Specific Uncoupling of GRB2 from the Met Receptor

DIFFERENTIAL EFFECTS ON TRANSFORMATION AND MOTILITY*

(Received for publication, December 13, 1995, and in revised form, March 25, 1996)

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The biological effects of hepatocyte growth factor/scatter factor are mediated by autophosphorylation of its receptor, the Met tyrosine kinase, on two carboxyl-terminal tyrosines. These phosphotyrosines (Y1349VH-VNV) are multifunctional docking sites for several effectors. Grb2, the adaptor for the Ras guanyl-nucleotide exchanger SOS, binds to Tyr1356 in the YVNV motif. By site-directed mutagenesis we either abrogated or duplicated the Grb2 consensus, without interfering with the other effectors. Loss of the link with Grb2 severely impaired transformation. The same mutation, however, had no effect on the "scattering" response, indicating that the level of signal which can be reached by Grb2-independent routes is permissive for motility. Duplication of the Grb2 binding site enhanced transformation and left motility unchanged. Thus, two Met-mediated biological responses, motility and growth, can be dissociated on the basis of their differential requirement for a direct link with Ras.

HGF/SF is a mesenchymal cytokine capable of inducing proliferation, motility (scattering), and tubulogenesis in epithelial cells (1). These complex biological responses depend on the activation of a series of signaling pathways. The interaction of HGF/SF with its receptor, the tyrosine kinase c-Met, triggers phosphorylation of two carboxyl-terminal tyrosines essential for signal transduction (Y1349VH-VNV). These phosphoryrosines are binding sites for the SH2 domains of p85, pp60src, phospholipase-Cγ, and Shc (2, 3). Y1356VNV has the unique ability, relative to Y1349VH-VNV, of binding also Grb2, the adaptor for the Ras guanyl-nucleotide exchanger SOS. This is due to the presence of an asparagine residue in the +2-position, which is the hallmark for Grb2 specificity (4).

The contribution of distinct signaling pathways to the biological effects of HGF/SF remains largely to be determined. However, a number of studies indicate that Ras is central to all Met-mediated responses. Ras can be activated either directly via Grb2/SOS recruitment to the activated HGF/SF receptor or indirectly, through Shc phosphorylation (3). Expression of a dominant-negative Ras or microinjection of Ras-neutralizing antibodies inhibit the motility signal of HGF/SF in MDCK cells (5, 6). Mutation of Tyr1356, which binds Grb2, drastically reduces the transforming potential of Tpr-Met, the oncogenic counterpart of the receptor (2, 7). The same mutation impairs Met-mediated motility and morphogenesis in MDCK cells (8, 9). However, removal of Tyr1356, which works as a multifunctional docking site for several effectors, not only results in the abrogation of the link with Grb2, but also in a significant reduction of overall signal transduction by the receptor (2). To evaluate the net contribution of the direct link with the Ras pathway in the HGF/SF response, we aimed at selectively uncoupling Grb2 from Met. This was done by disrupting the consensus for Grb2 binding via an asparagine to histidine substitution in position +2 of Tyr1356. The reciprocal substitution was introduced in position +2 of Tyr1349 to duplicate the Grb2 binding site. In this way we did not alter the potential of the phosphotyrosine residues to bind any other effector. These mutations were inserted in Tpr-Met, the oncogenic form of the HGF/SF receptor (10), and in Trk-Met chimeras. The transforming ability of Tpr-Met mutants was tested in a focus forming assay, using rat Fisher fibroblasts. The scatter response was tested in MDCK cells expressing the Trk-Met chimeras, following treatment with NGF. To efficiently promote transformation Met requires direct binding with Grb2, while the motility response seems to be independent from Grb2 binding.

EXPERIMENTAL PROCEDURES

Reagents, Cells, and Antibodies—All reagents, unless specified, were purchased from Sigma. Protein A covalently coupled to Sepharose was kindly provided by Dr. M. Prat (11). Monoclonal anti-Met antibodies were provided by Dr. M. M. Weber (University of Virginia, Charlottesville). Recombinant HGF/SF was provided by Dr. G. Gaulino (University of Torino) and was used in scatter experiments at a concentration of 50 units/ml. Nerve growth factor-2.5 S from mouse submaxillary glands was used at a concentration of 100 ng/ml for scatter assay, and 200 ng/ml to induce receptor phosphorylation. GST fusion proteins were large-scale produced and purified using pGEX expression vectors (Pharmacia) as
Site-directed Mutagenesis of TPR-MET cDNAs and Construction of the TPR-MET Expression Vector—Cloning of the full size MET and TPR-MET cDNAs has been reported previously (2, 14). To generate the new carboxy-terminal mutations an in vitro oligonucleotide-directed site-directed mutagenesis system (Promega) was used as previously reported (12). TPR-MET cDNAs, carrying the appropriate Tyr → Phe or Asn → His mutations, were reconstructed in the pMT2 vector, for transient expression in COS-1 cells.

The TPR-MET construct was generated by ligating two polymerase chain reaction products obtained as follows. A full-size TRK cDNA (13), kindly provided by M. Barbacid, served as a template for a polymerase chain reaction aimed at engineering the extracellular domain of the chimera. The sense oligo contained a Xbal site (underlined) and the initiation codon (5′-AGG GTC ACC GCA ATG GTG CGA GG-3′). The antisense oligo contained an EcoRI site (underlined) and corresponded to the last 6 residues of the TPR extracellular domain (5′-AGG CCG GAA TCC GTC CTT CTT CTC CAC-3′). The antisense oligo contained a KpnI site (underlined) and included the last residue of the Met protein plus the stop codon (5′-C TT GTT GAC ATG GTA CCA GCA CTA TGA T-3) oligonucleotides were synthesized on an Applied Biosystem 391 apparatus. The chimeric cDNA was reconstituted into a modified pMT2 vector containing a Xbal site (underlined). The carboxy-terminal mutations were inserted by subcloning in the pMT2 vector. All constructs were finally subcloned in pBAS expression site. Carboxy-terminal mutations were inserted by subcloning in the included the last residue of the Met protein plus the stop codon (5′-CCG GAA TCC GTC CTT CTT CTC CAC-3′). The antisense oligo contained an EcoRI site (underlined) and corresponded to the last 6 residues of the TPR extracellular domain (5′-AGG CCG GAA TTC ACA GGA TTC ATT-3′). The antisense oligo contained a KpnI site (underlined) and included the last residue of the Met protein plus the stop codon (5′-CTT GAG ATG GTA CCA GCA CTA TGA T-3′). Oligonucleotides were used to carry out an in vitro kinase assay with [γ-32P]ATP. Labeled proteins were separated on 8% SDS-PAGE. The gel was dried and exposed for autoradiography.

The ability of the N1358H Tpr-Met proteins to bind downstream effectors was tested in vitro (Fig. 1). Immunoprecipitates were used to carry out an in vitro kinase assay as above. WT, wild type; PLCγ, phospholipase Cγ.

RESULTS

Met kinase Activity Is Unaffected by Carboxy-terminal Point Mutations—To abrogate Grb2 binding, asparagine 1358 (which confers Grb2 specificity to phosphotyrosine 1356) was converted into lysidine (mutant N1358H), while to enhance Grb2 binding, histidine 1351 was converted into asparagine (mutant H1351N). We have previously shown that carboxy-terminal Tyr → Phe mutations (at residues Tyr1349, Tyr1356, and Tyr1349-1356) do not affect Met kinase activity (2). We verified that the Asn → His mutations did not interfere with the ability of Met to autophosphorylate using immunoprecipitates obtained from lysates of COS-1 cells expressing Tpr-Met mutants (Fig. 1, Panel A). Panel A of Fig. 1 shows that the auto-kinase activity is similar for all mutants. The migration rate of the different mutants varies and is inversely proportional to the number of tyrosine residues available for phosphorylation. Mutant Y1356F runs ahead of mutant Y1349F because Tyr1356 is more heavily phosphorylated in vitro than Tyr1349 (8). Mutant H1351N and N1358H migrate exactly like wild type Tpr-Met, indicating that the Asn → His mutations in +2 do not affect the level of phosphorylation of the upstream tyrosine residues. Similarly active is a Tyr → Phe mutant lacking, in addition to Tyr1349 and Tyr1356, also the most carboxy-terminal tyrosine residue (Y1349F/Y1356F/Y1365F).

Grb2 Binding Is Selectively Abrogated by the Asn → His Mutation in Position 1358—The ability of the N1358H Tpr-Met mutant to bind downstream effectors was tested in vitro (Fig. 1, Panel A). Panel A of Fig. 1 shows that the auto-kinase activity is similar for all mutants. The migration rate of the different mutants varies and is inversely proportional to the number of tyrosine residues available for phosphorylation. Mutant Y1356F runs ahead of mutant Y1349F because Tyr1356 is more heavily phosphorylated in vitro than Tyr1349 (8).
phospholipase-C, Grb2, and Shc (produced as GST fusion proteins) were used to precipitate wild type and the N1358H Tpr-Met mutant from lysates of transfected COS-1 cells. Panel B of Fig. 1 shows that all SH2s, except that of Grb2, were equally good at precipitating wild type and mutant Tpr-Met. This indicates that the Asn \( \rightarrow \) His mutation selectively abrogates binding with Grb2. Similar experiments carried out with the H1351N Tpr-Met mutant gave the same results as wild type Tpr-Met (not shown), indicating that the presence of the asparagine in +2 does not prevent all other effectors from binding to Tyr\(^{1349}\).

**Met-mediated Transformation Is Dependent on Direct Association with Grb2—**In Tpr-Met, the extracellular domain of Met is replaced with Tpr sequences, which provide two strong dimerization motifs (17). Dimerization causes constitutive activation of the Met kinase, which acquires the ability to transform rodent fibroblasts. We have previously shown that mutating Tyr\(^{1349}\) and Tyr\(^{1356}\) into Phe completely abrogates Tpr-Met-mediated transformation of rat Fisher cells (2). Tyr\(^{1356}\), which binds Grb2, is the more critical among the two tyrosines, suggesting that activation of the Ras pathway through Grb2 is central to Tpr-Met-mediated transformation. However, since this phosphotyrosine is a docking site for several SH2-containing molecules, the Tyr \( \rightarrow \) Phe mutation not only interferes with Ras activation but also impairs the ability to activate additional pathways, which could be equally important to transformation. To evaluate the net contribution of the direct link with Ras in transformation we tested in the same assay the new Grb2-specific Tpr-Met mutants, obtained by the Asn \( \rightarrow \) His substitutions. Table I shows that selective uncoupling of Grb2 from Tpr-Met drastically lowers its transforming efficiency, bringing it close to the level of the Y1356F mutant. This indicates that the ability to fully activate Ras is by far the most important feature in Tpr-Met-mediated transformation. The increase in the number of foci brought about by the introduction of a second Grb2 binding site (mutant H1351N) shows that the Tpr-Met transforming potential can be further enhanced by recruitment of additional Grb2/SOS complex.

Table I also shows that mutation of the most carboxyl-terminal tyrosine, Tyr\(^{1365}\), previously not tested, had no effect on Tpr-Met-mediated transformation, thus confirming our previous conclusion that phosphorylation of residues Tyr\(^{1349}\) and Tyr\(^{1356}\) is the main transductional switch for the HGF/SF receptor.

**Met-mediated Motility Does Not Require Grb2 Binding—**To test the effect of the Ras-specific mutations on motility, a Trk-Met chimera was constructed fusing the extracellular domain of human Trk with the transmembrane and intracellular domain of human Met. The fusion is illustrated in Fig. 2. The carboxyl-terminal mutations were inserted in the chimera, and MDCK cells were co-transfected with these constructs together with a marker for neo selection (pSV2neo). MDCK are the preferred cells for the scatter assay since, in the absence of the ligand, they grow in tight clusters. On overnight treatment with HGF/SF, MDCK cells dissociate and undergo striking morphological changes, taking on a more “fibroblastoid” appearance and emitting long filaments. G418 resistant MDCK clones were tested either by scatter assay following NGF treatment, or by kinase assay using antibodies specific for human Met. Only a low percentage of the G418-resistant clones expressed the Trk-Met protein (20%). Among these, we chose by Western blotting a set of clones expressing comparable amounts of chimeric receptor, using as a reference the level of Met protein present in A549 cells, which are of human origin, and respond to HGF/SF with motility (Fig. 3, panel A). The endogenous Met of MDCK cells could not be used as a standard, due to the lack of an appropriate antibody. All Trk-Met chimeras had a low level of basal phosphorylation, which did not affect their resting phenotype. Tyr1349 phosphorylation increased following addition of NGF (a representative example is shown in Fig. 3, panel B). A few clones expressing higher levels of the Trk-Met chimeric receptor showed stronger basal phosphorylation and a constitutively motile phenotype. These clones were excluded from the experiments. NGF treatment induced motility (scatter) in cells expressing the wild type Trk-Met chimera (Fig. 4). Cells expressing Trk-Met mutants Y1349F and Y1365F were also induced to scatter by NGF, in a manner indistinguishable from wild type (not shown). This indicates that the contribution of these tyrosines to motility, if any, is minor. Cells expressing mutant Y1356F (which is drastically impaired in its ability to transform cells) when treated with NGF did not fully scatter, although they underwent morphological changes. Most cells looked flattened, and only some acquired a “fibroblastoid” morphology (Fig. 4, see also Ref. 9). A completely amotile phenotype was apparent only in MDCK cells expressing the Trk-Met double mutant Y1349F/Y1356F (as indicated on Fig. 3A as Y1349–1356F) (Fig. 4). Surprisingly, mutation N1358H (which uncouples Grb2 from the chimeric receptor and is as drastic as Y1356F on transformation), did not interfere at all with motility. Several independent clones were tested, and they all showed a full scattering response (example in Fig. 4). Lastly, introduction of an asparagine in position +2 of Tyr\(^{1349}\), as expected, had no effect on motility (not shown).
These results prompted us to verify in the MDCK clones the effect of NGF stimulation on the activation of MAP kinase, a downstream step of the Ras-triggered kinase cascade. This event is mediated by MAP kinase phosphorylation, which can be visualized by a shift in mobility in SDS-PAGE. Extracts of MDCK cells expressing the mutant chimeras, before and after NGF stimulation, were run on SDS-PAGE and blotted with anti-MAP kinase antibodies. Fig. 5 shows that NGF induced a MAP kinase shift in all clones capable of motility, included those expressing the Trk-Met chimera N1358H. MAP kinase was only partially shifted in cells expressing the chimera Y1356F and not shifted at all in the amotile clones expressing the chimera Y1349F/Y1356F. These results indicate that in mutant N1358H phosphorylation of Tyr1356 allows other effectors to pass enough signal to cause a MAP kinase shift.

**DISCUSSION**

Following ligand binding, activated tyrosine kinase receptors dimerize and phosphorylate themselves on critical tyrosine residues becoming “docking devices” for SH2-containing molecules. Binding of SH2 domains to phosphotyrosine residues occurs via a spectrum of interactions, whose character varies from highly specific (i.e. platelet-derived growth factor receptor) to rather promiscuous (i.e. epidermal growth factor receptor). Activation of the HGF/SF receptor, in particular, results in phosphorylation of two carboxyl-terminal tyrosines (Y1349VHNATY1356VNV) which act as multifunctional docking sites for a number of SH2-containing effectors (2). The importance of these tyrosines in mediating the biological effect of HGF/SF is proven by the loss of Met-mediated transformation and motility upon mutation of both residues into phenylalanine (2, 7–9). Loss of Tyr1356 alone already significantly impairs Met function. With respect to Tyr1349, Tyr1356 is phosphorylated at higher stoichiometry (7), and, given the presence of an asparagine residue in position +2, has the unique ability of binding Grb2. This phosphorytrosine, however, is also involved in recruitment of other signaling molecules, and thus mutating it into phenylalanine affects overall Met-mediated signal transduction. In this work we wanted to evaluate the net contribution to growth and motility of the direct link between Met and Ras through Grb2. Grb2 binding was specifically abrogated, without interfering with other effectors, by mutating into histidine the asparagine residue in position +12 to Tyr1356 (Y1349VHNATY1356VHV). The reciprocal histidine to asparagine mutation in +2 of Tyr1349 was introduced to impart also to this multifunctional tyrosine the ability of binding Grb2 (Y1349VNVNATY1356VNV).

Disruption of the Grb2 consensus in Tpr-Met resulted in a reduction in the number of foci almost as severe as that caused by the loss of the corresponding tyrosine (Y1356). This suggest that the major contribution of Tyr1356 in Tpr-Met signal transduction is that of activating Ras through Grb2. When tested in a transcription assay from a Ras-responsive promoter to eval-

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**Fig. 3.** A, expression of Trk-Met chimeras in G418-resistant MDCK clones. Total protein extracts (approximately 200 μg/lane) of G418-resistant MDCK clones were separated on 8% SDS-PAGE, blotted, and probed with human-specific anti-Met antibodies. The upper band represents the mature (fully glycosylated) form of the chimeric protein. A549 human lung carcinoma cells were used as a reference for a physiological level of Met protein. In A549 cells the upper band represents the immature Met precursor, while the lower band represents the heavy chain of the mature receptor (29). B, tyrosine phosphorylation of Trk-Met chimeras is increased by treatment with NGF. The MDCK clone expressing the wild type chimera shown in panel A was kept in low serum for 24 h and treated with 200 ng/ml of NGF for 20 min at 37 °C. Anti-Met immunoprecipitates obtained from untreated (−) and NGF-treated (+) cells were run in 8% SDS-PAGE, blotted, and probed with anti-Met and anti-phosphotyrosine antibodies. Only the upper band (which shows a low basal level of tyrosine phosphorylation), responds to NGF treatment. The positions of 204, 121, 82, and 50 kDa molecular mass markers are indicated.

**Fig. 4.** NGF induces motility in MDCK cells expressing Trk-Met chimeras. The MDCK clones expressing wild type and mutant Trk-Met chimeras shown in Fig. 3 were treated with HGF/SF (25 units/ml) or NGF (100 ng/ml) overnight. Magnification × 10.
ulate their ability to activate the Ras kinase cascade, both Tpr-Met mutants N1358H and Y1365F were 50% as efficient as wild type. This level of residual signal is evidently not adequate for transformation. On the other hand, duplication of the Grb2 binding site in Tpr-Met increased the number of foci with respect to wild type. Accordingly, this mutant was also more efficient in inducing transcription from a Ras-responsive promoter. In conclusion, the number of foci obtained seems to reflect the intensity of the Ras signal elicited by the Tpr-Met mutants, strongly suggesting that full Ras activation is necessary for Met-mediated growth and transformation.

Surprisingly (considering their similar inhibitory effect on transformation), the mutation which specifically uncouples Grb2 from Tyr1356 in the Met receptor (N1358H) was permissive for a bona fide scatter response in MDCK cells, while the Y1365F Tpr-Met chimera induced a MAP kinase shift equivalent to that caused by wild type Tpr-Met, while the Y1365F Tpr-Met chimera induced only a partial MAP kinase shift. This suggests that when the N1358H mutation is present within the context of a receptor in MDCK cells, Tyr1356 mediates recruitment to the membrane of other effectors (i.e. Shc, phosphatidylinositol 3-kinase or phospholipase-Cγ) which feed into the MAP kinase cascade and activate it to a level permissive for motility. Our stable clones do not allow us to evaluate the proliferative potential of the N1358H chimeric receptor, since HGF/SF is not a mitogen for MDCK cells. It would be interesting to test this mutant chimera in cells capable of a proliferative response.

In MDCK cells Ras is necessary together with Rac, to induce "spreading" and actin reorganization in the first few hours of HGF/SF treatment. Clearly, the necessary threshold of Ras/Rac activation is reached upon Met activation whether the link with Grb2 is present or not. Progression from "spreading" into full scattering requires a third signal, distinct from Ras and Rac (6). Trk-Met mutants N1358H and H1351N are equally capable of going beyond the "spreading" stage and are able to elicit a full scatter response, indicating that also the third signal mentioned above is passed on regardless of the residue in position +2 of either tyrosine. A likely candidate for the third signal alluded to by Ridley et al. (6) is phosphatidylinositol 3-kinase. Phosphatidylinositol 3-kinase is activated following HGF/SF stimulation in MDCK cells (24), and scattering is inhibited by Wortmannin (25). Both our mutants are indeed competent to bind phosphatidylinositol 3-kinase, since the sequence YV(H/N)V is a phosphatidylinositol 3-kinase recognition motif, albeit of lower affinity with respect to the canonical YXXM sequence (12).

This work shows that Met-mediated transformation and motility can be dissociated on the basis of their differential requirement for a direct link with the Ras pathway. Mutant N1358H, which is likely to be impaired in mediating growth but is competent in transducing motility, seems particularly interesting in terms of its possible application to the study of the HGF/SF-Met pair in vivo. Targeting of the hgf/s or met genes by homologous recombination shows that they are essential for placental, liver, and muscle development, and result in embryonal death at E13.5–15.5 (26–28). Introducing in the mouse genome a "partial" loss of function mutation (such as the Asn→His substitution) which allows retention of some biological activity, may make it possible to circumvent the lethal phenotype resulting from the straight knock-out approach. This in turn may allow identification of HGF/SF-Met functions in later developmental stages.

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J. Biol. Chem. 1996, 271:14119-14123.
doi: 10.1074/jbc.271.24.14119

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