14, 15). The other mechanism of the association of Grb2 with c-Met is through the adaptor protein Shc (16). Phosphorylation of Tyr1349 and Tyr1356 is involved in the binding of Shc to c-Met, but Asn1358 is not required for the binding (17). Tyrosine phosphorylation of Shc at Tyr317 (YVNV) forms a consensus binding site for Grb2 (18), and the association of Grb2 with Shc promotes activation of Ras (19). Shc has been shown to be tyrosine-phosphorylated in cells stimulated with HGF (16).

In this study, we examined which mechanism is involved in the HGF-induced strong activation of ERK, which is required for cell cycle arrest in HepG2 cells. To this end, we constructed a chimeric receptor composed of the extracellular ligand-binding domain of the NGF receptor and the cytoplasmic domain of c-Met, and introduced a point mutation into this chimeric receptor by replacing Asn1358 with His, which specifically abrogates the direct binding of Grb2 to c-Met. We introduced this mutated receptor into HepG2 cells, and analyzed effects of the mutated receptor into HepG2 cells, and analyzed effects of the mutation on the ERK activation and cell proliferation. The results demonstrated that the direct binding of Grb2 to c-Met is crucial for the HGF-induced strong activation of ERK, which leads to inhibition of cell proliferation.

In addition to the translocation of Sos to the plasma membrane, the direct binding of Grb2 to c-Met contributes to the association of the docking protein Gab1 with c-Met, which amplifies the Ras/ERK pathway through the tyrosine phosphatase Shp2 (20, 21). Therefore, we further examined the involvement of Gab1 in the strong activation of ERK, using the RNAi method. The result demonstrated that Gab1 plays a crucial role in the strong activation of ERK, which is required for the HGF-induced inhibition of HepG2 cell proliferation.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Reagents were obtained as follows: anti-c-Met (sc161), anti-p16 (sc759), and anti-GST (sc459) antibodies from Santa Cruz Biotechnology; anti-trkA (06-574), anti-ERK2 (sc161), anti-p16 (sc759), and anti-GST (sc459) antibodies from Sigma; antiphospho-p44/42 MAPK (Thr202/Tyr204) antibody (05-157), and anti-Gab1 (06-579) antibodies from Upstate Biotechnology; anti-phosphotyrosine antibody (PY20) from Transduction Laboratories; anti-α-tubulin antibody (T0026) from Sigma; anti-phospho-p44/42 MAPK (Thr202/Tyr204) antibody (9106) from Cell Signaling Technology; horseradish peroxidase-conjugated anti-mouse (NA931) and anti-rabbit (NA934) Ig from Amersham Biosciences; recombinant human HGF from the Research Center of Mitsubishi Chemical Corp.; and NGF from Alomone Labs.

**Construction of TrkA/c-Met Chimeric Receptor cDNA Expression Plasmid**—The plasmid including rat TrkA cDNA (pRC/CMV-rat TrkA) was kindly provided by Dr. K. Suzuki of Novartis Pharm Corp. Human c-Met cDNA was amplified from RNA of HepG2 cells by RT-PCR, and cloned into pME18S. The TrkA cDNA was amplified by PCR using primer 1, 5′-ACACAA-AACGCTTCAGGGCTC-3′, and primer 3, 5′-GCAATCAATCCGAGATTTGCTATTGCTTCTTCTC-3′. The c-Met cDNA was amplified using primer 2, 5′-GGAGAAAGACGAGACTTCC-AAGGATTGATTC-3′, and primer 4, 5′-GCTCGTGTTGC-TACAGATTCC-3′. The PCR products were mixed and used as templates for PCR with primers 1 and 4. The obtained chimeric fragment was digested with HindIII and AccI, and ligated to cDNA fragments encoding the extracellular region of TrkA and the intracellular region of c-Met. The full-length chimeric receptor cDNA was inserted into an expression plasmid pCDNA5/FRT. Point mutations were introduced with a QuikChange site-directed mutagenesis kit (Stratagene).

**Immunoblot Analysis**—The cell lysates and precipitated immune complexes were washed twice with cold phosphate-buffered saline containing 1% EDTA and 0.2 mM Na3VO4. For the immunoblot analysis of other proteins, cells were lysed with lysis buffer (mobility shift) (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 2 mM Na2VO4, 50 mM NaF, and 30 mM tetrasodium pyrophosphate) containing 5 μg/ml leupeptin, 1 μg/ml pepstatin A, 2 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride. For the immunoblot analysis of other proteins, cells were lysed with lysis buffer (RIPA) (137 mM NaCl, 8.1 mM Na2HPO4, 0.3% Na3PO4, 2.68 mM KCl, 14.7 mM KH2PO4, 2 mM Na2VO4, 5 mM EDTA, 1% Nonidet P-40, and 0.5% deoxycholic acid) containing the same protease inhibitors. The lysates were cleared by centrifugation, and the protein concentration in the lysate was determined using the BCA protein assay reagent (Pierce). Equal amounts of cell lysate were incubated with antibody for 2 h at 4 °C, and then with a 50% slurry of protein A-Sepharose (Amer sham Biosciences) for 1 h at 4 °C. The immune complexes were precipitated and washed twice with washing buffer (10 mM Tris-HCl, pH 7.4, 159 mM NaCl, 2 mM Na2VO4, 0.1% Nonidet P-40, and 50 mM NaF).

**Immunoblot Analysis**—The cell lysates and precipitated immune complexes were boiled in Laemmli sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.001% bromphenol blue, and 5% 2-mercaptoethanol). The samples were then separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were incubated with the primary antibody for 1 h at room temperature and then with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Immunoreactive proteins were visualized with an enhanced chemiluminescence Western blotting detection system (ECL, GE Healthcare).

**Assay for in Vitro Binding to Immobilized GST Fusion Proteins**—The full-length Grb2 cDNA was obtained by PCR using primers, 5′-CGGGATCCATGGAGCCCATCGCC-3′ and 5′-GGAATTCAGACGCC-3′, and pME18S-HA-Grb2 (provided by Dr. M. Kato, Tokyo Institute of Technology) as a template. The PCR
product was digested with BamHI and EcoRI and inserted into the BamHI and EcoRI sites of pGEX-6P-2 (Amersham Biosciences). The GST fusion protein was expressed in *Escherichia coli* XL1-blue. COS7 cells were transfected with expression vectors encoding chimeric receptors using FuGENE6 transfection reagent (Roche Applied Sciences). At 48 h after transfection, cells were treated with NGF (100 ng/ml) for 5 min, and then cell lysates were prepared. The cell lysate was incubated with purified GST–Grb2 bound to glutathione-Sepharose 4B beads for 1 h at 4 °C. After the beads were washed twice with lysis buffer, the binding proteins were released by boiling in 2x Laemmli sample buffer, and then separated by SDS-PAGE, before being analyzed by immunoblotting.

**Isolation of HepG2 Cell Clones Stably Expressing the Chimeric Receptors**—The Flp-In<sup>TM</sup> System was obtained from Invitrogen. HepG2 cells were transfected with pFRT/LacZeo using FuGENE6 transfection reagent, and cultured for 48 h. The cells were then cultured in selective medium containing Zeocin (250 μg/ml). Cells resistant to Zeocin were selected and expanded. A cell clone was used as a Flp-In host cell clone. The host cell clone was cotransfected with the expression plasmids encoding the chimeric receptors and the Flp-expression plasmid pOG44 using JetPEI transfection reagent (Poly Transfection), and cultured for 48 h. The cells were then cultured in selective medium containing hygromycin B (350 μg/ml).

**SEAP Receptor Assay**—Cells were seeded at a density of 1.25 × 10<sup>5</sup> cells/well in six-well plates. After 24 h, the medium was replaced with fresh medium, and plasmid pRRE (collagenase-1)-SEAP or pSRE (c-fos)-SEAP (2.2 μg) was transfected into the cells using FuGENE6 transfection reagent. At 24 h after transfection, the cells were treated with HGF (50 ng/ml) or NGF (100 ng/ml) for 48 h. The culture medium was harvested, and subjected to a SEAP assay. The SEAP assay was performed using a SEAP reporter assay kit (Toyobo), and chemiluminescence was quantified on a Wallac 1420 ARVOx multi-label counter.

**Cell Proliferation Assay**—Cells were seeded at a density of 5 × 10<sup>4</sup> cells/well in 12-well plates and cultured in DMEM containing 10% FBS. After 24 h, the medium was replaced with fresh medium containing 10% FBS, and the cells were treated with HGF (50 ng/ml) or NGF (100 ng/ml) for 4 days. The cells were harvested after trypsinization, and the number of cells was counted using a hemocytometer.

**RNA Interference**—Using the small interfering RNA (siRNA) expression vector pSilencer 1.0-U6 (Ambion Inc.), vectors that target Gab1 mRNA were constructed. The Gab1 siRNA vectors siRNA-1 and 2 target the nucleotide residues at positions 165–183 in the coding region (5'-GCTTATCCTAGTATTTGAT-3') and 2206–2224 in the 3' non-coding region (5'-AGGACCTTCTGACATA-3') from the translation initiation codon, respectively. DNA oligonucleotides containing the sense sequence target, a hairpin loop, and the antisense target sequence were synthesized, annealed, and inserted into pSilencer 1.0-U6. The cells were seeded at a density of 2 × 10<sup>5</sup> cells/well in 6-well plates and cultured with DMEM containing 10% FBS for 24 h. The medium was replaced with fresh medium, and cells were treated with 4 μg of the expression plasmid mixed with 11.2 μl of jetPEI for 24 h. The medium was replaced with fresh medium, and cells were treated again with 4 μg of the expression plasmid mixed with 11.2 μl of jetPEI for 48 h. The medium was replaced with fresh medium, and cells were further cultured in the absence and presence of HGF (50 ng/ml).

**RESULTS**

**Construction of Mutated Receptors**—To distinguish transfected c-Met from the endogenous receptor in HepG2 cells, we constructed a cDNA encoding a chimeric receptor composed of the extracellular ligand-binding domain of the rat NGF receptor (TrkA), and the transmembrane and cytoplasmic domain of human c-Met, because NGF treatment did not affect the proliferation and morphology of HepG2 cells (data not shown), and the TrkA protein was not detected in HepG2 cells in an immunoblotting analysis (data not shown). To examine the requirement of the direct binding of Grb2 to c-Met for the HGF-induced strong ERK activation and the inhibition of proliferation in HepG2 cells, we introduced a point mutation into this chimeric receptor by replacing the asparagine 1358 of human c-Met with a histidine residue (N1358H) by site-directed mutagenesis of the cDNA. This mutation has been shown to specifically abrogate the direct binding of Grb2 to c-Met (14, 15). As a negative control for the tyrosine kinase activity of c-Met, we introduced a point mutation at the ATP binding site by replacing lysine 1110 with an alanine residue (K1110A). In addition, as a negative control for the signaling downstream from c-Met, we introduced point mutations at the multifunctional docking site of c-Met by replacing tyrosines 1349 and 1356 with phenylalanine residues (Y1349F/Y1356F). The cDNAs encoding the wild-type and mutated chimeric receptors were inserted into an expression vector, pcDNA5/FRT.

The inability of Grb2 to bind to the mutated receptors was confirmed by the GST pull-down method (12). The wild-type and mutated chimeric receptors were transiently expressed in COS7 cells, and the cells were treated with NGF. The expressed receptors were assayed for binding to the GST-fused Grb2. The wild-type receptor bound to GST-Grb2, whereas the mutated receptors did not (Fig. 1).

**Isolation of HepG2 Cell Clones Stably Expressing the Chimeric Receptors**—To obtain HepG2 cell clones equally expressing each receptor, the Flp-In System was used. The plasmid pFRT/lacZeo was transfected into HepG2 cells. Cell clones resistant to Zeocin were selected and expanded. A cell clone, which showed HGF-induced inhibition of proliferation and scattering similarly to parental HepG2 cells, was selected and used as a Flp-In host cell line. The expression plasmids encoding the wild-type or mutated receptors were cotransfected with the Flp-expression plasmid pOG44 into the host cell line. Cell clones resistant to hygromycin B were selected and expanded. They were subjected to an analysis of the expression of receptor proteins by immunoblotting. Two clones for the wild-type and each mutated receptor were selected for further analysis. Receptor proteins were highly expressed in all clones (Fig. 2). To examine the tyrosine phosphorylation status of the receptors, each cell clone was left untreated or treated with HGF or NGF, and phosphorylation was analyzed by Immunoprecipita-
Coupling of Grb2 to Gab1 in HGF-treated HepG2 Cells

FIGURE 1. The inability of Grb2 to bind to the mutated chimeric receptors. The binding of Grb2 to chimeric receptors was analyzed by the GST pull-down method. The wild-type (WT) and mutated chimeric receptors (NH, YF, and KA) were transiently expressed in COS7 cells, and cells were treated with NGF. NH, YF, and KA are the N1358H, Y1349F/Y1356F, and K1110A receptors, respectively. The expressed wild-type receptor was assayed for binding to GST alone (GST) or GST-fused Grb2 (GST-Grb2). The expressed mutated receptors were assayed for binding to GST-Grb2. The bound receptors were analyzed by immunoblotting (IB) with an anti-c-Met antibody followed by immunoblotting with an anti-phosphotyrosine antibody (PY20) (top panel). Tyrosine phosphorylation of the receptors was analyzed by immunoprecipitation (IP) with an anti-c-Met antibody followed by immunoblotting with an anti-phosphotyrosine antibody (PY20) (second panel). Expression of the receptors was verified by immunoprecipitation with the anti-c-Met antibody followed by immunoblotting with an anti-TrkA antibody (third panel). GST and GST fusion proteins were detected by immunoblotting with an anti-GST antibody (bottom panel).

FIGURE 2. The expression and phosphorylation of the chimeric receptors in HepG2 cells stably expressing the receptor. Two cell clones (01 and 02) expressing the wild-type and each mutated receptor were analyzed. Parental HepG2 cells (HepG2) and HepG2 cells expressing the chimeric receptors were treated with or without (−) HGF (H; 50 ng/ml) or NGF (N; 100 ng/ml) for 5 min. Lysates of the cells were immunoprecipitated with the anti-c-Met antibody and the immunoprecipitates were immunoblotted with the anti-phosphotyrosine antibody (PY20) (upper panel) and with the anti-TrkA antibody (lower panel).

FIGURE 3. The phosphorylation of ERK2 in HepG2 cells expressing the chimeric receptors. Cells were treated with or without HGF (50 ng/ml) or NGF (100 ng/ml) for 30 min. Lysates of the cells were immunoblotted with an anti-ERK2 antibody. Phosphorylation of ERK2 protein is indicated by a shift to a slower electrophoretic mobility.

No Up-regulation of p16 Expression in HepG2 Cells Expressing the N1358H Receptor—We previously demonstrated that the expression of p16INK4a, a Cdk inhibitor, was enhanced in HepG2 cells treated with HGF, and the enhancement is mediated by the strong activation of ERK (7). We also demonstrated that the enhancement of p16 expression plays a crucial role in the HGF-induced inhibition of the proliferation of HepG2 cells (7). Thus, the p16 gene is an important target gene downstream of the strong activation of the ERK pathway. The expression level of p16 was analyzed by immunoblotting using an anti-p16 antibody. The expression of p16 protein was enhanced in all cell clones treated with HGF. NGF treatment enhanced the phosphorylation of ERK2 in cells expressing the wild-type receptor, but not in cells expressing mutated receptors (Fig. 3). Second, transcriptional activity controlled by the RRE of the collagenase-1 promoter and the SRE of the c-fos promoter, which are mediated by ERK (22–24), was analyzed. We previously made constructs that contained the SEAP reporter gene under the control of RRE (collagenase-1) or SRE (c-fos) (5). We used these constructs in the present study. Transcriptional activity controlled by RRE (collagenase-1) and SRE (c-fos) was enhanced in all cell clones treated with HGF. NGF treatment also enhanced the transcriptional activity in cells expressing the wild-type receptor, but not in cells expressing the mutated receptors (Fig. 4). These results suggest that the direct binding of Grb2 to c-Met is required for the strong activation of the ERK pathway.

Failure of ERK Activation in HepG2 Cells Expressing the N1358H Receptor—ERK activity in HepG2 cells expressing the mutated receptors after ligand treatment was examined using two assay systems. First, the activity was assayed based on the phosphorylation of ERK2, which was detected by a mobility shift of phosphorylated ERK2 in an immunoblot analysis using an anti-ERK2 antibody. The phosphorylation of ERK2 was enhanced in HepG2 cells expressing the wild-type and mutated chimeric receptors after HGF treatment (Fig. 3), indicating that the signaling pathway linking c-Met to ERK was intact in all cell clones. NGF treatment enhanced the phosphorylation of ERK2 in cells expressing the wild-type receptor, but not in cells expressing mutated receptors (Fig. 3). Second, transcription activation controlled by the RRE of the collagenase-1 promoter and the SRE of the c-fos promoter, which are mediated by ERK (22–24), was analyzed. We previously made constructs that contained the SEAP reporter gene under the control of RRE (collagenase-1) or SRE (c-fos) (5). We used these constructs in the present study. Transcriptional activity controlled by RRE (collagenase-1) and SRE (c-fos) was enhanced in all cell clones treated with HGF. NGF treatment also enhanced the transcriptional activity in cells expressing the wild-type receptor, but not in cells expressing the mutated receptors (Fig. 4). These results suggest that the direct binding of Grb2 to c-Met is required for the strong activation of the ERK pathway.
Coupling of Grb2 to Gab1 in HGF-treated HepG2 Cells

We have previously shown that HGF induced strong activation of ERK in HepG2 cells, and its reduction restored the proliferation of HepG2 cells inhibited by HGF (5). Thus, it was assumed that the direct binding of Grb2 to c-Met is required for the HGF-induced inhibition of the proliferation of HepG2 cells, because a failure of the binding did not enhance the activation of ERK and the expression of p16. The proliferation of HepG2 cells expressing the chimeric receptors after ligand treatment was tested by counting cell numbers. Proliferation was inhibited in HepG2 cells expressing the wild-type and mutated chimeric receptors after HGF treatment (Fig. 6). NGF treatment inhibited the proliferation of cells expressing the wild-type receptor. In contrast, proliferation was not inhibited among cells expressing the mutated receptors (Fig. 6). These results suggest that the direct binding of Grb2 to c-Met is required for the HGF-induced inhibition of proliferation of HepG2 cells.

In addition to affecting proliferation, HGF induces scattering of cell colonies in HepG2 cells (5). It has been shown that the activation of ERK is required for HGF-induced cell scattering (25, 26). However, analysis of MDCK cells expressing the N1358H receptor showed that the direct binding of Grb2 to c-Met is not required for the activation of ERK and the HGF-induced scattering in MDCK cells (17). Thus, it is important to investigate whether the direct binding of Grb2 to c-Met is required for the scattering of HepG2 cells, because the inability of Grb2 to bind to c-Met abrogated the activation of ERK. The morphological change of HepG2 cells expressing the chimeric receptors after ligand treatment was examined using light microscopy. Scattering was observed in all cell clones after HGF treatment. NGF treatment induced scattering in HepG2 cells expressing the wild-type receptor, but did not change the morphology of cells expressing the mutated receptors (Fig. 7). These results suggest that the direct binding of Grb2 to c-Met is also required for the HGF-induced scattering of HepG2 cells.

Failure of Gab1 Phosphorylation in HepG2 Cells Expressing the N1358H Receptor—In addition to the translocation of Sos to the plasma membrane, the direct binding of Grb2 to c-Met contributes to the association of the docking protein Gab1 with c-Met, which amplifies the ERK pathway through the tyrosine phosphatase Shp2 in MDCK cells (20, 21). Thus, it was possible that the direct binding of Grb2 to c-Met induces the association of Gab1 with c-Met and its tyrosine phosphorylation in HepG2 cells. To test this possibility, we examined the tyrosine-phosphorylation status of Gab1 in HepG2 cells expressing the mutated receptors after ligand treatment. Each cell clone was left untreated or treated with HGF or NGF, and phosphorylation was analyzed by immunoprecipitation with an anti-Gab1 antibody followed by immunoblotting with the anti-phosphotyrosine antibody. Gab1 was phosphorylated in HepG2 cells expressing the wild-type receptor, but did not change the morphology of cells expressing the mutated receptors after ligand treatment. Each cell clone was treated with or without HGF (50 ng/ml) or NGF (100 ng/ml) for 48 h. The SEAP assay was performed as described under “Experimental Procedures.” The average fold-increase compared with untreated cells is indicated. Each value represents the mean ± S.D. of triplicate determinations from a representative experiment.

FIGURE 4. ERK-mediated transcriptional activation in HepG2 cells expressing the chimeric receptors. Cells were transiently transfected with the plasmid pRRE-SEAP or pSRE-SEAP. At 24 h after transfection, the cells were treated with or without HGF (50 ng/ml) or NGF (100 ng/ml) for 48 h. The SEAP assay was performed as described under “Experimental Procedures.”

FIGURE 5. Expression of p16 protein in HepG2 cells expressing the chimeric receptors. Cells were transiently transfected with the plasmid pRRE-SEAP or pSRE-SEAP. At 24 h after transfection, the cells were treated with or without HGF (50 ng/ml) or NGF (100 ng/ml) for 24 h. Lysates of the cells were immunoblotted with an anti-p16 antibody. Tubulin was used as a loading control.
tor, and treated with HGF. The siRNA-expression vectors efficiently reduced the level of endogenous Gab1 in HepG2 cells in the presence or absence of HGF (Fig. 9A). The phosphorylation level of ERK was about 60% in siRNA-transfected cells compared with mock-transfected cells (Fig. 9A). Accordingly, the HGF-induced up-regulation of p16 expression was reduced in siRNA-transfected cells (Fig. 9A).

Next, we examined the effect of the siRNA on the inhibited proliferation and scattering of HepG2 cells treated with HGF. For the proliferation assay, the number of cells was counted at 4 days after HGF treatment. The number of mock-transfected cells was reduced to about 50% by HGF treatment, compared with that of the untreated cells. Introduction of the siRNA-expression vectors for Gab1 restored the number to about 70% (Fig. 9B).

We next examined the effect of the siRNA on the inhibited proliferation and scattering of HepG2 cells treated with HGF. For the scattering assay, the morphological change of HepG2 cells was examined using light microscopy at 4 days after HGF treatment. HGF treatment induced scattering of mock-transfected cells. Introduction of the siRNA-expression vectors for Gab1 inhibited the HGF-induced scattering of HepG2 cells (Fig. 9C and data not shown). These results indicate that Gab1 plays a crucial role in the strong activation of ERK required for inhibition of the proliferation and scattering of HGF-treated HepG2 cells.

**DISCUSSION**

In this study, to investigate the mechanism of the association of Grb2 with c-Met in HGF-treated HepG2 cells, which induces strong activation of the ERK pathway, we constructed a chimeric receptor composed of the extracellular ligand-binding domain of the NGF receptor and the cytoplasmic domain of c-Met, then introduced a point mutation (N1358H) into this chimeric receptor, which specifically abrogates the binding of Grb2 directly to c-Met, but not through the adaptor protein Shc. We introduced the mutant receptor into HepG2 cells, and analyzed the ligand-induced activation of ERK. We demonstrated that the mutant receptor failed to mediate the activation of ERK, suggesting that the direct binding of Grb2 to c-Met is required for the HGF-induced strong activation of the ERK pathway in HepG2 cells, and the adaptor protein Shc is not involved in the activation.

Grb2 couples activated receptor tyrosine kinases to the Ras guanine nucleotide exchange factor Sos, promoting activation of Ras. The activated Ras initiates a protein kinase cascade, which leads to ERK activation through the protein kinases Raf and MEK (13). In addition to the coupling, the direct binding of Grb2 to c-Met is required for the full association of Gab1 with c-Met (15). This association induces tyrosine-phosphorylation of Gab1, which recruits multiple signaling molecules to Gab1.
The recruitment of the tyrosine phosphatase Shp2 enhances the Ras/ERK pathway (27). In the present study, we demonstrated that the N1358H chimeric receptor failed to induce a high level of tyrosine-phosphorylation of Gab1, suggesting that Gab1 is involved in the enhancement of the ERK pathway. Then, we examined its involvement using Gab1 siRNA, and found that the expression of Gab1 siRNA suppressed the HGF-induced phosphorylation of ERK. The phosphorylation level of ERK was about 60% in siRNA-transfected cells compared with mock-transfected cells. Because the efficiency of transient transfection of the plasmid expressing Gab1 siRNA was about 50%, the 40% suppression of the phosphorylation would rise to about 80% if the transfection efficiency was 100%. Thus, we conclude that Gab1, which associates with c-Met through Grb2, mainly contributes to the enhancement of the ERK pathway in the HGF-treated HepG2 cells.

We have previously shown that the HGF-induced inhibition of HepG2 cell proliferation requires strong activation of the ERK pathway (5). One of the molecular pathways that link the strong activation of ERK to the inhibition is the up-regulation of p16INK4a expression (7). In the present study, we demonstrated that the N1358H chimeric receptor did not induce the up-regulation of p16 expression and the inhibition of HepG2 cell proliferation after ligand stimulation. In addition, we demonstrated that the expression of Gab1 siRNA suppressed the HGF-induced up-regulation of p16 expression and inhibition of HepG2 cell proliferation. The Gab1 siRNA restored the number of HGF-treated cells to about 70% from about 50% in mock-transfected cells. Because the efficiency of transient transfection of the plasmid expressing Gab1 siRNA was about 50%, the 70% restoration would rise to about 90% if the transfection efficiency was 100%. These results suggest that the HGF-induced up-regulation of p16 expression and inhibition of HepG2 cell proliferation requires the activation of the ERK pathway, which is enhanced by Gab1 associated with c-Met through Grb2.

Based on the results presented in this study and from previous reports, we propose the model displayed in Fig. 10 to illustrate the role of Grb2 and Gab1 in HGF-induced strong activation of ERK. The association of HGF with c-Met elicits recruitment of the Grb2/Sos complex to c-Met. The translocation of Sos to the plasma membrane stimulates the exchange of GTP for GDP in Ras. In addition, the binding of Grb2 to c-Met stimulates the association of Gab1 with c-Met, which leads to the tyrosine-phosphorylation of Gab1. The phosphorylated Gab1 recruits and activates Shp2, and the activated Shp2 enhances the activation of Ras, possibly by preventing the binding of RasGAP to Gab1 (28). The highly activated Ras leads to the strong activation of ERK.
The expression of regulators of the cell cycle including p16, activated Ras enhances a protein cascade, which leads to the strong activation of RasGAP from Ras activation complexes. Similar mechanism may mediate the HGF-induced Ras activation. The highly activated Ras enhances a protein cascade, which leads to the strong activation of ERK. The activation of ERK changes the expression of regulators of the cell cycle including p16, which results in the cell cycle arrest at $G_1$. It has been shown that activation of ERK is required for growth factor-induced inhibition of proliferation of some cell lines (2, 29, 30). However, the mechanism of the activation has not been clarified. In this study, we found that coupling of Grb2 to Gab1 plays a key role in enhancing the activation of ERK in HGF-treated HepG2 cells. The findings provide novel insights into understanding the molecular mechanism of the activation of ERK required for growth factor-induced inhibition of cell proliferation.

The association of Gab1 with c-Met is essential for HGF-induced branching tubulogenesis of MDCK cells. Its full association requires Grb2 directly bound to c-Met. Furthermore, coupling of Gab1 to Shp2 is essential for HGF-induced activation of the ERK pathway, leading to the branching tubulogenesis of MDCK cells (20, 21). In the present study, we suggest that the association of Gab1 with c-Met through Grb2 is involved in the HGF-induced strong activation of the ERK pathway, leading to the inhibition of HepG2 cell proliferation. Thus, Gab1 may play a crucial role not only in branching tubulogenesis but also in the regulation of the proliferation of epithelial cells. HGF induces inhibition of the proliferation of HepG2 cells, whereas it induces stimulation of the proliferation of the human gastric carcinoma cell line MKN74. The activation of the ERK pathway by HGF is much weaker in MKN74 cells than in HepG2 cells (5). We found by an immunoblotting analysis that the level of Gab1 is higher in HepG2 cells than in MKN74 cells and HGF-induced tyrosine-phosphorylation of Gab1 is higher in HepG2 cells than in MKN74 cells (data not shown), raising the possibility that the high level of Gab1 induces the strong activation of the ERK pathway by HGF, which results in the inhibition of proliferation of carcinoma cells, while the low level of Gab1 induces the weak activation of the ERK pathway, which leads to the stimulation of proliferation. To test this possibility, further analysis of the level of Gab1 is needed in other carcinoma cell lines.

HGF-induced scattering of MDCK cells requires activation of the ERK pathway. MDCK cells expressing a chimeric receptor composed of the extracellular domain of the colony stimulating factor-1 receptor and the intracellular domain of N1358H mutant c-Met, scattered in response to colony stimulating factor-1, indicating that the direct binding of Grb2 is not required for the HGF-induced scattering of MDCK cells (17). The N1358H chimeric receptor still retains the ability to bind the adaptor protein Shc, thus coupling of Shc to Grb2 may mediate the activation of the ERK pathway, leading to scattering of MDCK cells (17). In the present study, we showed that the N1358H mutant receptor, which did not induce the activation of ERK, failed to mediate the scattering of HepG2 cells, suggesting that Shc does not mediate the activation of ERK in HepG2 cells. The expression level of Shc may not be enough to induce the activation of ERK in HepG2 cells. Alternatively, it is possible that the tyrosine-phosphorylation of Shc, which mediates the association of Grb2, is suppressed in HGF-treated HepG2 cells. The finding that the N1358H mutant receptor failed to mediate the scattering of HepG2 cells also suggests that the direct binding of Grb2 to c-Met is required for the scattering. Moreover, knockdown of Gab1 using siRNA inhibited the HGF-induced scattering of HepG2 cells, suggesting that coupling of Grb2 to Gab1 mediates the activation of the ERK pathway required for the scattering of HepG2 cells. However, the coupling is not essential for the scattering of MDCK cells (17). Further analysis is needed to understand the relationship between the level of ERK activation and scattering phenotype of epithelial cells.

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