Effect of Potent and Selective Inhibitors of the Grb2 SH2 Domain on Cell Motility*

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Cell motility has been correlated both with oncogenic invasiveness and metastatic potential. The development of selective inhibitors of motility has thus great potential importance. Grb2 is a SH2/SH3 domain-containing adaptor protein that links growth factor receptor tyrosine kinases to the Ras signaling pathway. We have developed specific small molecule inhibitors of the Grb2 SH2 domain as potential leads for drug discovery. Synthesis of the inhibitors and their effects on growth factor-induced growth in cells have been reported previously. In the current study, we establish that these inhibitors inhibit hepatocyte growth factor/scatter factor-induced A431 and Madin-Darby canine kidney cell motility and various cell motility-related events, including epidermal growth factor-induced ruffling of A431 cells and epidermal growth factor-induced translocation of the small GTPase Rac in these cells. We demonstrate for the first time a direct role for Grb2 in cell motility and indicate a new avenue for cancer therapeutics.

Acquisition of cell motility is a prerequisite to biological processes taking place in tissue remodeling. It has been described as a major event in morphogenesis and in pathological situations such as invasion and metastasis of tumor cells (1, 2). Most cell motility factors are also growth factors (GFs),¹ a most intriguing duality of function that implies the possibility of common or related signaling transduction pathways (3). Hepatocyte growth factor (HGF), also known as scatter factor (SF), is a pleiotropic GF that, besides promoting cell survival and proliferation, has the ability to dissociate epithelial sheets and to stimulate cell motility (3). HGF/SF predominantly acts as a paracrine factor. Nearly 90% of human malignant tumors arise from epithelial tissue, and most carcinoma cells express the c-Met/HGF receptor and are likely to use HGF/SF produced from stromal tissue (4, 5). On the other hand, GFs such as the epidermal growth factor (EGF) can enhance motility in an autocrine manner in several tumor cell lines (6). The EGF receptor (EGFR) has also been extensively studied. GF activation of EGFR influences a number of phenotypic properties in malignant cells in vitro, including mitogenesis and cell motility stimulation (7). Interestingly, overexpression of EGFR is correlated more strongly with metastasis and invasion than with tumor size (7).

Growth/motility factors such as EGF and HGF/SF, initiate a response by binding to receptors and thereby activating a tyrosine kinase domain located in the cytoplasmic portion of the receptor (8). This event leads to the autophosphorylation of the receptors on tyrosine residues. The presence of phosphotyrosine at specific sites on receptors is crucial for downstream signaling: within a specific sequence context, phosphotyrosines act as binding sites for many signal-transducing molecules within the cells (9, 10). The common element that confers the specific property of phosphotyrosine-containing sequence recognition to all these target molecules is the Src homology 2 (SH2) domain (11).

Grb2 is an adaptor protein made of one SH2 domain flanked by two SH3 domains. Grb2 interacts via its SH2 domain with activated receptors and via its SH3 domains to the nucleotide exchange factor Sos, which thus becomes activated as a positive regulator of Ras (12). Design of molecules that block the interaction between the phosphotyrosine-containing activated receptors and the SH2 domain of Grb2 should interrupt the Ras signaling pathway and may promise therapeutic leads for diseases like cancer, in which the Ras signaling pathway plays a major role (13). Grb2 may have other functions as well. Microinjection studies with anti-Grb2 antibodies in rat kidney cells suggest that Grb2 may play a role in signaling from receptor tyrosine kinases to the small GTPase Rac, a protein involved in motility-related events (14).

We have reported the rational drug design of CGP78850, a potent and selective inhibitor of Grb2 SH2 domain (15). We have already demonstrated that CGP78850 inhibits Grb2 SH2 domain binding and Ras activation in live cells and prevents anchorage independence of growth (16). CGP85793 is a prodrug derivative of CGP78850 with improved cell penetration (16).

In this study, using both compounds, we explored the possibility that Grb2 may also play a role in cell motility. Significantly, we demonstrate that inhibition of Grb2 SH2 domain prevents HGF/SF-induced A431 and MDCK cell motility and cytoskeletal rearrangements essential for this process. We also establish that EGF can no longer activate Rac in A431 cells.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—EGF (BT-201) was from Biomedical Technologies Inc., and recombinant human HGF/SF (249-HGF), was from R & D systems. Mouse monoclonal antibody specific for Grb2 (clone C1.4) was from Neomarkers. Rabbit polyclonal antibodies specific for Rac1 (C-14) were from Santa Cruz. Monoclonal anti-pan-Ras antibody (Pan-Ras Ab-3, OP40) was from Oncogene. GST-RBD and purified recombinant c-Met, as GST-Met fusion protein from baculoviruses, were kindly provided by J. Downwards and D. Stover, respectively. Drugs were dissolved in Me2SO as stock solution and diluted 1:1000 in culture medium right before use. For all experiments, appropriate controls were run to test the effects of the solvent in which stock drug

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‡ The abbreviations used are: GF, growth factor; EGF, epidermal growth factor; HGF/SF, hepatocyte growth factor/scatter factor; GST, glutathione S-transferase; SH2, Src homology 2; EGFR, EGF receptor; RBD, Ras-binding domain; MDCK, Madin-Darby canine kidney; ELISA, enzyme-linked immunosorbent assay.
solutions were prepared.  

Expression and Purification of Proteins—For the SH2 ELISA, the Grb2 SH2 domain (residues 55–152) was expressed and purified as described previously (17), GST-RBD encodes amino acids 1–149 of c-Raf-1 fused to GST. GST-RBD fusion proteins were expressed in *E. coli* and purified by binding to glutathione-agarose (Caledon Pharmaceuticals, New York). The GST-RBD fusion proteins were then diluted to 2 µg/ml in wash buffer (50 mM Tris, pH 7.5, 100 mM NaCl, 0.1% Tween). The plates were washed with wash buffer (100 mM Tris, pH 7.5, 100 mM NaCl) and the plates were washed as described above. Peroxidase activity was monitored at 655 nm on a plate reader. The EGFR ELISA assay has already been described (28). The results are expressed as the concentration at which half-maximal inhibition was observed (IC50, μM). The errors quoted correspond to the standard error in the fits of the data.

Assay for Detection of Activated Ras in Cells—MDCK cells were sero-starved for 20 h in Dulbecco's modified Eagle's medium and 0.1% bovine serum albumin. Compounds were added for 2 h at 37°C followed by treatment with HGF/SF (100 pM) for 15 min. MDCK cells were then washed with ice-cold phosphate-buffered saline and lysed in a solution containing 50 mM Tris, pH 7.5, 100 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1.5 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 80 µg/ml aprotinin, and 50 µg/ml leupeptin, 12 mM MgCl2. For affinity precipitation lysisates were incubated with GST-RBD prebound to glutathione-Sepharose for 30 min at 4°C with rocking. Bound proteins were eluted with SDS-polyacrylamide gel electrophoresis sample buffer, resolved on 12.5% acrylamide gels, and subjected to Western blotting. Blots were probed with anti-Ras antibodies. Proteins were detected using peroxidase-conjugated anti-mouse antibodies and visualized by ECL.

Cell Motility Assay—For the stimulatory effect on cell motility, cells were seeded at a density of 2 × 104 cells/well for MDCK cells and 4 × 104 cells/well for A431 cells on 6-well plates (Corning). After the cells had become attached to the plate, 50 µl HGF/SF were added, and the cultures were maintained for 24 h. The cells were then fixed with 300 µl of blocking buffer (blocking reagent for ELISA; Roche Molecular Biochemicals) and incubated for 30 min at room temperature. The plates were washed with Tris-buffered saline, 0.1% Tween, 0.1% Tween, and the proportion of isolated cells was determined. Migration was quantified by measuring cell density and counting isolated cells in 20 randomly selected fields. Mean and S.E. were calculated as the concentration at which half-maximal inhibition was observed (IC50, μM). The errors quoted correspond to the standard error in the fits of the data.

**RESULTS**

Inhibition of Grb2 SH2 Domain/c-Met Association by CGP78850 in Vitro—Phosphoprotein recognition by SH2 domain-containing proteins is thought to derive its specificity from phosphorylation per se and from the surrounding amino acid sequence (9). The recognition motif for Grb2 is pXpY (17). Proteins that interact with Grb2 contain a pXpY motif, including EGFR, SHC, and c-Met (18, 19). CGP78850 was designed by molecular modeling to contain essential elements of this Grb2 SH2 recognition motif, along with other features that enhance Grb2 binding affinity (15). As such CGP78850 has become a potent inhibitor of Grb2/EGFR and Grb2/SHC interactions in *in vitro* assays, in cell extracts, and in live cells (16). We measured here the ability of CGP78850 to also inhibit the interaction between the activated c-Met, expressed as a GST-Met fusion protein, with an immobilized Grb2 SH2 domain. GST-Met bound with high affinity to purified Grb2 SH2, a SH2/SH3-containing molecule, that the motogenic effects induced by HGF/SF in MDCK cells are initiated by its interaction with its cell surface receptor, the c-Met protein, which results in activation of Ras (19–21). Molecular analysis of the signaling pathways involved in the motogenic activity induced by HGF showed that Ras activation was essential (22) but that binding of Grb2 to the phosphorylated receptor was dispensable (23). Because SHC is associated with signaling by the Met protein, activation of Ras could be accomplished by the SHC-Grb2-Sos complex (20). We tested whether CGP78850, which is capable of inhibiting both Grb2-Met and Grb2-SHC interactions, could inhibit Ras activation in cultured MDCK cells. We used an assay that exploits the known specificity of the interaction between Ras-GTP and the RBD of Raf-1 in order to detect activated Ras in HGF/SF-stimulated MDCK cells (24). In this experiment, activated Ras was inhibited by pretreatment of cells with CGP78850 at 100 µM (Fig. 1). The data imply that Ras activity is dependent on Grb2 in MDCK cells stimulated with HGF/SF and that the SHC-Grb2-Sos pathway may be the dominant one coupling activated Met to Ras.

Grb2-SH2 Inhibitors Inhibit HGF/SF-stimulated Colony Scattering—We next examined whether inhibition of Grb2 function would directly inhibit the motility of various species of cells. HGF/SF has a marked stimulatory activity on motility of MDCK cells but not on their growth (5). In addition to high expression of EGFR, A431 cells also express the c-Met receptor (5). Assays were set up by adding varying concentrations of CGP78850 or its prodruge derivative CGP87593 (data not shown) to established islands of MDCK or A431 cells 90 min before stimulation with HGF/SF, with subsequent examination for scattering 24 h later. Fig. 2 shows a typical assay. CGP78850 completely prevented scattering of A431 and MDCK cells at 100 µM. Cells remained in close apposition with no separation of cells at the edge of the colonies and were not distinguishable from cells in the unstimulated controls. At 10 µM of CGP78850, there was a higher proportion of smaller islands and free cells (Fig. 2). At 1 µM, CGP78850 seemed to

### Table I

| Protein | CGP78850 | Ac-EpYING-NH2 |
|---------|----------|--------------|
| Met     | 0.11 ± 0.03 | 4.95 ± 0.12 |
| EGFR   | 0.041 ± 0.015 | 4.33 ± 0.75 |

**FIG. 1.** Serum-starved MDCK cells were treated without (lane 2) or with 100 pM HGF/SF (lanes 3–6) for 10 min before cell lysis. CGP78850 was added 2 h before stimulation (lanes 4–6). Lysates were subjected to affinity precipitation with GST-RBD. Ras proteins were detected by immunoblotting with monoclonal anti-Ras antibodies. Lane 1 contains whole cell lysate (WCL).
have no effect on HGF/SF-stimulated cells, and cell sheets were dispersed into single cells or small clusters of cells as in the stimulated controls (Fig. 2). MDCK cells showed the most pronounced change after addition of HGF/SF, and these cells were chosen as model target cells for quantification of the effect of CGP78850 on cell motility. Fig. 3 shows the changes in terms of cell density in MDCK cells. As expected cell density was high in the unstimulated controls and dropped after addition of HGF/SF. Fig. 3 also gives the percentage of isolated cells separated from the colonies. This was uniformly low throughout in the unstimulated controls, but the numbers of isolated cells rose sharply after addition of HGF/SF with the sudden disintegration of the cell sheets. However, in the presence of CGP78850, there was no significant difference in the number of individual cells or in cell density between unstimulated control cells and sequential treatment with 100 μM CGP78850 followed by HGF/SF. At 10 μM of compound, cell density was the same as in the unstimulated control cells, but the number of isolated cells rose. At 1 μM of CGP78850, there was no significant difference in the number of individual cells or in cell density compared with the stimulated control cells (Fig. 3).

**Requirement of Grb2 for EGF-induced Membrane Ruffling and Rac Activation**—EGF and HGF/SF have been shown to cause the formation of membrane ruffles (21, 25). Membrane ruffling formation is due to extensive actin polymerization and is one of the cytoskeletal changes associated with cell motility. To see whether inhibition of Grb2 SH2 domain was accompanied by changes in membrane ruffling formation, the subcellular localization of Grb2 and actin was determined in A431 cells by double immunofluorescence using Grb2 antibody and phalloidin, respectively (Fig. 4). In serum-deprived A431 cells, Grb2 labeling was found mostly associated with the plasma membrane in areas of cell-cell contacts, and F-actin was localized around the cell periphery. Upon treatment with EGF for 2 min, spectacular bursts of ruffling activity occurred as seen by actin staining, and Grb2 was confined to the membrane ruffles. In...
cells pretreated with CGP85793, addition of EGF did not lead to membrane ruffle formation, and Grb2 localization was the same as in serum-deprived cells (Fig. 4). The inability of EGF to induce membrane ruffles in A431 cells treated with CGP85793 suggests that EGF signaling to the actin cytoskeleton is compromised in these cells.

As previously demonstrated, the reorganization of actin fiber assembly is under the control of the Rac and Rho proteins, the former being indispensable for the formation of ruffles and the latter for the formation of stress fibers (26). In mouse dermal fibroblasts Rac has been shown to be recruited from the plasma membrane upon GF stimulation concomitant with its activation (27). In serum-deprived A431 cells Rac as detected by immunofluorescence was found only in the cytosol (Fig. 5). Upon EGF stimulation, Rac colocalized with Grb2 in the membrane ruffles but not in cells treated with CGP85793 where Rac remained cytosolic (Fig. 5), indicating that EGF stimulation does not activate Rac in cells treated with Grb2 SH2 inhibitors. The results of the present experiments show that Grb2 functions in Rac-related pathways downstream from EGFR and suggest that Grb2 controls EGF-induced membrane ruffle formation through Rac.

DISCUSSION

A parameter often associated with epithelial invasiveness is motility. Cell motility requires several distinct steps that must occur in a coordinated fashion for cellular translocation to occur. Following the establishment of adhesion to the underlying substratum, the cell must be able to form protrusions, establishing new adhesions, and be able to break older adhesions for translocation to occur (3). The fact that so many GFs can, at least in some cells, stimulate movement as well as growth suggests that there may be some link in the early stage of signal transduction. Grb2 is a SH2/SH3 domain-containing adaptor protein that links receptor tyrosine kinases to the Ras signaling pathway and mitogenesis (18). Earlier studies have shown that the injection of an antibody against Grb2 into normal rat kidney-derived fibroblasts had an effect on the cytoskeletal rearrangements induced by EGF and platelet-derived growth factor (14), suggesting the possibility that Grb2 functions in pathways involving cell motility.

Scattering of MDCK and A431 cells is indeed prevented by CGP78850 or its produg derivative CGP85793. Because regulated changes in actin microfilament organization underlie cell motility, we have also monitored early changes in the cytoskeleton of A431 cells. Membrane ruffles and lamellipodia are found at the leading edges of motile cells and are believed to play a fundamental role in migration (28, 29). Membrane ruffling formation is due to extensive actin polymerization. In this respect it is of interest to mention that EGF has been demonstrated as causing actin polymerization (30). The strong colocalization of Grb2 to membrane ruffles and the observation that Grb2 SH2 inhibitors prevented membrane ruffle formation support a role for Grb2 in cell motility and provide evidence that Grb2 is essential for tyrosine kinase signaling to the cytoskeleton.

The GTPase that appears most likely to control cell surface protrusive activity associated with translational locomotion is Rac (21). Overexpression of constitutively active Rac promotes the formation of ruffling lamellae in fibroblasts (26). In quiescent A431 cells, we observed Rac in the cytosol, correlating with the inactive state and its translocation to the cell periphery to regions of ruffling activity upon stimulation. CGP85793 completely blocked this translocation, indicating that Rac translocation is dependent on Grb2 binding to phosphoproteins. However, the different localization of Grb2 (mostly membranous) and Rac (cytosolic) before stimulation suggests that Grb2 effect on Rac translocation is indirect and may occur through Ras. Moreover, our demonstration that Rac and Grb2 are recruited and colocalize upon stimulation to the sites of ruffling activity suggests that Grb2 may be involved in the two aspects of GF regulation of Rac activity, i.e., not only in the recruitment of Rac from the cytosol to the membrane but also, as for Ras, in its guanine nucleotide exchange activity, possibly, as recently proposed, directly through Sos Dbl homology domain (31).

We establish for the first time that Grb2 is involved directly in the regulation of cell motility. The present study also demonstrates that, at least in A431 cells, Grb2 plays a role in cell motility-related phenomena, including Rac regulation and cytoskeletal rearrangements downstream of receptor tyrosine kinases. Our Grb2 SH2 inhibitors are now prototypes for a new class of therapeutic agents that may control the ability of tumor cells to move and traverse interstitial barriers.
Grb2, a SH2/SH3-containing Molecule, Regulates Cell Motility

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