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A Peptide Representing the Carboxyl-terminal Tail of the Met Receptor Inhibits Kinase Activity and Invasive Growth*

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Interaction of the hepatocyte growth factor (HGF) with its receptor, the Met tyrosine kinase, results in invasive growth, a genetic program essential to embryonic development and implicated in tumor metastasis. Met-mediated invasive growth requires autophosphorylation of the receptor on tyrosines located in the kinase activation loop (Tyr^{1234}–Tyr^{1235}) and in the carboxyl-terminal tail (Tyr^{1349}–Tyr^{1356}). We report that peptides derived from the Met receptor tail, but not from the activation loop, bind the receptor and inhibit the kinase activity *in vitro*. Cell delivery of the tail receptor peptide impairs HGF-dependent Met phosphorylation and downstream signaling. In normal and transformed epithelial cells, the tail receptor peptide inhibits HGF-mediated invasive growth, as measured by cell migration, invasiveness, and branched morphogenesis. The Met tail peptide inhibits the closely related Ron receptor but does not significantly affect the epidermal growth factor, platelet-derived growth factor, or vascular endothelial growth factor receptor activities. These experiments show that carboxyl-terminal sequences impair the catalytic properties of the Met receptor, thus suggesting that in the resting state the nonphosphorylated tail acts as an intramolecular modulator. Furthermore, they provide a strategy to selectively target the *MET* proto-oncogene by using small, cell-permeable, peptide derivatives.

The Met tyrosine kinase is a high affinity receptor for hepatocyte growth factor (HGF/scatter factor)[1] (1, 2). Both Met and HGF are expressed in numerous tissues, although their expression is confined predominantly to cells of epithelial and mesenchymal origin, respectively. Signaling via this ligand-receptor pair triggers a unique biological program in target cells leading to "invasive cell growth." The latter results from the integration of multiple biological responses to HGF such as cell proliferation, survival, motility, invasion of extracellular matrices, and formation of tubular structures (branched morphogenesis) (3–6). During mouse development the coordinated control of the invasive growth program by the HGF/Met pair is essential, since knock-out experiments involving either the ligand or the receptor result in embryonic lethality due to defects in migration of myoblasts, implantation of placenta, and liver development (7–9).

c-*MET* was originally identified as the cellular counterpart of a transforming gene, *TPR-MET*, resulting from a chromosomal rearrangement (10, 11). A direct genetic link between *MET* and human cancer has been established by the identification of activating mutations in the c-*MET* gene in hereditary papillary renal carcinomas (12). Deregulated activation of the invasive growth phenotype by the *MET* oncogene confers invasive and metastatic properties to cancer cells (13, 14).

Binding of growth factors has been shown to induce receptor dimerization and is associated with autophosphorylation on tyrosine residues both within and outside the catalytic domain in the receptor dimer (15–17). Whereas the former are required for catalytic activity (catalytic tyrosines), the latter can serve as high affinity binding sites (docking tyrosines) for effector or adaptor molecules that recruit signal transducers to the receptor (18–20).

As for other tyrosine kinase receptors, activation of Met results in autophosphorylation of both "catalytic" and "docking" tyrosines. The major Met phosphorylation site is represented by tyrosines Tyr^{1234} and Tyr^{1235} (21). These are located within the activation loop of the kinase domain and are part of a three-tyrosine motif (Tyr^{1230}, Tyr^{1234}, and Tyr^{1235}) conserved in other kinase receptors. Both Tyr^{1234} and Tyr^{1235} are essential for full activation of the enzyme (22). Upon phosphorylation of these residues, the enzymatic activity of the Met kinase is up-regulated in an autocatalytic fashion (21, 23).

When the Met receptor is activated, in addition to phosphorylation of the catalytic tyrosines, two other tyrosines (Tyr^{1349}-Tyr^{1356}) located in the carboxyl-terminal tail of the receptor become phosphorylated (24–26). These tyrosine residues mediate coupling of the receptor with several SH2-containing effectors, including the Grb2/SoS complex (25, 26), the p85 regulatory subunit of phosphatidylinositol 3-kinase (24), Stat-3 (27), and the multiadaptor protein Gab1 (28–30). Tyr^{1349} and Tyr^{1356} are strictly required for Met-mediated invasive growth. Substitution of both tyrosines with phenylalanine does not affect the receptor kinase activity but completely abolishes proliferation, motility, invasion, and tubulogenesis (26, 31–33).

Selective inhibition of tyrosine kinase receptors can be useful to study their activation mechanisms, to dissect their signaling pathways, and to interfere with their biological effects. A number of receptors (fibroblast growth factor receptor, Ret, epidermal growth factor receptor, Kit/Steel, Met) are directly involved in human diseases including cancer, skeletal, and other developmental disorders (34–37). Therefore, the development of molecules capable of selective inhibition of tyrosine kinase receptors has a number of potential applications.

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[1] The abbreviations used are: HGF, hepatocyte growth factor; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; VEGF, vascular endothelial growth factor; PAGE, polyacrylamide gel electrophoresis; MAP, mitogen-activated protein; PRC, papillary renal carcinomas.

In the current study we sought to identify peptides capable of blocking both the kinase activity and the biological properties of the Met receptor. Previous kinetic and crystallographic studies suggest that receptor tyrosine kinases (*i.e.* IR, fibroblast growth factor receptor) can be inhibited by sequences corresponding to autophosphorylation sites located in the kinase activation loop (38–40). In view of these observations we exploited the use of sequences derived from the activation loop to interfere with the biochemical and biological properties of the Met receptor. Unexpectedly, we found that activation loop peptides do not act as Met inhibitors. We reasoned that sequences containing autophosphorylation sites of the carboxyl-terminal tail could be an alternative approach to modulate receptor activity. Accordingly, we show that a tail peptide inhibited Met kinase activity *in vitro*, blocked ligand-dependent phosphorylation and signal transduction, and impaired Met-induced invasive growth in transformed epithelial cells. The Met tail peptide does not significantly affect the EGF, PDGF, or VEGF receptor activities, demonstrating that the inhibitory mechanism is selective. These data provide evidence that peptides containing carboxyl-terminal sequences can efficiently work as inhibitors of the Met tyrosine kinase, and suggest that in the resting state the carboxyl-terminal domain may act as an intramolecular modulator of this receptor.

EXPERIMENTAL PROCEDURES

Reagents, Peptides, Antibodies, and Cell Culture—All reagents used were from Fluka (FlukaChemie AG) and Sigma. Reagents for SDS-PAGE were from Bio-Rad. HGF and macrophage-stimulating protein were obtained as described (52, 53). EGF and PDGF were from Sigma. VEGF was provided by Dr. Bussolino. Cell-permeable peptides derived from Met sequences in tandem with the Antennapedia sequence were obtained from Genosys Biotechnologies. Anti-phosphotyrosine, anti-Gab1, and anti-EGF receptor antibodies were purchased from Upstate Biotechnology, Inc. Anti-Active MAP kinase antibody was obtained from Promega, anti-PDGF receptor was from Transduction Laboratories. Anti-VEGF was from Santa Cruz Biotechnology. Anti-Met and anti-Ron antibodies were obtained as (53, 54). Anti-fluorescein antibody was from Amersham Pharmacia Biotech. A549 cells were from ATCC. GTL16 and MLP-29 (mouse liver progenitor cells) have been previously described (47). Human endothelial cells cells were kindly provided by Dr. Bussolino. Cultures of mammalian cells were maintained in Dulbecco's modified Eagle's medium or RPMI supplemented with 10% serum (Sigma) (or 20% in the case of human endothelial cells cells) in a humidified atmosphere of 5% CO_2, air.

Cell Delivery of Peptides—200 μg of each peptide were incubated in 0.01 M NH_4HCO_3, pH 9.0, at a final concentration of 200 μM in the presence of 100 μg/ml fluorescein isothiocyanate (Sigma) for 3 h at room temperature. Efficiency of fluoresceination was verified by 22% SDS-PAGE followed by Western blotting with anti-fluorescein antibodies. To assess cell permeability of peptides, fluoresceinated peptides were added to culture medium at a final concentration of 20 μM, and after 2 h, cells were fixed and examined by fluorescence microscopy. To calculate the intracellular concentration of Antennapedia peptides, cells were incubated with the Ant-$Tyr^{1234-1235}$- and Ant-$Tyr^{1349-1356}$-fluoresceinated peptides for 1 h, washed twice with phosphate-buffered saline, and lysed. Total lysates were analyzed by 22% SDS-PAGE. The presence of the fluoresceinated peptide in the lysate was revealed directly using the Fluorimager system (Molecular Dynamics). The concentration of the peptide was evaluated by comparison with lysates containing known amount of fluoresceinated peptide.

In Vitro Auto- and Substrate Phosphorylation—The Met or the Tpr-Met tyrosine kinase were immunoprecipitated with anti-Met antibodies from GTL-16 or from transfected COS cells lysates in the absence of sodium orthovanadate to allow dephosphorylation. After extensive washing, immunoprecipitates were subjected to autophosphorylation in kinase buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 12.5 mM $MgCl_2$) in the presence of 20 μM peptide, 10 μM cold ATP, and [γ-^{32}P]ATP (5 μCi/sample) for 20 min at 4 °C. The reaction was stopped by adding boiling Laemmli buffer, and samples were analyzed by 8% SDS-PAGE. Gels were dried and exposed for autoradiography. In kinetic experiments, 5 μM myelin basic protein (Sigma) was also included in the reaction as a substrate together with increasing concentrations of peptides, 40 μM cold ATP, and [γ-^{32}P]ATP (5 μCi/sample). The reaction was performed at 4 °C for 25 min and stopped by adding Laemmli buffer. Samples were separated by 8–12% SDS-PAGE followed by analysis with the PhosphorImager STORM (Molecular Dynamics). The intensity of bands corresponding to phosphorylated Met and myelin basic protein was quantitated using the program ImageQuant (Molecular Dynamics).

In Vitro Interaction of Peptides with the Receptor—A549 cells were lysed in EB buffer (100 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 10% glycerol, 1% Triton X-100) including 1 mM sodium orthovanadate and protease inhibitors. Cell lysates were incubated with 20 μM peptide for 1 h; heparin-Sepharose was then added for 2 h to allow peptide binding to heparin. Samples were washed extensively with EB buffer including 1 M LiCl and analyzed by SDS-PAGE followed by Western blotting with anti-Met antibodies. Alternatively, peptides were coupled to Affi-Gel 10, an activated affinity support from Bio-Rad, by incubation in Me_2SO for 4 h at 4 °C. 100 mM ethanolamine was added to block any other active groups, and interaction with the receptor was assayed as above. The binding of immobilized peptides with the Met catalytic domain was detected using lysates from Sf9 cells expressing the isolated Met kinase domain.

Cell Starvation and Treatment with Peptides—A549 cells were starved in serum-free medium for 48 h; MLP-29 cells were maintained in 2% serum for 3 days and then starved for 16 h in serum-free medium; human endothelial cells cells were starved for 12 h in serum-free medium with the addition of 2% bovine serum albumin. After starvation, cells were treated with peptides at the indicated concentrations for the indicated time and stimulated with the appropriate factor (80 ng/ml HGF, 100 ng/ml macrophage-stimulating protein, 100 ng/ml EGF, 100 ng/ml PDGF, 20 ng/ml VEGF) for 10 min at 37 °C. Cells were then lysed in EB buffer including sodium orthovanadate and protease inhibitors. Immunoprecipitation was performed with the appropriate antibodies, and samples were analyzed by SDS-PAGE followed by Western immunoblotting as indicated. To evaluate MAP kinase phosphorylation, 100 μg of total lysates/sample were separated on a 12% polyacrylamide gel and analyzed by Western blotting with anti-active MAP kinase antibodies.

Scatter Assay—3.5×10^3 cells/well (24 well cluster: Costar) were seeded in medium containing 2% serum. Cells were pretreated for 2 h with peptides at a final concentration of 20 μM and incubated with HGF (20 ng/ml) in the presence of peptides. After 12 h, cells were fixed with glutaraldehyde (11% in phosphate-buffered saline) and stained with crystal violet.

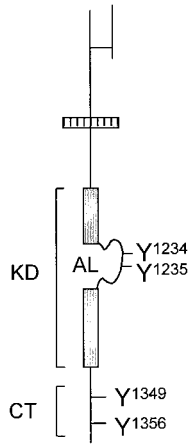
Invasion Assay—10^5 cells were seeded in medium containing 10% serum on the upper side of a porous polycarbonate membrane (8.0-μm pore size) coated with 1.2 mg/ml artificial basement membrane, Matrigel® (Collaborative Research, Waltham, MA) containing laminin, collagen type IV, proteoglycans, and growth factors. Cells were treated with 20 μM peptides for 2 h and incubated with 20 ng/ml HGF in the presence of peptides. After 24 h of incubation, cells on the upper side of the filters were mechanically removed. Cells that had invaded the Matrigel® and migrated to the lower side of the filter were fixed with glutaraldehyde (11% in phosphate-buffered saline) and stained with crystal violet. The crystal violet was solubilized with 10% acetic acid, and the concentration was evaluated as absorbance at 590 nm.

Tubulogenesis Assay—Invasive growth in collagen was assessed in tridimensional collagen gels as described (47). 10^5 cells/ml were suspended on ice in gel solution containing eight parts type I collagen 2 mg/ml stock solution (G Collagen, Seromed), 1 part 10× Dulbecco's modified Eagle's medium, and 1 part Hepes, 0.5 M, pH 7.4. The cold mixture was placed into microtiter plate wells and allowed to polymerize for 15 min at 37 °C before adding 0.1 ml of Dulbecco's modified Eagle's medium supplemented with 20% serum. Cells were cultured for 24–36 h until they formed spherical structures, then treated with 20 μM peptides for 2 h and incubated with 20 ng/ml HGF in the presence of peptides. Cells were cultured for 5 days and examined under a phase-contrast microscope.

RESULTS

Design of Cell-permeable Peptides Containing Met Autophosphorylation Sites—Activation of the Met receptor results in autophosphorylation of specific tyrosine residues located both in the kinase activation loop (Tyr^{1234}–Tyr^{1235}) and in the carboxyl-terminal tail (Tyr^{1349}–Tyr^{1356}) (21, 25). A number of peptides were synthesized corresponding to the Met autophosphorylation sites (Fig. 1). All peptides contained the Antennapedia internalization domain at the amino terminus (41). This sequence has been shown to freely translocate across biological

A



B

Ant	*RQIKIWFQNRRHKWKK*
Ant-$Y_{1234-1235}$	*RQIKIWFQNRRHKWKK* - GLARDMYDKEYYSVHNKTG
Ant-$Y_{1349-1356}$	*RQIKIWFQNRRHKWKK* - IGEHYVHVNATYVNVKCVA
Ant-$F_{1349-1356}$	*RQIKIWFQNRRHKWKK* - IGEHFVHVNATFVNVKCVA
Ant-Scramble	*RQIKIWFQNRRHKWKK* - IVAVNCGVHYEHTNVKVYA
Ant- Y_{1349}	*RQIKIWFQNRRHKWKK* - IGEHYVHVNAT

FIG. 1. **Cell-permeable peptides containing Met autophosphorylation sites.** *A*, schematic representation of the functional domains of the Met tyrosine kinase receptor. The tyrosine kinase domain (*KD*) is indicated by a *gray box*. Tyr^{1234} and Tyr^{1235} are the catalytic tyrosines located in the activation loop (*AL*) of the kinase domain. Tyr^{1349}–Tyr^{1356} are located in the carboxyl-terminal tail (*CT*) of the receptor and upon phosphorylation, generate docking sites for signal transducers. *B*, list of peptides corresponding to the Met autophosphorylation sites. Peptides were synthesized with an amino-terminal internalization sequence derived from the Antennapedia protein.

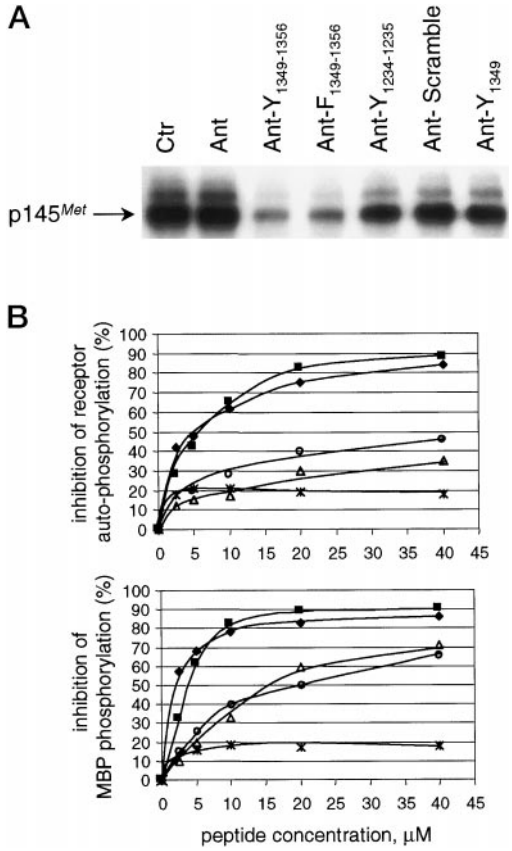


FIG. 2. **Tail peptides inhibit Met kinase activity.** *A*, Met receptor was immunoprecipitated from GTL16 cells, and immunocomplexes were subjected to *in vitro* kinase assay in the presence of the indicated peptides and [γ-^{32}P]ATP under conditions described under "Experimental Procedures." Autophosphorylated receptors were separated by SDS-PAGE and analyzed by autoradiography. *B*, immunoprecipitated Met receptors were subjected to kinase assays in the presence of the indicated peptides and analyzed as in *panel A*. The inhibitory effect of the peptides was evaluated by measuring either receptor autophosphorylation and phosphorylation of the exogenous substrate myelin basic protein (*MBP*). After densitometric analysis, values were expressed as percentage of inhibition. ♦, Ant-$Tyr^{1349-1356}$; ■, Ant-$Phe^{1349-1356}$; △, Ant-$Tyr^{1234-1235}$; ○, Ant-scramble; ×, Ant.

membranes with minimal cell toxicity (42). An unphosphorylable version of the Tyr^{1349}–Tyr^{1356} peptide was obtained by substituting the tyrosines with phenylalanine residues (Ant-$Phe^{1349-1356}$). Two additional peptides were synthesized including the Antennapedia control peptide (Ant), a shorter version of the Tyr^{1349}–Tyr^{1356} peptide lacking tyrosine 1356 (Ant-Tyr^{1349}), and a scrambled Tyr^{1349}–Tyr^{1356} peptide (Ant-scrambled).

Peptides Containing Met Carboxyl-terminal Sequences Inhibit the Kinase Activity—We investigated the possibility that peptides derived from the activation loop and the carboxyl-terminal tail could be used as inhibitors of the Met receptor. Peptides including Tyr^{1234}–Tyr^{1235} and Tyr^{1349}–Tyr^{1356} were compared for their ability to inhibit the receptor kinase activity in autophosphorylation assays performed *in vitro*. The Met receptor was immunopurified from GTL16 cells and incubated with [γ-^{32}P]ATP in the presence of increasing concentrations of the indicated peptide. We observed that the tail peptide efficiently inhibited Met autophosphorylation whereas the activation loop peptide had little effect (Fig. 2, *panel A* and *B*). To exclude a nonspecific effect of the tail peptide, either a scrambled Tyr^{1349}–Tyr^{1356} peptide version or a truncated peptide lacking Tyr^{1356} was included in the experiments. These peptides had little effect on the kinase activity of the Met receptor, showing that inhibition by the tail peptide depends on its primary amino acid sequence. The effect of the Tyr^{1349}–Tyr^{1356} peptide could be due to ATP depletion (resulting from progressive peptide phosphorylation) or to direct inhibition of the kinase reaction (resulting from interaction of the peptide substrate with the catalytic active site). To test this, a nonphosphorylable peptide analogue was designed by replacing the tyrosine with phenylalanine residues unable to accept kinase-catalyzed phosphoryl transfer from ATP. The Phe-containing tail peptide inhibited Met autophosphorylation *in vitro* as efficiently as the parental Tyr counterpart, suggesting that direct binding to the active site could be responsible for the Tyr^{1349}–Tyr^{1356} inhibitory potential (Fig. 2, *panel A* and *B*).

The effect of peptides on substrate phosphorylation was next measured using myelin basic protein. At low peptide concentrations, only the Tyr^{1349}–Tyr^{1356} and the Phe^{1349}–Phe^{1356}-containing peptides inhibited the reaction. As expected, at higher concentrations the competitive effect of the Tyr^{1234}–Tyr^{1235} derivative was also detected (Fig. 2, *panel B*).

These experiments show that peptides containing carboxyl-terminal sequences efficiently inhibit the Met kinase activity *in vitro* through a mechanism that does not rely on substrate competition.

Peptides Containing Met Carboxyl-terminal Sequences Bind the Receptor in Vitro—The possibility that the carboxyl-terminal domain may interfere with the kinase activity by interacting directly with the kinase domain of the receptor was evaluated. Peptides were immobilized on heparin-Sepharose that has been shown previously to bind the Antennapedia moiety

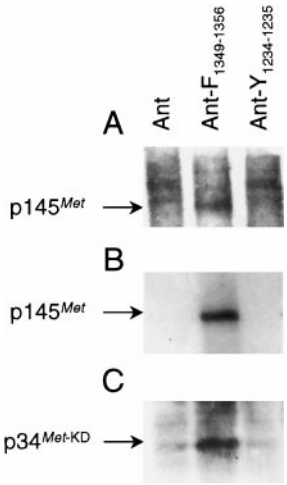


FIG. 3. **Tail peptides bind the Met receptor *in vitro*.** The indicated peptides were immobilized on heparin-Sepharose (*A*) or coupled to an agarose affinity support (Affi-Gel) (*B*) and incubated with lysates of GTL-16 cells. The amount of associated receptor was determined by Western blotting with anti-Met antibodies. *Arrows* indicate the position of the 145-kDa Met receptor β-chain ($p145^{Met}$). *C*, tail peptides coupled to heparin-Sepharose were incubated with lysate of Sf9 cells expressing the isolated Met kinase domain ($p34^{Met\text{-}KD}$). The amount of associated Met-KD protein was determined by Western blotting with anti-phosphotyrosine antibodies.

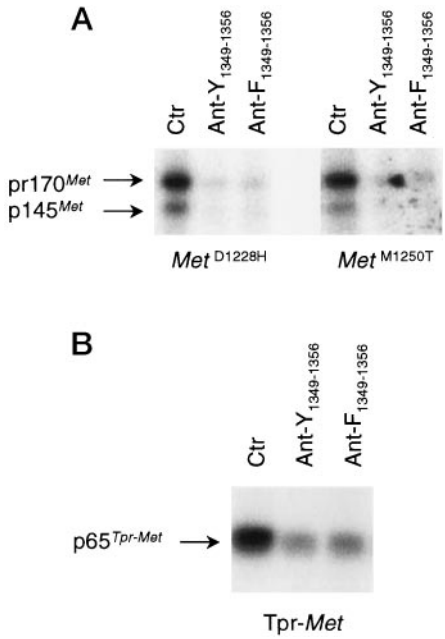


FIG. 4. **Tail peptides inhibit the constitutively activated Tpr-Met and Met-PRC kinases.** Met-PRC mutants (*A*) and Tpr-Met (*B*) were immunoprecipitated from transfected COS cells, and immunocomplexes were subjected to *in vitro* kinase assay (see Fig. 2) in the presence of the indicated peptides and [γ-^{32}P]ATP. Autophosphorylated receptors were separated by SDS-PAGE and analyzed by autoradiography. *Arrows* indicate bands corresponding to Met-PRC (the precursor and the β-chain) and to Tpr-Met ($p65^{Tpr\text{-}Met}$). *Ctr*, control.

(43). Cell lysates containing the Met protein were incubated with the immobilized peptides, and the associated receptor was visualized by immunoblotting with anti-Met antibodies. Fig. 3 shows that the carboxyl-terminal peptides bind the Met receptor most likely by interacting with its catalytic region. To verify this hypothesis, we performed similar experiments using the isolated catalytic domain (amino acid 1044–1347) lacking the juxtamembrane domain and the carboxyl-terminal tail docking sites. The isolated Met kinase domain, containing a polyhistidine tag, is catalytically active and constitutively tyrosine-phosphorylated when expressed in Sf9 cells (data not shown). Immobilized tail peptides were incubated with lysates containing the catalytic domain. The associated kinase domain protein was visualized by immunoblotting with anti-Ptyr antibodies (Fig. 3, *panel C*). To further characterize the interaction mechanism, we performed the same experiment with tyrosine-phosphorylated peptides. The phosphorylated tail peptide interacts with the Met catalytic domain with a slightly increased efficiency when compared with its unphosphorylated counterpart (data not shown).

Carboxyl-terminal Peptides Inhibit the Kinase Activity of Oncogenic Met Mutants—Oncogenic forms of the Met receptor have been described including Tpr-Met and Met-PRC mutants. In Tpr-Met the extracellular domain of Met is replaced by Tpr sequences, which provide two strong dimerization motifs. Dimerization causes constitutive activation of the Tpr-Met kinase, which acquires transforming and metastatic properties (10, 11, 14, 44). Met-PRC^{M1250T} and Met-PRC^{D1228H} are Met variants identified in papillary renal carcinomas (PRC) in which critical residues located in the kinase domain are mutated (12). Tpr-Met and Met-PRC mutants were expressed in COS cells, and association experiments with immobilized tail peptides were performed as described in the previous paragraph. Both the Tpr-Met and Met-PRC mutants were found to interact with the immobilized peptides (data not shown). To evaluate the inhibitory potential of tail peptides on the mutant forms of Met, the corresponding proteins were immunopurified from transfected COS cells with anti-Met-specific antibodies. Peptides were then compared for their ability to inhibit the kinase activity in autophosphorylation assays performed *in vitro* in the presence of the indicated peptide (20 μM). We observed that the tail peptides inhibit the kinase activity of Met mutants (Fig. 4). These data suggest that the tail peptides could be used as inhibitors of the Met oncogenic potential.

Efficient Cell Delivery of Met-derived Peptides Containing the Antennapedia Internalization Sequence—To verify that the Antennapedia-containing peptides could efficiently translocate across the plasma membrane, peptides were labeled with fluorescein (see "Experimental Procedures"). When added to cell cultures, peptides were recovered in the intracellular compartment of epithelial cells after as little as 15 min of incubation (Fig. 5). The amount of internalized peptides increased with its concentration in the culture medium with saturation at 25 μM (data not shown). Peptides were detectable intracellularly for up to 16 h. No significant differences were detected among the internalization efficiency of the different peptides. To evaluate the delivery efficiency, two different peptides were added to the culture medium (final concentration 20 μM), and the intracellular peptide concentrations were calculated (see "Experimental Procedures"). The cytosolic peptide concentration was found to be in the micromolar range (10–20 μM), which is sufficient to achieve inhibition of the Met receptor kinase activity (see Fig. 2). The relatively high intracellular peptide concentration is similar to that of the peptides originally added to the culture medium. This can be explained considering that translocation of the peptides across the plasma-membrane occurs in the absence of a receptor (42). In this situation the peptides can diffuse freely across the membrane, and equilibrium can be reached.

Carboxyl-terminal Peptides Impair HGF-dependent Met Autophosphorylation in Intact Cells—The ability of the tail peptides to inhibit Met autophosphorylation *in vitro* suggests that they could also be used to impair ligand-dependent activation of the receptor in intact cells. To verify this hypothesis, Met-expressing epithelial cells were serum-starved and treated with the peptides before ligand stimulation. Cells were incu-

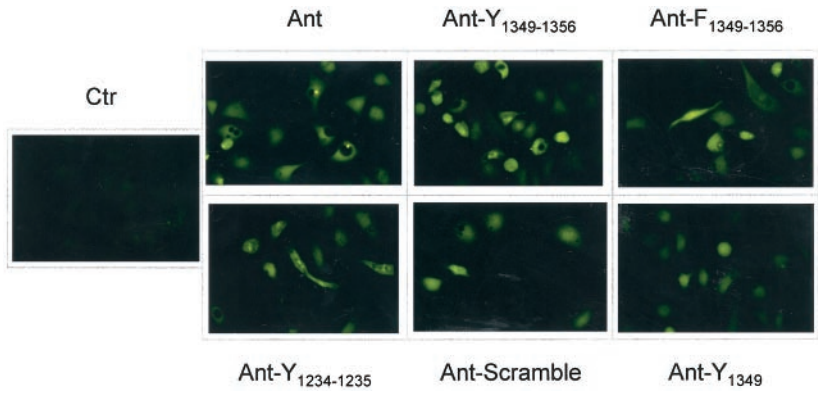


FIG. 5. **Cellular internalization of the Antennapedia-Met peptides.** Peptides were labeled at the amino terminus with fluorescein under conditions described under "Experimental Procedures." Labeling efficiency was verified by SDS-PAGE followed by Western blotting with anti-fluorescein antibodies. Subconfluent monolayers of A549 cells were treated for 2 h with the indicated peptides (20 μM), washed, and analyzed by fluorescence microscopy. *Ctr*, control.

bated with recombinant HGF, and the level of Met tyrosine phosphorylation was evaluated by immunoblotting with anti-phosphotyrosine antibodies (Fig. 6, *panel A*). In agreement with the data obtained *in vitro*, both the Tyr and Phe versions of tail peptides efficiently blocked ligand-dependent Met autophosphorylation, whereas the activation loop peptide was ineffective. In addition, the scrambled and the truncated tail peptides had little effect on ligand-dependent Met phosphorylation, confirming the specificity of the inhibitory effect (Fig. 6, *panel A*). Dose-response experiments showed that the inhibitory potential was dependent on peptide concentration with almost complete inhibition observed at 20 μM (Fig. 6, *panel B*). The effect of the peptides was reversible as shown by recovery of HGF-dependent Met phosphorylation 16 h after treatment (Fig. 6, *panel C*).

Met Tail Peptides Inhibit the Closely Related Ron Receptor but Do Not Significantly Affect the EGF, PDGF, or VEGF Receptor Activities—The inhibitory effect of the Met tail peptide on ligand stimulation of other receptor tyrosine kinases was evaluated. We tested a highly homologous receptor such as Ron and other distant Met relatives such as the EGF, PDGF, and VEGF receptors. Cells expressing the receptor of interest were serum-starved, treated with the Met or control peptides, and stimulated with the appropriate ligand. The receptors were immunoprecipitated, and tyrosine phosphorylation was measured by immunoblotting with anti-phosphotyrosine antibodies (Fig. 7). Interestingly, the Met carboxyl-terminal peptides significantly impaired ligand stimulation of the Ron receptor, whereas they had either no or a minor effect on activation of the EGF and VEGF and PDGF receptors. These data show that the Met peptide acts through a mechanism that is similar for Met and Ron; furthermore they indicate that the inhibitory activity of the Met peptide is selective.

Tail Peptides Inhibit Met-dependent Downstream Signaling—The HGF/Met pair triggers invasive growth by activation of a cascade of downstream signaling events. After ligand phosphorylation Met binds and phosphorylates the multiadaptor protein Gab1, which in turn recruits and activates a number of SH2-containing effectors (28–30). The signal is then transmitted to the nucleus via activation of various pathways including the MAP kinase cascade (25). We assessed whether the peptides that impair Met kinase activity also inhibited receptor signaling. *Panel A* of Fig. 8 shows that in cells treated with the Tyr or the Phe version of the tail peptide, Met-dependent Gab1 phosphorylation is impaired. This is also the case for activation of the p42 MAP kinase as evaluated using activation specific antibodies (Fig. 8, *panel B*). These data show that in addition to blocking the Met kinase activity, tail peptides also interrupt downstream signaling initiated by the HGF/Met interaction.

Tail Peptides Inhibit HGF-mediated Cell Migration, Invasiveness, and Branched Morphogenesis—HGF/Met promote a highly integrated biological program leading to "invasive

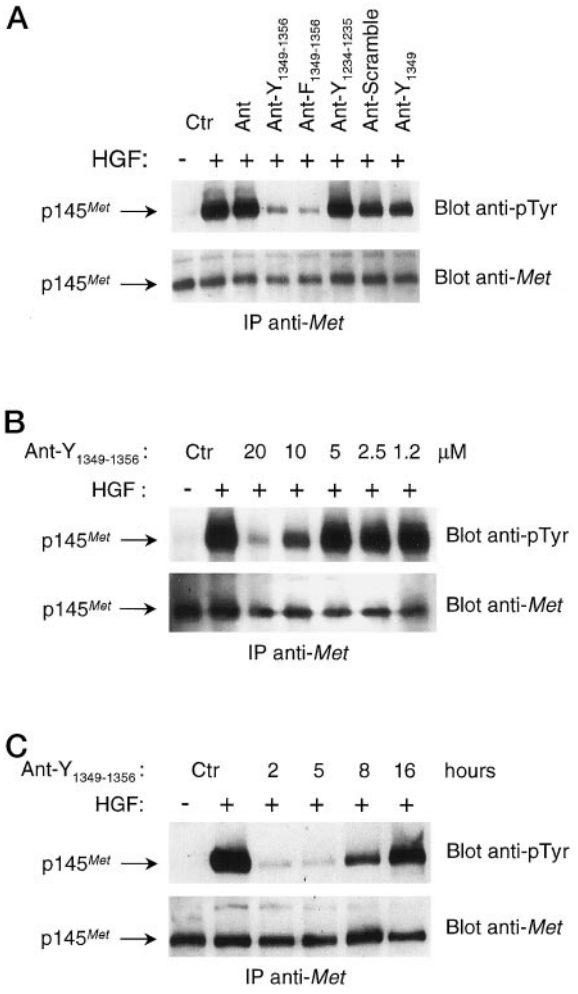


FIG. 6. **Inhibition of ligand dependent Met phosphorylation by tail peptides.** *A*, effect of peptides derived from the activation loop or the carboxyl-terminal tail on ligand-dependent Met phosphorylation. A549 cells were serum-starved for 3 days and treated with the indicated peptides at a final concentration of 20 μM. Two hours later cells were treated with recombinant HGF for 15′ and then lysed. Met phosphorylation was evaluated by immunoprecipitation (*IP*) of the receptor with anti-Met antibodies followed by Western blotting with anti-pTyr antibodies. The identity of the phosphorylated protein was confirmed by reprobing the same blot with an anti-Met antibody. *Ctr*, control. *B*, dose-response activity of the Ant-Tyr$^{1349-1356}$ peptide on ligand-dependent Met phosphorylation. Serum-starved A549 cells were treated with the tail peptide at the indicated concentrations and stimulated with HGF. Met phosphorylation was assessed as described in *A*. *C*, time course activity of the Ant- Tyr$^{1349-1356}$ peptide on ligand-dependent Met phosphorylation. Serum-starved A549 cells were treated for 2 h with the tail peptide (20 μM). At the indicated time points, cells were stimulated with HGF, and Met phosphorylation was assessed as above. The *arrows* indicate the position of the Met receptor β-chain (p145^{Met}).

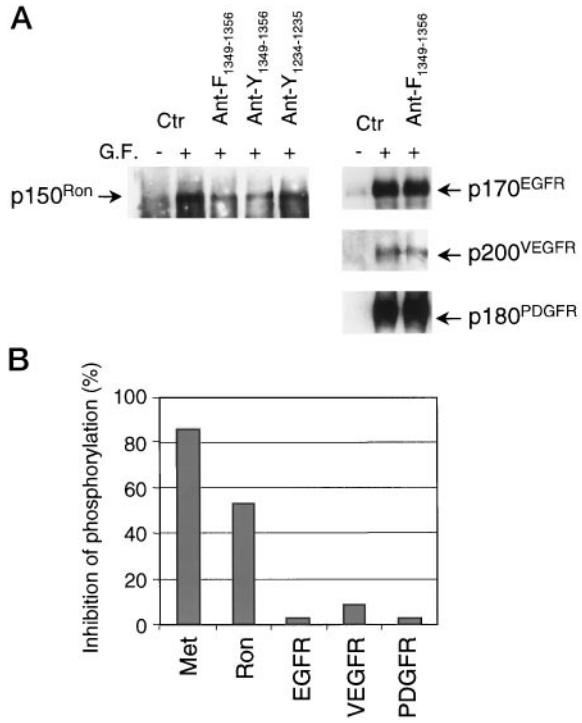


FIG. 7. **Effect of the tail Met peptide on ligand-dependent phosphorylation of different receptor tyrosine kinases.** *A*, cell lines expressing different receptors were serum-starved, incubated with the indicated peptides (20 μM) for 2 h, and stimulated with the appropriate ligands as described under "Experimental Procedures." Phosphorylation of the EGF receptor (*EGFR*) was assessed in human epithelial cell (A549)-stimulated EGF. Phosphorylation of Ron, VEGF receptor (*VEGFR*), and PDGF receptor (*PDGFR*) was evaluated, respectively, in mouse liver progenitor cells (MLP-29) stimulated with macrophage-stimulating protein, in human endothelial cells stimulated with VEGF, and in mouse fibroblasts stimulated with PDGF. Phosphorylation was detected by immunoprecipitation with receptor-specific antibodies followed by Western blotting with anti-Tyr(P) antibodies. The PDGF receptor was immunoprecipitated with anti-Tyr(P) antibodies. The identity of the phosphorylated proteins was confirmed by Western blotting with specific antibodies. *Ctr*, control. *G.F.*, growth factor. *B*, quantitative analysis of the Met peptide inhibitory activity is expressed as a percentage of inhibition compared with receptor phosphorylation in the absence of peptide.

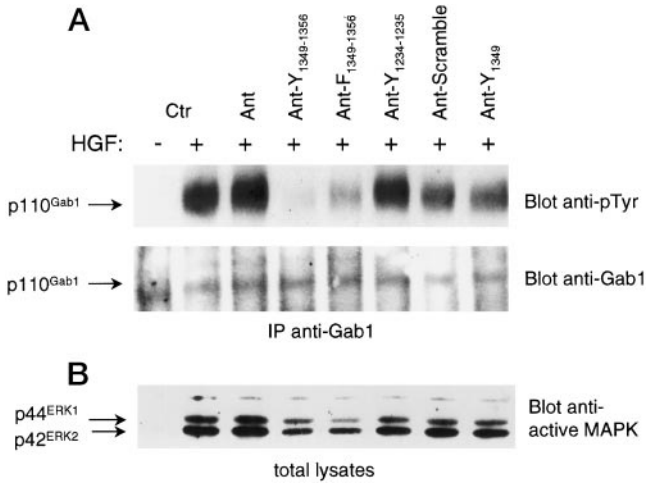


FIG. 8. **Tail peptides inhibit Met-dependent phosphorylation of Gab1 and MAP kinase activation.** *A*, effect of peptides derived from the activation loop or the carboxyl-terminal tail on Met-dependent Gab1 phosphorylation. A549 cells were serum-starved and incubated with the indicated peptides at a final concentration of 20 μM. Two h later cells were treated with recombinant HGF for 15 min and then lysed. Lysates were immunoprecipitated (*IP*) with anti-Gab1 antibodies and analyzed by Western immunoblotting with anti-Tyr(P) (*pTyr*) antibodies. The identity of the phosphorylated protein was confirmed by reprobing the same blot with anti-Gab1 antibodies. *Arrows* indicate the position of Gab1 protein ($p110^{Gab1}$). *B*, effect of activation loop or tail peptides on Met-dependent MAP kinase activation. Total lysates from A549 cells treated as in *panel A* were analyzed by SDS-PAGE and Western immunoblotting with anti-active MAP kinase antibodies. *Arrows* indicate bands corresponding to the MAP kinase active forms $p42^{ERK1}$ and $p44^{ERK2}$. *Ctr*, control.

growth," which is characterized by cell proliferation, migration, and invasion of extracellular matrix (3, 4, 45). The most complex response of HGF/Met is morphogenesis, which requires the coordinated accomplishment of each of the aforementioned biological activities (46, 47). The effects of the peptides on HGF-mediated motility, invasion, and morphogenesis were assessed (Fig. 9). The effect on cell migration was evaluated on normal epithelial cells (MLP29) in a "scatter assay." Cells were pretreated with the peptides and stimulated with HGF. Dissociation and migration of cells (scattering) was then monitored. Both the Tyr and Phe versions of the tail peptide partially inhibited HGF/Met-mediated scattering, whereas the activation loop peptide was ineffective (Fig. 9, *panel A*). The partial inhibitory effect of peptides can be explained by previous data indicating that even low levels of Met activation are sufficient to induce scattering in epithelial cells. The effect of peptides on invasion was assessed by the Matrigel® invasion assay using epithelial transformed cells (A549) (Fig. 9, *panels B* and *C*). This kind of experiment evaluates the ability of HGF-stimulated cells to cross reconstituted basal membranes made of laminin, collagen type IV, and proteoglycans. The inhibitory effect was more pronounced than that previously observed in the scatter assay. This indicates that the level of kinase inhibition achieved by the tail peptide is sufficient to block Met-mediated invasiveness, whereas it results in partial inhibition of cell motility. To measure the effects of the peptides on morphogenesis, MLP-29 cells were grown in a tridimensional type-1 collagen matrix for 3 days. By this time spherical aggregates of cells were observed that, after HGF stimulation, differentiated and formed tubular-like structures (Fig. 9, *panel D*). In the presence of the tail peptides this response was dramatically impaired. As in the invasion assay, the activation loop peptide had no effect. We also found that the Phe version of the tail peptide inhibited morphogenesis more efficiently than its Tyr counterpart. This data correlates with the relative activities of the two peptides in blocking the Met kinase activity *in vitro* (*cf.* Fig. 2, *panel B*).

DISCUSSION

Tyrosine kinase receptors are involved in human diseases including cancer, metabolic disorders, and developmental defects (34–37). On the basis of their mechanism of action, there are at least two possible strategies to inhibit receptor tyrosine kinases. On one side the catalytic process can be targeted by developing inhibitors of the enzymatic activity. On the other, receptor coupling to signal transducers can be blocked using molecules that bind the SH2 domain of the effector proteins. The finding that multiple receptors are coupled to overlapping arrays of SH2 effectors makes it difficult to interfere with signaling by a single receptor without simultaneously affecting others.

In this work we sought to target the biochemical and biological properties of the Met receptor by interfering with the mechanism of receptor autophosphorylation. On the basis of previous crystallographic studies we initially used peptides containing autophosphorylation sites located in the activation loop (39, 40). Unexpectedly, the activation loop peptide did not block Met autophosphorylation, although it interfered with substrate phosphorylation at high concentrations. Molecular

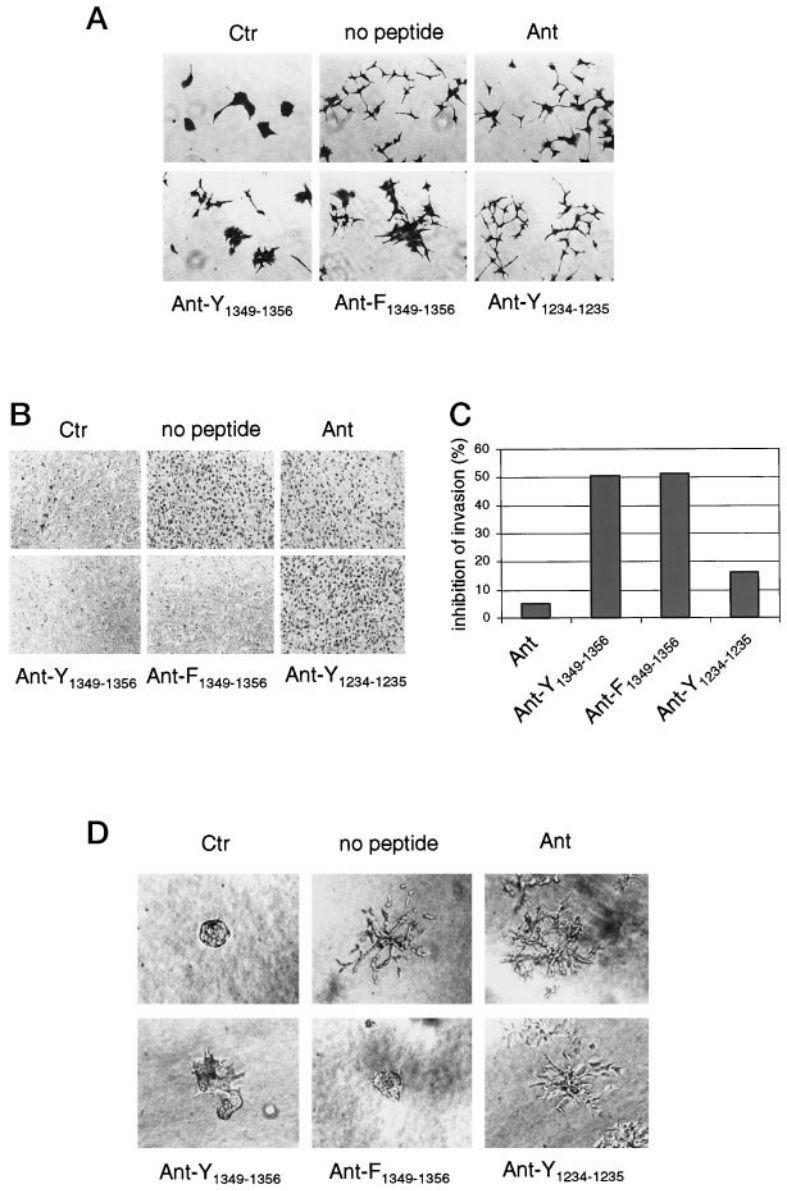


FIG. 9. **Tail peptides inhibit Met-mediated cell migration, invasion, and branched morphogenesis.** *A*, the effect of Met peptides on HGF-induced cell migration was evaluated using the scatter assay. Liver progenitor cells (MLP29) were seeded on coverslips, treated with the indicated peptides (20 μM) for 2 h, and then stimulated with HGF. Cells were allow to migrate 12 h, fixed, and stained with crystal violet. Micrographs show representative cell fields. *B*, the effect of Met peptides on HGF-induced cell invasion was evaluated by the Matrigel® Transwell assay. This assay measures the ability of cells to invade reconstituted basal membranes made of laminin, collagen type IV, and proteoglycans (Matrigel®). Epithelial cells (A549) were seeded in the upper compartment, treated with the indicated peptides for 2 h, and then stimulated with HGF. After a 24-h incubation, cells that had crossed the Matrigel® and attached to the lower side of the filter were fixed and stained with crystal violet. Micrographs show representative cell fields. *C*, cell invasion was quantitated as described under "Experimental Procedures." Values are expressed as percentage of inhibition. *D*, the effect of peptides on HGF-induced branched morphogenesis was evaluated in a tridimensional network of collagen type I. MLP29 cells were seeded in collagen gels and grown until they formed spheric structures. Cells were treated with the indicated peptides (20 μM) for 2 h and then stimulated with HGF. The morphogenic effects was detected 24 h after HGF treatment and was most evident after 72 h. Micrographs of representative fields were taken after 48–72 h. *Ctr*, control.

modeling of the Met cytoplasmic domain suggested that the tail could actually get in contact with the catalytic pocket (data not shown). We therefore exploited the possibility that tail sequences could modulate the kinase activity of the Met receptor. A tail-derived peptide blocked both auto and substrate phosphorylation of the Met receptor. Receptor tyrosine kinases preferentially phosphorylate tyrosine residues followed by a hydrophobic residue in the +3 position (48). The tyrosines located in the Met activation loop do not match the optimal consensus for phosphorylation. Conversely, tyrosines located in the carboxyl-terminal tail are predicted to be optimal substrates of the Met receptor. In agreement with this, we reported recently that Met preferentially phosphorylates peptides derived from the carboxyl-terminal tail compared with peptides derived from the activation loop (49). Substrate selectivity may therefore account for the differential effect on the Met kinase activity displayed by activation loop and tail peptides.

The inhibitory potential of the tail peptide requires the presence of a unique amino acid sequence (Ile-Gly-Glu-His-Tyr^{1349}-Val-His-Val-Asn-Ala-Thr-Tyr^{1356}-Val-Asn-Val-Lys-Cys-Val-Ala), because either a peptide truncated at tyrosine 1356 or a scramble version were inactive. The finding that a Phe^{1349}–Phe^{1356} nonphosphorylable analogue also blocked the Met kinase activity suggested that the inhibitory mechanism relies on peptide binding to the active site rather then on ATP depletion. This was confirmed by experiments demonstrating the interaction of the immobilized tail peptide with the Met receptor.

The crystal structures of the insulin and fibroblast growth factor receptors indicate that, in the inactive state, the activation loop blocks the access of the substrate peptide to the catalytic site loop (39, 40). This situation may be different in Met. The inhibitory activity of peptides containing sequences from the Met carboxyl-terminal tail suggest that this region modulates the function of the receptor. One possibility is that, in the inactive receptor state, the tail interferes with access of the substrate to the catalytic pocket of the enzyme. Ligand-induced dimerization may unleash the kinase activity by releasing this autoinhibitory mechanism. Alternatively, the tail could impair receptor phosphorylation by interacting with another moiety of the catalytic domain. We are currently performing crystallographic studies to verify this hypothesis.

Interestingly the Met tail peptide also inhibited Ron, a Met-related receptor, but not the EGF, VEGF, and PDGF receptors. Among the receptor tyrosine kinases, Ron has the highest sequence homology with Met. In particular, the amino acid sequence surrounding the tail phosphorylation sites (Y-

hydrophobic-X-hydrophobic-X_3-Y-hydrophobic-X-hydrophobic) is conserved between Met and Ron (25). The finding that the inhibitory activity was restricted to receptors of the Met family implies a common mechanism. These experiments further indicate that intramolecular peptide sequences can be utilized to selectively target the catalytic properties of tyrosine kinase receptors.

The membrane internalization properties of the Antennapedia homeodomain were used to transduce the Met inhibitory peptides into epithelial cells. Once internalized, the tail peptide blocked both ligand-dependent autophosphorylation and downstream Met signaling. In particular, the peptides impaired Gab1 phosphorylation and MAP kinase activation. The effects were dose-dependent and reversible, confirming the specificity of the inhibitory process.

A number of biological assays were used to study whether peptides derived from the Met tail could interfere with HGF-induced invasive growth as measured by cell motility, invasion, and branched morphogenesis. Met-mediated invasion and tubulogenesis were severely impaired by the tail receptor peptide. Interestingly, motility was only partially affected, suggesting that a low level of receptor activation is sufficient to induce cell flattening and dissociation. This is in agreement with previous data showing that Met receptor mutants unable to promote invasion and tubulogenesis are still competent in inducing motility (31, 50, 51). Met-triggered invasive growth is required for embryonic development, whereas its inappropriate activation confers to cancer cells invasive and metastatic properties. Selective inhibitors of this process could be useful in understanding the HGF/Met biology and in targeting the invasive metastatic potential of Met-expressing cells. By demonstrating that tail sequences act as inhibitors of the Met tyrosine kinase, this study provides an approach to interfere with the biological effects triggered by the *MET* proto-oncogene.

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A Peptide Representing the Carboxyl-terminal Tail of the Met Receptor Inhibits Kinase Activity and Invasive Growth

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