Potent Blockade of Hepatocyte Growth Factor-stimulated Cell Motility, Matrix Invasion and Branching Morphogenesis by Antagonists of Grb2 Src Homology 2 Domain Interactions*

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Hepatocyte growth factor (HGF) stimulates mitogenesis, motogenesis, and morphogenesis in a wide range of cellular targets during development, homeostasis and tissue regeneration. Inappropriate HGF signaling occurs in several human cancers, and the ability of HGF to initiate a program of protease production, cell dissociation, and motility has been shown to promote cellular invasion and is strongly linked to tumor metastasis. Upon HGF binding, several tyrosines within the intracellular domain of its receptor, c-Met, become phosphorylated and mediate the binding of effector proteins, such as Grb2. Grb2 binding through its SH2 domain is thought to link c-Met with downstream mediators of cell proliferation, shape change, and motility. We analyzed the effects of Grb2 SH2 domain antagonists on HGF signaling and observed potent blockade of cell motility, matrix invasion, and branching morphogenesis, with ED50 values of 30 nM or less, but only modest inhibition of mitogenesis. These compounds are 1000–10,000-fold more potent anti-motility agents than any previously characterized Grb2 SH2 domain antagonists. Our results suggest that SH2 domain-mediated c-Met-Grb2 interaction contributes primarily to the motogenic and morphogenic responses to HGF, and that these compounds may have therapeutic application as anti-metastatic agents for tumors where the HGF signaling pathway is active.

Hepatocyte growth factor (HGF) stimulates mitogenesis, motogenesis, and morphogenesis in a wide range of cellular targets including epithelial and endothelial cells, hematopoietic cells, neurons, melanocytes, as well as hepatocytes (reviewed in Refs. 1–3). These pleiotropic effects play fundamentally important roles during development, organogenesis, and tissue regeneration. For example, HGF is essential for the normal development of both liver and placenta (reviewed in Ref. 4), contributes to neural development (reviewed in Ref. 5) and branching morphogenesis in various organs (reviewed in Ref. 6), and promotes kidney and lung regeneration (7, 8).

The biological responses to HGF are mediated by its cell surface receptor, c-Met, a transmembrane tyrosine kinase. Upon HGF binding, several tyrosines residues within the c-Met intracellular domain are phosphorylated, some of which are essential for catalytic activity, and some of which mediate the binding of signaling proteins such as the p85 subunit of phosphoinositide 3-kinase (PI3K), phospholipase C-γ, Shc, Gab1, and Grb2 (reviewed in Ref. 9). In epithelial cells, PI3K activity is required for both HGF-stimulated scatter and mitogenesis (10, 11), Gab1 is sufficient for tubulogenesis (12), and Grb2 binding appears to be required for HGF-stimulated cell motility and branching tubulogenesis (13–15). The small GTP-binding proteins Ras, Rho, and Rac are required for HGF-stimulated cytoskeletal rearrangements and subsequent cell motility (16, 17).

In addition to its critical roles in normal development and adult homeostasis, HGF signaling is strongly linked to cancer, including colon, breast, lung, thyroid, and renal carcinomas; melanoma; and several sarcomas; as well as glioblastoma (reviewed in Ref. 18). The inappropriate expression of c-Met in certain mesenchymal cells can lead to a carcinogenic transformation in which the tumor cells express both mesenchymal and epithelial markers (19). Inherited mutations in c-Met that result in constitutive activation of the HGF signaling pathway are associated with human renal papillary carcinoma (20–22). Importantly, in addition to its mitogenic activity, the ability of HGF to initiate a program of cell dissociation and increased cell motility coupled with increased protease production has been shown to promote cellular invasion through extracellular matrix substrates, and is correlated with tumor metastasis in vivo (reviewed in Refs. 6, 9, and 18). Increased extracellular matrix proteolysis, cell dissociation, and increased cell motility are essential for tumor metastasis, and HGF may be unique among extracellular signaling molecules in its ability to initiate all of these events.

Artificial inhibitors of protein and lipid kinases, phosphatases, and protein-protein interactions have been used extensively to examine the roles of individual intracellular effector molecules in specific HGF-stimulated activities, and to explore their potential as anticancer drugs. Many intracellular effectors interact with receptor tyrosine kinases through conserved amino acid sequence modules, such as the Src homology 2 (SH2) domain (reviewed in Refs. 23 and 24). SH2 domains directly recognize phosphotyrosine (Tyr(P)) (25), with additional secondary binding interactions within two or three amino acids C-proximal to the Tyr(P) residue introducing differential affinity toward SH2 domain subfamilies (26, 27). These and other observations have led to the development of potent tripeptide inhibitors of SH2 domain interactions. Modification of the Tyr(P) residue to phosphonomethyl phenylala-
nine, or related structures, protects this moiety from phosphatases (28, 29), whereas other modifications have been identified that increase inhibitor affinity and the potential for cell membrane penetration (30).

In this study we describe the effects of several artificial tripeptide-based inhibitors of the Grb2 SH2 domain on HGF-stimulated mitogenesis, motogenesis, and extracellular matrix invasion in epithelial and hematopoietic target cell models. Grb2 is thought to link HGF-stimulated c-Met activation with the activation of Rho, Ras, and Rac (17), and to regulate critical steps in early embryonic development, as well as in malignant transformation (31). We show that these compounds potently block HGF-stimulated cell motility, matrix invasion, and branching morphogenesis, but not HGF-stimulated mitogenesis, with ED_{50} values of 1–30 nM. The anti-motility and anti-invasion effects were characterized using four different assay systems, with no evidence of toxicity, or loss of contractility required for cellular functions other than locomotion. The active compounds did not alter HGF-stimulated c-Met tyrosine kinase activation, and their potency for inhibiting HGF-stimulated motility correlated well with their affinity for binding to Grb2 in vitro. Overall, our results suggest that c-Met-Grb2 interaction contributes primarily to the motogenic and morphogenic cellular responses to HGF, and that these compounds may have therapeutic application as anti-metastatic drugs in tumors where the HGF signaling pathway is active.

EXPERIMENTAL PROCEDURES

Materials—Human HGF protein and the cDNA for human c-Met in the pMOG vector were gifts from Dr. G. Vande Woude. The truncated HGF isoform HGF/NK1 was produced in a bacterial expression system, purified, and refolded as previously described (32). HGF/NK1 produced by this method stimulates all of the major biological responses of full-length HGF (33). The Grb2 SH2 domain antagonists designated 1–4 (Fig. 1) were synthesized and purified as described (30, 34).

Cultured Cell Lines and cDNA Transfections—The human mammary epithelial cell line 184B5 (35) was maintained in RPMI 1640 + 10% fetal bovine serum (FBS) and 5 ng/ml epidermal growth factor (Becton Dickinson). Ball/MK keratinocytes (35) were maintained in low calcium Eagle's minimal essential medium and/or modified Eagle's medium (FBS and 5 ng/ml mouse epidermal growth factor (R&D Systems). The human gastric carcinoma cell line Okajima (36) was maintained in RPMI + 15% FBS. The murine interleukin-3-dependent cell line 32D (37) was cultured in RPMI 1640 + 15% FBS and 5% WEHI-3B conditioned medium. 32D/c-Met cells were generated by co-transfection of 32D cells with pMOG/c-Met expression plasmid, resulting in neomycin resistance as described (32). The human leiomysarcoma cell line SK-LMS-1 and Madin-Darby canine kidney (MDCK) cells were maintained in DMEM + 10% FBS. TAC-2 (38), a normal mammary gland epithelial cell line, was cultured in high glucose DMEM (Life Technologies, Inc.) supplemented with 10% FBS.

Immunoprecipitation and Immunoblot Analysis—c-Met autophosphorylation was analyzed by immunoprecipitation and immunoblotting as described (32). c-Met-Grb2 interaction was analyzed by co-immunoprecipitation of c-Met with agarose-conjugated antibodies against Grb2 (Santa Cruz Biotechnology), followed by immunoblot detection using antibodies against c-Met (Santa Cruz Biotechnology), phosphotyrosine (TyrP); Upstate Biotechnology), and Grb2 (Upstate Biotechnology). Briefly, intact cells were serum-depleted for 16 h in the presence or absence of Grb2 antagonists. Cells were then growth factor-treated for 10 min as indicated, and lysed in cold buffer containing non-ionic detergents and protease and phosphatase inhibitors. After immunoprecipitation for 2 h on ice, immunocomplexes were washed, eluted with SDS sample buffer, resolved by SDS-polyacrylamide gel electrophoresis, transferred to Immobilon P (Millipore), and detected by ECL (Amerham Pharmacia Biotech). HGF/NK1-stimulated PI3K activation in intact cells after 16 h of incubation in the presence or absence of Grb2 antagonists was analyzed by co-immunoprecipitation of the p85 subunit of PI3K with agarose-conjugated anti-TyrP, followed by immunoblot detection using antibodies against PI3K (Upstate Biotechnology). Cells were treated similarly to assess Akt and MAP kinase activation, except that SDS-polyacrylamide gel electrophoresis-resolved whole cell lysates were immunoblotted using anti-phospho-Akt and anti-Akt, or anti-phospho-MAP kinase (New England Biolabs), respectively.

Mitogenic Assays—Mitogenic assays were performed using 184B5 cells or 32D/c-Met cells as described (35, 37). Briefly, cells were serum-deprived for 24 h (184B5) or 4 h (32D/c-Met) before the addition of growth factors and/or antagonists for 16 h (184B5) or 36 h (32D/c-Met). Cells were incubated with 3H-thymidine for 6 h, and DNA synthesis was measured by liquid scintillation counting.

Cell Migration Assays—The migration of Okajima, 184B5, and SK-LMS-1 cells in modified Boyden chambers was measured using Biocoat Cell Environment control inserts (8-μm pore size; Becton Dickinson). Lower chambers contained DMEM + 0.1% bovine serum albumin, to which HGF (50 ng/ml) and/or Grb2 inhibitors were added. Cells were grown in 0.1% bovine serum albumin, added to upper chambers (2 × 10^5 cells/ml) with growth factors and/or inhibitors, and incubated for 16 h at 37 °C. Cells on the upper surface of each filter were removed with a cotton swab, whereas cells that had traversed to the bottom surface of the filter were fixed and stained using Diff-Quik (Dade Diagnostics), and counted using brightfield microscopy. Mean values from 10 randomly selected unit areas were calculated for each of triplicate wells. The ratio of growth factor-treated to control migrating cells is designated on the y axis as "migration (fold increase)." 32D/c-Met cell migration was assayed using a modified Boyden chamber with 5-μm pore size Nuclepore filters (Corning). HGF/NK1 (300 ng/ml) and/or Grb2 inhibitors were added to both chambers, and cells were applied to the upper chamber at a final density of 2 × 10^5 cells/ml. After 8 h at 37 °C, cells that migrated to the lower chamber were counted with an automated cell counter (Coulter, Inc.), and migration was expressed as described for Okajima cells.

Extracellular Matrix Invasion Assays—SK-LMS-1 cell invasion was analyzed using Matrigel Invasion Chambers (Becton Dickinson) and quantitated essentially as described for Okajima cell migration across uncoated membranes. Briefly, SK-LMS-1 cells were seeded at 1 × 10^4 cells/chamber in DMEM + 5% FBS. Chambers were placed into 24-well culture plates containing DMEM + 5% FBS alone, or with HGF (10 ng/ml) and/or compounds 1–4 as indicated for 40 h at 37 °C. The ratio of growth factor-treated to control invading cells is designated on the y axis as "invasion (fold increase)."

MDCK cell invasion into three-dimensional collagen gels was analyzed as described (39). Briefly, type I collagen (1.5 mg/ml; Cohesion Technologies) was mixed with 10× minimal essential medium and sodium bicarbonate (11.76 mg/ml) at a ratio of 8:1:v/v/v on ice, and 0.4-ml aliquots were dispensed into 16-mm tissue culture wells and allowed to gel at 37 °C for 20 min. Cells were seeded onto gels (1 × 10^6 cells/well) in 0.4 ml DMEM + 0.4 ml of growth medium containing HGF and/or compounds 1 or 4 as indicated. After 5 days, cells were fixed in situ, and cells that migrated below the fibronecrosis barrier were counted. Each day 10 randomly selected fields (1 × 1.4 mm) were counted microscopically using a 20× phase contrast objective. Depth of cellular invasion into the collagen gel was quantitated in the same 10 fields per treatment group using a calibrated fine focusing micrometer. Values shown in Table I are the mean number of invading cells/field or mean invasion depth/cell in the collagen gels ± S.E.

MDCK Cell Scatter Assays—MDCK cell scatter, observed as the dispersion of single cells from tightly grouped colonies, was assayed as described (32). Briefly, MDCK cells were seeded into 24-well plates (2 × 10^4 cells/well) in DMEM containing various concentrations of inhibitors and/or HGF (30 ng/ml). Cells were incubated for 16 h at 37 °C. The scatter of fixed and stained cells was observed by brightfield microscopy; images were captured at 12.5× total magnification.

Branching Morphogenesis Assays—TAC-2 cells were suspended in three-dimensional collagen gels as described (38) at 1 × 10^6 cells/ml in collagen and incubated in complete medium containing HGF and/or Grb2 inhibitors as indicated. After 3 days, the cultures were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, and brightfield microscopic images of three or more randomly selected fields per experimental condition were digitally recorded in each of three separate experiments. The total length of the tubular structures (cord length) in each colony present in each optical field was measured using IPLab software (Scanalytics, Inc.). Cord length was considered "0" for spheroid colonies and for elongated colonies with length to diameter ratios less than 2. The mean values for each experimental condition were compared with controls using Student's unpaired t test.

RESULTS AND DISCUSSION

Grb2 Antagonists Inhibit Grb2/c-Met Interaction in Intact Cells—The structures of the Grb-2 SH2 domain binding antagonist series described in Fig. 1, the affinity of each compound for binding to Grb2 SH2 domains in vitro
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have been described (30, 34). Each compound contains a common backbone structure corresponding to the invariant Grb2 SH2 domain binding motif pY-X-N, with distinct modifications to the Tyr(P)-mimetic moiety that render each resistant to phosphatases, but maintain the net charge (~2) of this moiety. Compound 4 also has these features, but differs from the others in that it has greater than 100-fold lower affinity for binding to Grb2 SH2 domains in vitro (30). The similar charge and overall structure of compound 4 to compounds 1–3 thus made it an excellent negative control throughout the course of the biological characterization of these compounds.

To rule out the possibility that the Grb2 antagonists might interfere with HGF binding or receptor activation, we examined HGF-stimulated c-Met autophosphorylation in intact Okajima cells in the presence and absence of various concentrations of compound 1 (Fig. 2). As shown in panel A, HGF/NK1-stimulated tyrosine phosphorylation of the 145-kDa c-Met β subunit was well above the level observed in control, untreated cells (compare left and right upper panels). The lower panels confirm that equal amounts of c-Met protein were present in each lane (Fig. 2A). No differences in the level of HGF/NK1-stimulated c-Met autophosphorylation were observed in cells treated with up to 1 μM compound 1 (Fig. 2A). Identical results were obtained for compound 2, as well as for both compounds on HGF-stimulated c-Met activation in intact 32D/c-Met cells (data not shown). Additional evidence that these compounds did not block HGF-c-Met interaction or subsequent c-Met activation is provided in the analysis of HGF-stimulated mitogenesis described below.

Direct evidence that these compounds blocked c-Met-Grb2 interaction was obtained from co-immunoprecipitation/immunoblot analysis (Fig. 2B). Intact Okajima cells were treated with Grb2 inhibitors for 16 h, then briefly stimulated with HGF/NK1 before lysis, immunoprecipitation with anti-Grb2 antibodies, and immunoblotting with anti-c-Met, anti-Tyr(P), or anti-Grb2 (Fig. 2B). Consistent with previous studies describing the phosphorylation-dependent binding of Grb2 to Tyr-1356 in c-Met (14), HGF/NK1 stimulated the co-immunoprecipitation of the 145 kDa c-Met β subunit with Grb2 (Fig. 2B). When cells were pretreated for 16 h with 30 nM compound 1 prior to HGF/NK1 stimulation, the amount of HGF receptor that was co-immunoprecipitated with Grb2 was reduced by ~50% (Fig. 2B, right lanes). Thus, the effective concentration of this compound for blocking c-Met/Grb2 interaction was almost identical to its relative affinity for Grb2 SH2 domain binding measured in vitro (34). At 300 nM compound 1, Grb2-c-Met interaction was completely abolished (Fig. 2B). The lower panel of Fig. 2B confirms that equal amounts of Grb2 protein were present in each lane. Similar results were obtained using compound 2 (data not shown).

Despite complete inhibition of Grb2-c-Met interaction by the active compounds in intact cells, other SH2 domain-mediated interactions such as HGF-stimulated PI3K activation were not affected (Fig. 2C). The ability of HGF/NK1 to stimulate anti-Tyr(P)-immunoprecipitability of the PI3K p85 subunit, as well as activation of the downstream effector Akt, was identical in 32D/c-Met cells treated with compound 1 at 300 nM for 16 h and control cells (Fig. 2C). Consistent with their known dependence on PI3K, HGF-stimulated activation of the MAP kinases ERK1 and ERK2 was also completely unaffected by compound 2 after incubation of intact 32D/c-Met cells for 16 h with antagonist concentrations as high as 1 μM (Fig. 2D). Together, our data demonstrate that these antagonists act selectively on Grb2 SH2 domain binding interactions in vivo at concentrations more than 100-fold over biologically effective doses.

To characterize the mechanism by which the Grb2 SH2 domain antagonists entered intact cells, we analyzed HGF/NK1-stimulated Grb2-c-Met interaction by co-immunoprecipitation following treatment of cells with compound 1 for varying time periods (Fig. 2E). Very short (10 min) exposure of cells to 300 nM compound 1 had no effect on Grb2-c-Met interaction (data not shown). With increasing time of exposure to this treatment, Grb2-c-Met association diminished gradually over 6 h; association was inhibited by more than 50% after 2–4 h (Fig. 2E). Similar experiments using 10-fold lower concentrations of compound 1 also revealed a gradual increase in inhibition, reaching 50% inhibition after 8 h (data not shown). Although these experiments are not definitive, the simple time- and concentration-dependent inhibition of Grb2-c-Met interaction observed is consistent with passive diffusion of these compounds across the plasma membrane.

Grb2 Antagonists Fail to Block HGF-stimulated Mitogenesis—Three well established cultured cell systems representing hematopoietic and epithelial HGF targets were used to evaluate the effects of the Grb2 SH2 domain antagonists on HGF-stimulated DNA synthesis: 32D/c-Met cells (37), 184B5 human mammary epithelial cells (35), and Balb/MK mouse keratinocytes (35). Although substantial inhibition of Grb2-c-Met interaction was observed with as little as 30 nM compound 1, none of the compounds blocked HGF or HGF/NK1-stimulated DNA synthesis in 32D/c-Met cells or either epithelial cell line at concentrations up to 300 nM (data not shown). When tested over a much broader concentration range of 1–100 μM, compound 2 had little significant effect on 32D/c-Met cells, but at 100 μM, 50% inhibition of DNA synthesis was observed in 184B5 mammary epithelial cells, and 20% inhibition in Balb/MK keratinocytes. Results with compounds 1 and 3 were similar to those observed for compound 2. Even at doses in the micromolar range, no adverse effects of the compounds on cell morphology or proliferation rate were observed for up to 3 days.

Compounds 1–3 bind to the Grb2 SH2 domain with affinities
in the low nanomolar range in vitro, whereas compound 4 binds with at least 100-fold lower affinity (30, 34). The results of immunoprecipitation and immunoblot analyses demonstrated that, within 16 h, effective concentrations of these compounds gain access to the cytosol and bind selectively to intracellular Grb2 protein. In light of this, our mitogenicity studies suggest that SH2-domain-mediated Grb2-c-Met interaction is not crucial for HGF-stimulated DNA synthesis in epithelial or hematopoietic HGF target cells. The modest inhibitory effects of high doses of these compounds on 184B5 cells and Balb/MK keratinocytes may reflect a limited dependence of mitogenic signaling on Grb2-c-Met interaction in these cell types. Alternatively, it may have resulted from the less selective antagonism of SH2 domain-mediated interactions other than those of Grb2 that might be anticipated at doses 1000–10,000-fold above their affinity for the Grb2 SH2 domain.

The role of SH2-domain-mediated c-Met-Grb2 interaction in HGF-stimulated mitogenesis is controversial. Our conclusions differ from certain prior studies in which mutation of c-Met Tyr-1356, or neighboring residues was used to assess HGF signaling through Grb2 (13, 40). It is important to note that several downstream effectors of HGF signaling interact with c-Met in the region of Tyr-1356, and thus mutation of this site may block c-Met interaction with effectors other than Grb2 that are required for mitogenesis. Moreover, different oncogenic isoforms of c-Met show varying dependence on Grb2 for transforming activity (41), which is often taken as an index of mitogenic pathway activation. In particular, Trp-Met has been used extensively to assess the role of Grb2 in HGF mitogenic signaling, and shows the greatest dependence on Grb2 for its transforming activity (41). The different subcellular localizations of Trp-Met and c-Met, as well as other possible differences such as biological half-life, could contribute to differences in their relative dependence on Grb2 for mitogenic signaling. Our observations of unaltered HGF-stimulated PI3K and MAP kinase activation in the presence of the Grb2 SH2 domain antagonists are consistent with the well established dependence of HGF mitogenic signaling on these intracellular effectors. Clearly the Grb2 SH2 domain antagonists described here afford a valuable independent approach for further investigation of the role of Grb2 in growth factor and cytokine signaling generally.

Grb2 Antagonists Inhibit Cell Motility, Matrix Invasion, and Tubulogenesis—The effect of the various Grb2 SH2 domain antagonists on the migration of Okajima human gastric carcinoma cells is shown in Fig. 4A. Grb2 SH2 domain antagonists 1–3 each reduced HGF-stimulated Okajima cell migration in a dose-dependent manner, with approximate ED_{50} values in the range of 1–10 nM (Fig. 4A). In contrast, compound 4 was as much as 100-fold less potent in blocking ligand-stimulated cell migration, consistent with its more than 100-fold lower binding affinity for Grb2 in vitro (Fig. 3A). The active compounds were equally effective in blocking HGF-stimulated migration by human mammary 184B5 cells, as well as Balb/MK keratinocytes (data not shown).

**Fig. 2.** Immunoprecipitation and immunoblot analyses of the effects of Grb2 SH2 antagonists on HGF/NK1 pathway activation and c-Met-Grb2 interaction. A, serum-deprived Okajima cells were pre-treated with various concentrations of compound 1 for 16 h, then stimulated briefly with HGF/NK1 (right panels, NK1), or left untreated (left panels, control). Upper panels show the detection of tyrosine-phosphorylated c-Met, whereas the amount of c-Met protein present in each lane is shown in the lower panels. B, serum-deprived Okajima cells were pre-treated with various concentrations of compound 1 for 16 h, then stimulated briefly with HGF/NK1 (right panels, NK1), or left untreated (left panels, control). The amount of c-Met that was co-immunoprecipitated with Grb2 is shown in the upper panel; the middle panel shows anti-Grb2-precipitated tyrosine-phosphorylated c-Met, whereas the lower panel shows the level of Grb2 protein present in all lanes. C, serum-deprived 32D-c-Met cells were left untreated or pretreated with compound 1 for 16 h, then stimulated briefly with HGF/NK1 (right two lanes, NK1), or left untreated (left two lanes, control). The amount of Akt protein present in all lanes of the top panel shows the level of Akt protein present in all lanes of the middle panel only. D, serum-deprived 32D-c-Met cells were left untreated or pretreated with various concentrations of compound 2 for 16 h, then stimulated briefly with HGF/NK1 or left untreated, as indicated. The amount of active MAP kinase present in whole cell lysates is shown by immunoblotting with anti-phospho-MAP kinase antibody. E, serum-deprived 32D-c-Met cells were left untreated or pretreated with compound 1 (300 nM) for various time periods, then stimulated briefly with HGF/NK1 or left untreated, as indicated. The amount of c-Met that was co-immunoprecipitated with anti-Grb2 is shown in the upper panel, whereas the lower panel shows the amount of Grb2 protein present in each sample.
Similar to the effects of the active Grb2 SH2 domain antagonists on the HGF-stimulated migration of epithelial cells, potent blockade of HGF/NK1-stimulated 32D/c-Met cell migration was also observed (Fig. 3B). As reported previously, HGF/NK1 stimulated cell migration in this system almost 20-fold over untreated control cells (Ref. 37 and Fig. 3B). Compounds 1–3 each reduced HGF/NK1-stimulated cell migration in a dose-dependent manner with ED50 values in the 1–10 nM range, whereas compound 4 was far less potent in blocking 32D/c-Met cell migration (Fig. 3B). Interestingly, we noted that the various basal migration levels observed among the different cell types analyzed were not suppressed by the active SH2 domain antagonists, i.e., only HGF-stimulated cell migration was blocked by these compounds. This suggests that steady-state levels of cell motility are independent of SH2 domain-mediated Grb2 interactions, and that the compounds studied here act selectively on the link between c-Met and the locomotive machinery.

Consistent with their effects on HGF and HGF/NK1-stimulated cell migration, compounds 1–3 potently inhibited inva-
mation through Matrigel by SK-LMS-1 cells (Fig. 3C). This highly invasive leiomyosarcoma cell line exhibits a complex response to HGF that includes increased protease production (42), decreased tissue inhibitor of metalloproteinase-3 expression (43), and increased motility, and has been exploited as a model of HGF-stimulated metastasis in vivo (42). Compounds 1–3 displayed ED_{50} values in the range of 10–30 nM, whereas compound 4 was only modestly effective at 300 nM (Fig. 3C). The 3–10-fold higher doses of compounds 1–3 needed to effectively block matrix invasion compared with cell migration alone suggest that certain aspects of matrix invasion may be less Grb2-dependent than cell motility per se. Nonetheless, the overall potency of compounds 1–3 in this cultured cell model system warrants further evaluation of their potential use as anti-metastatic drugs in vivo.

HGF exerts a unique and potent effect on the morphology, dispersion, and movement of MDCK epithelial cells, an effect known as “scatter” (Ref. 17; reviewed in Ref. 4). During this process compact cell colonies initially spread centrifugally; the subsequent disruption of intercellular junctions allows membrane ruffling, lamellipodia extension, and increased cell motility (17). As shown in Fig. 4, HGF-stimulated MDCK cell scatter was blocked by the Grb2 SH2 domain antagonists 1–3 at 10 nM each, whereas compound 4 had no detectable effect at this concentration. Although the active compounds did not appear to block the initial HGF-stimulated spreading of MDCK cells, they appeared to dramatically reduce the number of single cells, i.e. those assumed to have the highest level of motility (Fig. 4). The ability of MDCK cells to invade three-dimensional collagen matrices, a prerequisite for HGF-stimulated branching morphogenesis, was also assessed in the presence of compounds 1 and 4 (Table I). After 5 days in culture in the absence of HGF, MDCK cells remain as a monolayer on the surface of the collagen gel, but HGF stimulates a high proportion of these cells to invade the gel (35–50 μm mean depth of invasion; Table I), as reported previously (39). Compound 1 (100 nM) reduced both the number of invading cells, as well as the mean depth of invasion per cell, by at least 50%, whereas compound 4 had no significant effects (Table I). MDCK cell viability throughout the 5-day culture period was unchanged in the presence or absence of the Grb2 SH2 domain antagonists.

In a variety of epithelial and endothelial HGF target cells, increased migration and extracellular matrix invasion also contribute to HGF-driven branching (or tubular) morphogenesis (reviewed in Ref. 4). To determine whether Grb2 SH2 domain antagonists might inhibit this process, we used an in vitro model of ductal morphogenesis in which mammary gland-derived epithelial (TAC-2) cells grown within a three-dimensional collagen gel are induced to form branching duct-like structures by HGF (38). When grown in collagen gels for 3 days under control conditions, TAC-2 cells formed small, irregular cell aggregates (Fig. 5A, left panel). Treatment for 3 days with HGF (20 ng/ml) resulted in the formation of long, branching tubular structures (Fig. 5A, middle panel). In marked contrast, co-addition of compound 1 (30 nM) and HGF to TAC-2 cultures blocked the elongation and branching of ductlike structures (Fig. 5A, right panel). Quantitative analysis of tube formation revealed that, in the presence of compounds 1–3, HGF-induced elongation of epithelial tubes was significantly inhibited (p < 0.0001) in a dose-dependent manner, with submaximal inhibitory effects observed at 30 nM inhibitor, and maximal effects at 3 μM (Fig. 5B and data not shown). Compound 4 had no significant effect on tubulogenesis at these concentrations (Fig. 5B and data not shown). Together, these data demonstrate that Grb2 SH2 domain antagonists 1–3 potently block HGF-stimulated migration and matrix invasion by a variety of cell types derived from both normal and tumor tissues, as well as HGF-driven branching morphogenesis in a well characterized mammary epithelial cell model.

Like its role in HGF-stimulated mitogenesis, the role of Grb2-c-Met interaction in HGF-stimulated cell motility is controversial. Consistent with the findings reported here, loss of HGF-stimulated cell motility has been associated with abrogation of Grb2-c-Met interaction achieved through mutation of c-Met Tyr-1356 (14). However, loss of motility has not been consistently associated with mutagenesis of the c-Met Grb2 binding site (41, 44, 45). We also note that each of the afore-

**Table I**

| Treatment group | Mean invading cells/field | Mean invasion depth/cell |
|-----------------|---------------------------|-------------------------|
| Control         | 0                         | 0                       |
| HGF             | 29.7 ± 2.6                | 35.6 ± 2.7              |
| HGF + compound 1| 12.6 ± 1.9                | 17.9 ± 1.9              |
| HGF + compound 4| 34.3 ± 2.7                | 30.8 ± 2.9              |

**Fig. 5. Effects of Grb2 SH2 domain antagonists on HGF-stimulated branching tubulogenesis by TAC-2 cells.** A. TAC-2 cells were left untreated or pretreated with compound 1 (30 nM) for 18 h, then harvested and resuspended in collagen gels as described under “Experimental Procedures.” After 3 days, control cells formed small colonies of irregular cell aggregates with short, poorly defined branching structures (left panel); whereas cells incubated with HGF (20 ng/ml) for 3 days (middle panel) formed elongated tubular structures characterized previously (38). Compound 1-pretreated cells maintained in compound 1 and HGF for 3 days were similar to control cells in overall appearance (right panel). Phase contrast images are shown (original magnification, ×125). B. Quantitative analysis of TAC-2 tubulogenesis. Cells pretreated for 18 h with various concentrations of compounds 2, 3, or 4 as indicated on the x axis (nM) were suspended in collagen gels and further incubated with the same concentrations of compounds and in the presence (filled bars) or absence (open bars) of HGF (20 ng/ml). After 3 days the cultures were fixed and colony cord length was measured as described under “Experimental Procedures.” Values on the y axis are mean cord length per field ± S.E. Values for all concentrations of compounds 2 and 3 are significantly different (p < 0.001) from cultures treated with HGF alone; three or more experiments were performed for all conditions.
mentioned reports were limited to a single cell line (14, 41, 44, 45). We observed that Grb2 SH2 domain antagonists inhibited HGF-stimulated motility of Okajima, 184B5, SK-LMS-1, 32D/c-Met, and MDCK cells at concentrations close to their affinity for binding Grb2 in vitro. This analysis clearly demonstrates that SH2 domain-mediated Grb2-c-Met interaction is required for HGF-stimulated cell motility.

While this work was in progress, another study reported the inhibition of growth factor stimulated cell motility by Grb2 SH2 domain antagonists with structures similar, but not identical, to those used here (46). The compounds characterized in that study were biologically active in the range of 10–100 μM (46); thus, the compounds described here appear to be 1000–10,000-fold more potent anti-motility agents than any Grb2 SH2 domain antagonists described to date. Moreover, the antagonists described here do not require prodrug derivatization for cellular phosphatases and are the most potent non-phospho-place of a phosphate group. These moieties confer resistance to those used here (46). The compounds characterized in that study were biologically active in the range of 10–100 μM (46).

REFERENCES
1. Michalopoulos, G. K., and DeFrances, M. C. (1997) Science 276, 60–66
2. Rubin, J. S., Bottaro, D. P., and Aaronson, S. A. (1993) Biochim. Biophys. Acta 1155, 357–371
3. Zarnegar, R., and Michalopoulos, G. K. (1995) J. Cell Biol. 129, 1177–1180
4. Birchmeier, C., and Gherardi, E. (1998) Trends Cell Biol. 8, 404–410
5. Streit, A. C., and Stern, C. D. (1997) J. Biol. Chem. 272, 20778–20785
6. Zhu, H., Naujokas, M. A., Fixman, E. D., Torossian, K., and Park, M. (1994) Cell 77, 261–271
7. Nakamura, T. (1993) J. Biol. Chem. 268, 27780–27787
8. Streit, A. C., and Stern, C. D. (1997) Ciba Found. Symp. 212, 355–365
9. Bardelli, A., and Comoglio, P. M. (1997) Ciba Found. Symp. 212, 133–144
10. Birchmeier, W. (1996) Nature 384, 173–176
11. Roninson, I., Tremblay, E., and Elliott, B. (1996) J. Biol. Chem. 271, 24850–24855
12. Heidt, K. M., DiCesare, S., Sachs, M., Brinkmann, V., Behrens, J., and Birchmeier, W. (1996) J. Biol. Chem. 271, 22311–22317
13. Fournier, T. M., Kamikura, D., Teng, K., and Park, M. (1996) J. Biol. Chem. 271, 22311–22317
14. Hartmann, G., Weidner, K. M., Schwarz, H., and Birchmeier, W. (1994) J. Biol. Chem. 269, 21936–21939
15. Ridley, A. J., Comoglio, P. M., and Hall, A. (1995) Mol. Cell. Biol. 15, 1110–1122
16. Vande Woude, G. F., Jeffers, M., Cortner, J., Alvord, G., Tsarfaty, I., and Resaud, J. (1997) Ciba Found. Symp. 212, 119–130
17. Tsarfaty, I., Rong, S., Resaud, J. H., Rulon, S., da Silva, P. P., and Vande Woude, G. F. (1994) Science 263, 88–101
18. Schmidt, L., Duh, P. M., Chen, F., Kishida, T., Glenn, G., Choyke, P., Scherer, S. W., Zhuang, Z., Lubensky, I., Dean, M., Allikmets, R., Chidambaram, A., Bergerheim, U. R., Felts, J. T., Casadevall, C., Zamaron, A., Bernues, M., Richard, S., Lips, C. J., Waltzer, M. M., Tsu, L. C., Gei, L., Orcutt, M. L., Stockhouse, T., Zbar, B., et al. (1997) Nat. Genet. 16, 68–73
19. Schmidt, L., Junker, K., Nakaigawa, N., Kipps, T., Weidner, C., Miller, M., Lubensky, I., Neumann, H. P., Brauch, H., Decker, J., Vocke, C., Brown, J. A., Jenkins, R., Richard, S., Bergerheim, U., Gerrard, B., Dean, M., Linehan, W. M., and Zbar, B. (1999) Oncogene 18, 2343–2350
20. Jeffers, M., Schmidt, L., Nakaigawa, N., Webb, C. P., Weidner, C., Kishida, T., Zbar, B., and Vande Woude, G. F. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 11445–11450
21. Jeffers, M., Nakaigawa, N., Webb, C. P., Weidner, C., Zbar, B., and Vande Woude, G. F. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 11445–11450
22. Towle, T., and Scott, J. D. (1997) Science 278, 2075–2080
23. Towle, T., and Saxton, T. M. (1999) Cell 95, 675–678
24. Matsuda, M., Mayer, J. B., Fuku, Y., and Hanafusa, H. (1990) Science 248, 1537–1539
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