Motogenic and Morphogenic Activity of Epithelial Receptor Tyrosine Kinases

Martin Sachs,* K. Michael Weidner,* Volker Brinkmann,* Ingrid Walther,* Axel Obermeier,‡
Axel Ullrich,* and Walter Birchmeier*

*Max-Delbrück-Center for Molecular Medicine, 13125 Berlin, Germany; and ‡Max-Planck-Institute for Biochemistry, 82152 Martinsried, Germany

Abstract. Receptor tyrosine kinases play essential roles in morphogenesis and differentiation of epithelia. Here we examined various tyrosine kinase receptors, which are preferentially expressed in epithelia (c-met, c-ros, c-neu, and the keratin growth factor [KGF] receptor), for their capacity to induce cell motility and branching morphogenesis of epithelial cells. We exchanged the ligand-binding domain of these receptors by the ectodomain of trkA and could thus control signal by the new ligand, NGF. We demonstrate here that the tyrosine kinases of c-met, c-ros, c-neu, the KGF receptor, and trkA, but not the insulin receptor, induced scattering and increased motility of kidney epithelial cells in tissue culture. Mutational analysis suggests that SHC binding is essential for scattering and increased cell motility induced by trkA. The induction of motility in epithelial cells is thus an important feature of various receptor tyrosine kinases, which in vivo play a role in embryogenesis and metastasis. In contrast, only the c-met receptor promoted branching morphogenesis of kidney epithelial cells in three-dimensional matrices, which resemble the formation of tubular epithelia in development. Interestingly, the ability of c-met to induce morphogenesis could be transferred to trkA, when in a novel receptor hybrid COOH-terminal sequences of c-met (including Y14 to Y16) were fused to the trkA kinase domain. These data demonstrate that tubulogenesis of epithelia is a restricted activity of tyrosine kinases, as yet only demonstrated for the c-met receptor. We predict the existence of specific substrates that mediate this morphogenesis signal.

IN the last decade, a large variety of receptor tyrosine kinases were molecularly characterized (for reviews see Ullrich and Schlessinger, 1990; van der Geer et al., 1994). Several of these receptors were first discovered because of their transforming potential and were therefore associated with mediating mitogenic signals (Cooper et al., 1984; Ullrich et al., 1984; Takahashi et al., 1985; Birchmeier et al., 1986). However, it has recently become evident that tyrosine kinase receptors can also regulate decisive events in development; i.e., these receptors control cell movement, morphogenesis, and differentiation (Montesano et al., 1991a,b; Peles et al., 1993; Schuchardt et al., 1994; Bladt et al., 1995, Cheng et al., 1995; Drescher et al., 1995; Meyer and Birchmeier, 1995; Werner et al., 1994; Yang et al., 1995; for a recent review see Birchmeier and Birchmeier, 1993).

Previously, we and others have investigated the morphogenic activity of receptor tyrosine kinases on epithelial cells. It is noteworthy that several tyrosine kinase receptors exhibit exclusive or prevalent expression on epithelial cells. These include c-met, c-ret, c-ros, c-neu (c-erbB2), and the keratinocyte growth factor receptor (KGFR) (Miki et al., 1991; Sonnenberg et al., 1991, 1993; Peles et al., 1992; Schuchardt et al., 1994). The corresponding ligands, scatter factor/ hepatocyte growth factor (SF/HGF), keratinocyte growth factor (KGF), and neuregulin, were found to be preferentially synthesized by mesenchymal cells (Stoker et al., 1987; Sonnenberg et al., 1993; Rubin et al., 1989; Orr-Urtreger et al., 1993, Meyer and Birchmeier, 1995). SF/HGF has been described as a pleiotropic factor with various activities on epithelial cells in culture: (a) it promotes growth of primary hepatocytes and other epithelial cells (Miyazawa et al., 1989; Nakamura et al., 1989; Zarnecki and Michalopoulos, 1989); (b) it can induce motility and invasiveness of epithelial and endothelial cells (Stoker et al., 1987; Weidner et al., 1990; Bussolino et al., 1992;
male sterility due to abnormal development of epididymal matrices (Montesano et al., 1991a,b; Berdichevsky et al., 1994; Brinkmann et al., 1995; Soriano et al., 1995). SF/HGF also controls branching morphogenesis of mouse mammary glands in organ culture and is expressed at appropriate times in the mammary gland during development (Yang et al., 1995). The various signals of SF/HGF are mediated by the c-met tyrosine kinase, as has been shown by the use of a hybrid receptor (Weidner et al., 1993; cf. also Bottaro et al., 1991; Naldini et al., 1991; Komoda and Kitamura, 1993; Zhu et al., 1994).

KGF was characterized as a growth factor for skin epithelial cells and is a member of the FGF family (Rubin et al., 1989), which reacts with the FGFR2-IIb receptor (Miki et al., 1991). The c-neu (c-erbB2) receptor, together with two closely related molecules, c-erbB3 and c-erbB4, mediates the signaling of a recently described factor, neu-regulin (Holmes et al., 1992; Wen et al., 1992; Carraway and Cantley, 1994). Neuregulin (also named neu differentiation factor, heregulin, glial growth factor, or ARIA) is produced by mesenchymal and nerve cells (Orr-Urtreger et al., 1993; Meyer and Birchmeier, 1994) and acts on epithelial, muscle, and glial cells (Falls et al., 1993; Marchionni et al., 1993; Meyer and Birchmeier, 1995). Other epithelial receptor tyrosine kinases exist, to which no ligands were assigned yet, such as c-ros or c-ret (Riethmacher et al., 1994; Schuchardt et al., 1994).

The importance of receptor tyrosine kinases and their ligands in mesenchymal-epithelial interactions has recently been supported by genetic experiments. Targeted muta-
tions in the SF/HGF and the c-met genes in mice lead to an identical phenotype, embryonal lethality caused by severe deficiencies in the development of embryonal liver and placenta (Schmidt et al., 1995; Uehara et al., 1995). Furthermore, SF/HGF and c-met −/− animals do not form particular muscles because of a defect in migration of myogenic precursor cells from the somites to these target sites (Bladt et al., 1995). A dominant-negative KGF receptor under the control of the human lung surfactant protein C promoter induced severe defects in lung development (Petters et al., 1994). This transdominant receptor also interfered with the development of the skin when expressed in keratinocytes in vivo and prevented normal wound healing (Werner et al., 1993, 1994). A targeted mutation in the c-ret gene of the mouse induced severe hypoplasia or aplasia of the kidney and defects in development of the enteric nervous system (Schuchardt et al., 1994). Targeted mutation of the neu-regulin, c-erbB2, and c-erbB4 genes in transgenic mice leads to embryonal death at day 10 due to severe heart defects. In addition, cranial ganglia are absent, and the Schwann cell population in the trunk is greatly reduced (Meyer and Birchmeier, 1995; Lee et al., 1995; Gassmann et al., 1995). Targeted mutation of c-ros leads to male sterility due to abnormal development of epididymal epithelia (Riethmacher-Sonnenberg et al., 1996).

A major question concerns the specific signal transduction pathways involved in growth, morphogenesis, and differentiation of epithelia that are mediated by tyrosine kinase receptors. Considerable evidence has been accumulated that relates to signal transduction by activated (oncogenic) tyrosine kinases. For instance, one of five COOH-terminal tyrosine residues of neu/erbB2 (Y1253) is responsible for transducing the mitogenic signal, a biochemical pathway that includes ras, mitogen-activated protein kinase, and transactivation of c-jun (Ben-Levy et al., 1994). In c-met, Y1354 (Y15), and to a lesser degree Y1347 (Y14), are involved in mitogenic signaling. These sites bind GRB-2, phospholipase Cγ (PLCγ), c-src, and phosphatidylinositol 3 (PI3) kinase and other substrates (Ponzetto et al., 1994; Fixman et al., 1995). A c-ret pathway used in mitogenic signaling has also been characterized, and this activity is changed in germline mutations of MEN2A and MEN2B patients (Santoro et al., 1994; 1995). Ligand-dependent substrate activation of receptor tyrosine kinases with morphogenic signals have also been studied: for instance, the COOH-terminal domain of let-23 (an EGF receptor homologue in Caenorhabditis elegans) could be partitioned into subdomains with different cell type-specific functions (Aroian et al., 1994). In the NGF receptor (trkA), mutation of Y490 (an SHC binding site) and a deletion of a conserved sequence in the juxtamembrane domain abolished NGF-induced neurite outgrowth in PC12 cells (Obermeier et al., 1994; Stephens et al., 1994; Peng et al., 1995). Ligand-induced dissociation and branching morphogenesis of c-met in epithelial cells were affected by mutation of Y1354 (Y15) and Y1363 (Y16) (Weidner et al., 1995). In the PDGFβ receptor, the kinase insert contains the structural requirements for the motility response in endothelial cells (Wenström et al., 1994), which are binding sites for PI3 kinase (Fantl et al., 1992; Kashishian et al., 1992). Neurite outgrowth of PC12 cells could also be induced by the PDGFβ receptor; here, PI3 kinase and PLCγ are involved (Vetter and Bishop, 1995).

In the present investigation, we have analyzed the potential of several epithelial receptor tyrosine kinases to induce motility and morphogenic signals in MDCK epithelial cells. We found that the signal for branching morphogenesis of kidney epithelial cells is specific for c-met, whereas dissociation and increased motility can be transmitted by several tyrosine kinase receptors. A morphogenic signal could be created by fusing the COOH-terminal tail of c-met to the kinase domain of the trkA receptor, indicating that Y14 to Y16 of c-met are critical in mediating a signal that induces morphogenesis.

Materials and Methods

Construction and Expression of the trkA Receptor Hybrids

The trkA-cDNA (encoding the human NGF receptor, plasmid pLM6) was obtained from Dr. L. Parada (National Cancer Institute, Frederick, MD). The cDNAs of the human insulin receptor (IR) as well as the trk-NGF receptor were generated as follows: fragment I encoding the extracellular portion of the human insulin receptor (pINS3, pCMV-ET-Y751F, and pCMV-ET-Y785F) were as described (Obermeier et al., 1993). The cDNA of the murine c-ret tyrosine kinase (plasmid pMR1N), the rat c-neu proto-oncogene (pSVneuN), and the mouse keratinocyte growth factor receptor (plasmid KGF-PCR127) were provided by Dr. C. Birchmeier (MDC, Berlin, Germany), Dr. M.F. Rajewsky (Institute of Cell Biology, Essen, Germany), and Dr. S.A. Aaronson (National Institutes of Health, Bethesda, MD), respectively. The construction of the trk-met hybrid is described in Weidner et al. (1993).
tion of trkA (pLM6) with an XbaI and an NheI site at the 3'-end was digested with XbaI, blunt ended with Mung bean nuclease, and digested with NheI. This fragment was ligated with fragment II encoding the transmembranous and the cytoplasmic portion of c-ros. Fragment II was made in two steps: (a) PCR with the primers 5'-ATAACAGAAGAACATC- TACAATCAATGATTG and 5'-CCTCGAAGAACATCAGTCT and pMRIN as template to anneal an internal EcoRI site at the 5'-end of the transmembranous region of c-ros. (b) This fragment was fused to the COOH-terminal part of c-ros using an internal AvrII site. For in-frame fusion with fragment I, the EcoRI site in fragment II was partially filled with dATP, blunt ended with Mung bean nuclease, and ligated as NheI fragment.

For construction of the trk-neu hybrid, a COOH-terminal fragment of the extracellular part of trkA and the transmembrane and cytoplasmic part of c-neu was amplified with the primers 5'-GGTCCAGGTGCCCAATGTCATACTTACTATCATAGTT and 5'-CCITCAGGAACTCAATCTGCCG were used with the templates pLM6 and KGFR-pCEV27. The resulting fragments were purified, mixed, and used as templates for a second PCR with the primers W3 and SI. The generated chimeric fragment was digested with BamHI and SpeI, purified, and ligated into a modified pUC118 vector with MscI and SpeI sites. Ligation with a NH2-terminal KpnI–BamHI fragment of trkA resulted in the full-length hybrid.

For trkKGFR, the primers W3, 5'-GCCATGAATAAGCTTTCGTTTGCTTCTGTCCAG, 5'-AAGAAGGACGAAATATCATCGGCCCC- CATAGG, and 5'-GTGTTGCTGCTAAGATTAATGTTTAACAAC- TGCCG were used with the templates pLM6 and KGFR-pCEV27. The amplified fusion fragment was digested with BamHI and XbaI and ligated with the NH2-terminal EcoRI–BamHI fragment of trkA. The trkIR hybrid and the trk-Y490F mutant were made using a similar strategy: for the trkIR hybrid, the primers W3, 5'-GGGGCCGATGATAATTGTCGGCTCCATCACCG, and 5'-AATGATGAATGTCTGTCCYFCTCTCCACCG, and 5'-CGGTGGAGAAGAAGGACGAAACATTCATCATT, and 5'-GGTG- GTGGTGACTAGTTCATACAGGTACATCCAG were used. The amplified trkIR fusion fragment was digested with BamHI and SpeI, purified, and ligated into pUC118. The NH2-terminal KpnI–BamHI fragment of trkA was ligated into this plasmid and resulted in the full-length hybrid. For the trk-Y490F mutant, primers W3, 5'-CA- CAGGCACTACGAGAATTTATCGGTCTCAGGG, and 5'-ATCGAGAACCCCAATTCCTCATGATCTAGCCGCCC- CCTACCT, and 5'-GTGTTGCTGCTAAGATTAATGTTTAACAAC- TGCCG were used with pLM6 as template. An internal BamHI–NheI fragment containing the Y490 codon in trkA was replaced by the fragment containing the Y490F mutation. For constructing trkY751F and trk-Y785F, the internal NheI site in the trkA cDNA and the plasmids pCMV-ET751F and pCMV-ET-785F were used to mutate the wild-type codons Y751 and Y785. An NheI site at the 3'-end of the cDNAs of trkA, trk-Y751F, and trk-Y785F was introduced by ligation with an NheI linker.

The NheI fragment of the trkA was then replaced by the corresponding fragments of the mutated trkA hybrids. For constructing the two trkA receptors containing the COOH-terminal tail of c-met, the primers S2, 5'-GGAGAAGATTGAGACACGGGCGGTGACACTC, 5'-TGTG- CACCACCGCCTGCTCCTCCTTCCACAG, and 5'-GGAGACA- CTATCACGATCTGATGCTGTTTCCCTGCCCCCATC with the templates pLM6 and the trk-met hybrid were used. After amplification of the trk-met fusion fragment, the tail of trkA could be replaced by the c-met tail from the corresponding trk-met hybrid.

The correct sequences of the hybrid and mutant receptor cDNAs were confirmed by sequencing using T7 DNA polymerase (Pharmacia, Uppsala, Sweden). The hybrid receptor cDNAs were cloned into the pBAT expression vector (Nagafuchi and Takeichi, 1988) and stably introduced into MDCK epithelial cells by calcium phosphate cotransfection (Graham and van der Eb, 1973) with pSV2neo. G418-resistant cell clones were assayed for NGF-induced cell dissociation, branching morphogenesis, and expression of the various receptors.

Figures

Figure 1. Structure of various hybrid tyrosine kinase receptors. (a) The ectodomain of trkA is fused to the transmembrane and cytoplasmic domains of c-met, c-neu, c-ros, the KGFR, or the IR, respectively. Ellipses, cysteine residues in the ectodomain; boxes, location of the tyrosine kinase domains within the cytoplasmic part. (b) Nucleotide and amino acid sequences of the fusion points between the extracellular and the transmembrane region (underlined, hydrophilic sequence of the trkA ectodomain; bold type, adjacent transmembrane region of the various fused receptors). For construction of the receptors, see Materials and Methods.
Figure 2. Effect of various hybrid receptors on the cellular phenotype of MDCK epithelial cells in culture. The hybrid receptors trk-met, trk-neu (c-erbB2), trk-ros, and trk-KGFR induced NGF-dependent scattering of the cells (middle column), as did SF/HGF (right column). The concentrations of NGF and SF/HGF were 50 ng/ml. Control, untreated cells (left column). Cell morphology was analyzed after 24 h. Bar, 20 μm. For the efficiency of successful transfections, see Table I.

2.5% glutaraldehyde (Sigma, Deisenhofen, Germany), and small blocks containing representative cell aggregates were cut from the gel. After postfixation with OsO₄, the blocks were contrasted with tannic acid and uranyl acetate. The specimens were dehydrated in a graded ethanol series and embedded in Epon 812. Ultrathin sections (50-70 nm) were contrasted with lead citrate and analyzed in an electron microscope (EM 10; Zeiss). Semithin sections (0.5 μm) were stained with toluidine blue and analyzed with a light microscope (Axiophot; Zeiss).
Immunoprecipitation and Western Blotting

For examining tyrosine autophosphorylation of the various tyrosine kinase hybrids, 5 × 10^6 MDCK cells were incubated for 40 min at 37°C in the presence or absence of NGF (50 ng/ml) in DME, 0.5% BSA, 7 μM phenyl arsenate oxide, washed with PBS, and extracted for 1 h at 4°C with RIPA-kinase lysis buffer (Weidner et al., 1993). After clearing by ultracentrifugation at 100,000 g, immunoprecipitation was carried out using protein A-Sepharose (Pharmacia) and a 1:20 dilution of polyclonal antibodies directed against the COOH terminus of mouse c-met, human c-neu, human trkA (Santa Cruz Biotechnology Inc., Santa Cruz, CA), human IR (Transduction Laboratories, Lexington, KY), and mouse c-ros (kindly provided by Dr. C. Birchmeier). After washing with RIPA-kinase lysis buffer and high salt buffer, the immunoprecipitates were separated by 6% SDS-PAGE. The proteins were transferred to nitrocellulose membranes (Amersham Int., Little Chalfont, UK), probed with antiphosphotyrosine mAb PY-20 (Upstate Biotechnology Inc. [UBI], Lake Placid, NY), and stained using the enhanced chemiluminescence system (ECL; Amersham Intl., Little Chalfont, UK). Alternatively, the various receptors were immunoprecipitated with the mAb TTM-9.9.2 directed against the NH2 terminus of human trkA (kindly provided by Dr. M. Barbacid, Bristol-Meyers-Squibb Princeton, NJ).

Results

Construction and Biological Activities of Chimeras between the NGF Receptor (trkA) and Various Epithelial-specific Tyrosine Kinases

We have previously shown that a chimeric receptor containing the extracellular sequences of the NGF receptor and the transmembrane and cytoplasmic sequences of c-met transmits c-met-specific signals in the presence of NGF (Weidner et al., 1993). Here we have constructed a series of hybrids of trkA and the kinase domains of c-neu (c-erbB2), c-ros, KGFR, and IR (Fig. 1 a). In all, the first hydrophobic amino acid of the transmembrane domain of the receptors was fused in frame to the sequences encoding the last (acidic) amino acid of the extracellular sequence of trkA (Fig. 1 b). The hybrid cDNAs were cotransfected with a neomycin resistance gene into MDCK cells; for each cDNA transfection 30-70 independent G418-resistant clones were selected. The hybrids were first examined for NGF-induced cell dissociation (Fig. 2; Table I and II). We found that the chimeric c-met, c-neu, c-ros, and KGFR receptors induced dissociation and scattering of the cells in the presence of NGF (Fig. 2, middle column). 20-40% of the G418-resistant clones were responsive to NGF, which is similar as previously reported with trk-met (Table I; cf. also Weidner et al., 1993). The cells transfected with different chimeric receptors were also reactive to SF/HGF (Fig. 2, right column). Cells transfected with the trk-IR construct were not dissociated by NGF (Table II). However, our trk-IR hybrid was active in other cells (Isakoff et al., 1995). We should also note that neuregulin, the ligand of c-erbB3 and c-erbB4, which are coreceptors of c-neu (c-erbB2) (Karuagaran et al., 1996; Carraway and Cantley, 1994), does not scatter MDCK epithelial cells. Also, acidic FGF that binds to the NGF receptor (Miki et al., 1991) does not dissociate the cells (data not shown).

We have not examined MDCK cells for the expression of these receptors. Surprisingly, the transfected wild-type trkA receptor also dissociated MDCK epithelial cells in the presence of NGF (Fig. 3). This finding allowed us to examine the substrate requirements for dissociation of MDCK cells since trkA with mutated substrate binding sites have been described: Y490, Y751, and Y785 of trkA are binding sites for SHC, PI3 kinase, and PLCγ, respectively (Obermeier et al., 1994). We found that loss of SHC binding of trkA due to mutation of tyrosine 490 to phenylalanine prevents NGF-induced cell dissociation (Fig. 3; and Table II). The cells were still reactive to SF/HGF. Mutations of the other two tyrosine residues of trkA, Y751 and Y785, had no inhibitory effect. Thus, signals mediated by various receptor tyrosine kinases were still reactive to SF/HGF (Fig. 2, Table I and II). We should also note that neuregulin, the ligand of c-erbB3 and c-erbB4, which are coreceptors of c-neu (c-erbB2) (Karuagaran et al., 1996; Carraway and Cantley, 1994), does not scatter MDCK epithelial cells. Also, acidic FGF that binds to the NGF receptor (Miki et al., 1991) does not dissociate the cells (data not shown).

Table II. Biological Effects of Various Hybrid Receptors on MDCK Cells

| Hybrid receptors | Dissociation, motility | Branching morphogenesis |
|------------------|------------------------|------------------------|
| trk-met          | +                      | +                      |
| trk-neu          | +                      | +                      |
| trk-ros          | +                      | +                      |
| trk-KGFR         | +                      | +                      |
| trk-IR           | +                      | +                      |
| trkA             | +                      | +                      |
| trk-Y490F (SHC)  | +                      | +                      |
| trk-Y751F (PI3K) | +                      | +                      |
| trk-Y785F (PLCγ)| +                      | +                      |
| trk-met          | +                      | +                      |
| trk-met Y14F     | +                      | +                      |
| trk-met Y15F     | +                      | +                      |
| trk-met Y16F     | +                      | +                      |
| trk-met Y15F/Y16F| +                      | +                      |
| trk-met Y14F/Y15F| +                      | +                      |
| trk-met Y14-16 of met | +                   | +                      |
| trk-Y490F-Y14-16 of met | +               | +                      |
| trk-Y15F of met  | +                      | +                      |
| trk-Y16F of met  | +                      | +                      |
| trk-Y14/16F of met| +                      | +                      |
| trk-Y14/15F of met| +                      | +                      |

*The downward pointing arrow indicates reduced biological activity (cf. Weidner et al., 1995).
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Figure 3. Effect of various Y → F mutants of trkA on the phenotype of MDCK epithelial cells. The Y751F and Y785F mutants of trkA induced NGF-dependent scattering of the cells in a similar manner as the wild-type trkA receptor. In contrast, the Y490F mutation in the trkA receptor abolished NGF-dependent scattering of the cells. MDCK cells were cultured with NGF (50 ng/ml), SF/HGF (50 ng/ml), or without factor (control). Cell morphology was analyzed after 24 h. Bar, 20 μm. For efficiency of successful transfection, see Table I.

Figure 4. Expression and autophosphorylation levels of the various hybrid tyrosine kinase receptors in the NGF-responding cell clones (Fig. 4). We found that tyrosine phosphorylation of c-met, c-neu, c-ros, and the IR was clearly dependent on the presence of NGF (cf. arrows).
in Fig. 4 a). All hybrid receptors had the expected molecular mass, i.e., trk-met, 145 kD; trk-ros, 151 kD; trk-neu, 165 kD; and trk-IR, 144 kD. Wild-type trkA and its mutants showed identical molecular masses of 140 kD (Fig. 4 b). In general, the clones transfected with one particular hybrid cDNA and selected for NGF-induced scattering expressed similar amounts of receptor molecules and showed similar levels of receptor phosphorylation (cf. c-neu and c-ros in Fig. 4 a). Immunoprecipitations of the hybrid receptors with an mAb against the NH2 terminus of trkA also showed similar amounts of reacting protein (data not shown). Wild-type and mutant trkA receptors were also expressed and phosphorylated at similar levels in various cell clones (Fig. 4 b). We have also examined other receptor chimeras such as trk-ret and trk-ddr (the discoidin domain receptor) (cf. Johnson et al., 1993; Lai and Lemke, 1994; Alves et al., 1995); these receptors were expressed in MDCK cells, but did not display NGF-dependent tyrosine phosphorylation, and were therefore not used for further experiments.

The Specificity of c-met for Branching Morphogenesis

Branching morphogenesis of the various MDCK cell transfecants, which responded to NGF by cell scattering, was examined by culturing the cells in three-dimensional gels of collagen type I (cf. Materials and Methods). In the absence of ligand, the cells formed slowly growing, hollow cysts (Fig. 5, left column). We observed that only the transfected trk-met hybrid, but not trk-ros, trk-neu, and wild-type trkA, induced branching morphogenesis in response to NGF (Fig. 5, middle column; cf. also Table II). The trk-met hybrid also clearly promoted proliferation of the cells in collagen; we have here not quantified this activity (cf. Weidner et al., 1993). Again, all transfected cell clones responded with branching morphogenesis in the presence of SF/HGF, indicating that the signaling capacity for branching morphogenesis was intact (Fig. 5, right column). The trk-KGFR and trk-IR hybrids did also not induce NGF-dependent branching (Table II).

Previous results of our laboratory have indicated that mutations of Y15 (Y1354), Y16 (Y1363), and, to a lesser degree, Y14 (Y1347) of c-met lead to the disturbance of branching morphogenesis of MDCK cells (Weidner et al., 1995; cf. Table II). Here, we attempted to create a trkA receptor with the ability to induce branching morphogenesis; to do this we fused to it the COOH-terminal sequence of c-met encoding Y14-16 (Fig. 6, cf. Materials and Methods). This new receptor chimera (trk-Y14-16 of met) indeed induced scattering and, surprisingly, also morphogenesis of MDCK cells in the presence of NGF (Fig. 7; Table II). Detailed inspection indicated that the tubules induced by the chimeric receptor are identical to the ones formed in the presence of SF/HGF (compare Fig. 7, b and c). Analysis of tissue sections showed that the NGF-induced branches consist of single layers of cells surrounding a central lumen. The cells of the tubules are well polarized, showing basally localized nuclei and apical microvilli (Fig. 7, d and e). This new trk-met receptor did not induce scattering and morphogenesis when Y490 of trkA was mutated (Table II). Mutation of Y15 (Y1354) or Y16 (Y1363) of the tail of c-met in the new trk-met receptor abolished morphogenesis but not scattering. The data thus demonstrate that the novel combination of the trkA tyrosine kinase domain linked with COOH-terminal sequences of c-met is sufficient to induce a morphogenic response when activated by the ligand.

Discussion

Developmental studies have indicated that various receptor tyrosine kinases can mediate different signals in epithelia. Here we have analyzed various epithelial receptor tyrosine kinases for their potential to induce biological responses in kidney epithelial cells, such as the ability to form branching tubules or to induce cell scattering and increased cell motility. Branching morphogenesis is particularly relevant in the development of many epithelial organs, which occurs in kidney, lung, breast, prostate, or salivary gland. Interestingly, we found that the tyrosine kinase c-met, which is the receptor for SF/HGF, induces tubule formation and branching of kidney epithelial cells exclusively. Various other tyrosine kinase receptors such as c-neu,
Figure 5. Effect of various hybrid receptors on NGF-induced branching morphogenesis of MDCK cells in collagen gels. Only the trk-met receptor conferred NGF-dependent branching morphogenesis in a similar manner as activation of the endogenous c-met receptor by SF/HGF. MDCK cell clones were precultured in collagen gels (cf. Materials and Methods), incubated with NGF (400 ng/ml), SF/HGF (50 ng/ml), or without factor (control), and analyzed for branching morphogenesis. Bar, 180 μm.
c-ros, the KGFR, or trkA did not display this activity but were able to dissociate and to scatter epithelial cells. This latter is a surprising result, since scattering was thought to be specific for SF/HGF in these cells. Only one other protein, "scatter factor–like factor" has been shown to dissociate MDCK cells (Bellusci et al., 1994a). Interestingly, the ability to form branching tubules could be newly created when the COOH terminus of c-met (which contains the major substrate binding sites) was fused to the COOH terminus of the kinase domain of trkA. Thus, the COOH-terminal part of c-met mediates the signals required for the formation of branched tubules in kidney cells and may bind novel, as yet unidentified substrates.

We have previously demonstrated that the two functions, scattering and morphogenesis of epithelial cells, can be induced by separate means: the two-kringle variant of SF/HGF, which is a small splice form containing only the NH$_2$-terminal hairpin structure and the first two-kringle

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Figure 6. Structure of the trkA hybrid with the COOH terminus of c-met. The region surrounding the fusion point is enlarged below; it shows the COOH-terminal leucine (L) of the trkA kinase domain, which is fused to the c-met tail (bold type) containing Y14 (Y1347), Y15 (Y1354), and Y16 (Y1363). Y14 (Y1347) of c-met is dashed in the enlargement.

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Figure 7. Effect of trkA with the COOH-terminal tail of c-met on NGF-induced branching morphogenesis of MDCK cells in collagen gels. Branching morphogenesis was induced with NGF (b; 400 ng/ml) and SF/HGF (c; 50 ng/ml); a is the control without factors. Branching structures were sectioned and inspected by light (d) and electron microscopy (e). lu, lumen of branched structures; co, collagen matrix. Bars: (a–c) 184 μm; (d) 33 μm; and (e) 3 μm.
domains, promoted only dissociation of the cells but not the formation of branched tubules or growth (Hartmann et al., 1992; data not shown). Here we were now able to separate these two distinct pathways on the receptor level (Fig. 8). We found that several receptor tyrosine kinases can scatter epithelial cells, and we have previously reported that v-src (expressed as a temperature-sensitive mutant) also leads to scattering of MDCK cells (Behrens et al., 1993). It appears that the action of the substrate SHC is essential for this response: mutation of the SHC binding site Y490 in trkA, but not mutation of the binding sites for PI3 kinase, PLCγ, rasGAP, c-src, and others (Ponzetto et al., 1994). However, these substrates can also bind to other receptor tyrosine kinases that confer only scattering; for instance, trkA has binding sites for SHC, PI3 kinase, and PLCγ (Obermeier et al., 1994); c-neu (c-erbB2) has putative binding sites for rasGAP, PLCγ, syp, GRB-2, and SHC (Segatto et al., 1993; Carraway and Cantley, 1994; Ricci et al., 1995). It is therefore suggested that tubule formation of epithelial cells induced by c-met requires the activation of additional, as yet unknown substrates. The sequence flanking Y16 (Y1363, VAPYPSLL), which affects morphogenesis (Weidner et al., 1995), has in fact an unusual sequence and might represent a new substrate binding site. We have recently begun a search for new substrates that may mediate the morphogenesis response of c-met by using the yeast two-hybrid system (cf. Gustafson et al., 1995; Pelicci et al., 1995) and have identified substrates that bind specifically to c-met (Weidner, K.M., M. Sachs, S. DiCesare, and W. Birchmeier, manuscript in preparation). Alternatively, cell motility and branching morphogenesis might be due to differences in signal intensity and/or signal persistence ("shouting vs whispering") (cf. Dikic et al., 1994; Traverse et al., 1994; Marshall, 1995). For instance, strong signaling might be required for scattering followed by reduced activity during reformation of the epithelial structures. Alternatively, morphogenesis might require stronger signaling than scattering. Our present work has demonstrated, however, that branching morphogenesis by c-met does not require Y1001. This tyrosine residue in the juxtamembrane region of c-met negatively regulates the receptor activity; its mutation leads to constitutive scattering and fibroblastoid morphology of epithelial cells (Weidner et al., 1995). In conclusion, we suggest here that scattering of epithelial cells requires the activation of the substrate SHC, whereas new, as yet unidentified substrates induce branching morphogenesis.

Figure 8. Scheme of the different modules in trkA and c-met required for scattering and branching morphogenesis. In the receptor chimera trk-Y14-16 of met (middle), the ligand-binding region, the juxtamembrane region including the SHC binding site for motility (Y490), plus the kinase domain originate from trkA. The COOH terminus (black bar) containing Y14 (1347), Y15 (Y1354), and Y16 (Y1363) is from c-met and promotes branching morphogenesis.
Interestingly, we could transfer signaling specificity of c-met, i.e., particularly the unique activity of the receptor to induce formation of branching tubules, upon the trkA receptor by transferring COOH-terminal sequences of c-met that are active in substrate binding. Exchanges of sequences in receptor tyrosine kinases were previously reported for the kinase insert domains of PDGFα and β receptors (Arvidsson et al., 1992) and PDGFβ and FGFR receptors (Wenström et al., 1994). It was thus possible to transfer the activity of membrane ruffling upon the new receptors. Our exchange involved distantly related tyrosine kinases and an element (the COOH terminus of c-met) that is structurally entirely different from the corresponding element in trkA. This new finding is again a strong indication of a modular arrangement of the elements in tyrosine kinases, i.e., the kinase domain, on the one hand, and various substrate binding regions with multiple functions on the other.

The recent gene ablation experiments of SF/HGF and c-met in mice have revealed an important role of this signaling system in epithelial development (Schmidt et al., 1995; Uehara et al., 1995). Embryonic liver and placenta are affected in these mutants. A function of SF/HGF in the development of epithelia has also been demonstrated by organ culture experiments with kidney and mammary gland explants (Woolf et al., 1995; Yang et al., 1995). We have recently also shown that SF/HGF, besides controlling branching of kidney, breast, and prostate epithelia cells, induces further characteristic epithelial organoids such as crypts and alveoli (Brinkmann et al., 1995). Considering the relevance of morphogenesis of epithelia for the development of many eukaryotic organs, the identification of specific substrates that control these processes in vivo will therefore be an important task in the future.

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References

Alves, F., W. Vogel, K. Mosiss, B. Millura, H. Hoffer, and A. Ulrich. 1995. Distinct structural characteristics of discoidin I subfamily receptor tyrosine kinases and complementary expression in human cancer. Oncogene. 10(30-31):1339-1348.

Arao, R.V., G.M. Lea, and P.W. Sternberg. 1994. Mutations in the Caenorhabditis elegans let-23 EGF-like gene define elements important for cell-type specificity and function. EMBO J. 13:636-646.

Arvidsson, A.K., C.H. Heldin, and L. Claesson-Welsh. 1992. Transduction of circular membrane ruffling by the platelet-derived growth factor beta receptor is dependent on its kinase insert. Cell Growth & Differ. 3:881-888.

Bellucci, S., B. Gaozos, G. Marighi, M. Vigni, H. Waitches, and B. Tainsky. 1995. Essential role for the c-met receptor in the migration of myogenic precursor cells into the limb bud. Nature (Lond.). 376:768-771.

Bellucci, S., G. Mosis, G. Gaozos, P. Comoglio, T. Nakamura, J.-P. Thiery, and J. Jouanneau. 1994a. Creation of an activated human ros gene. Mol. Cell. Biol. 6:3109-3116.

Blasi, F., F. Strehl, F. Wittinghofer, and F. Sampieri. 1995. Essential role for the c-met receptor in the migration of myogenic precursor cells into the limb bud. Nature (Lond.). 376:768-771.

Blinski, V., D. Immanuel, J. Wu, N. Li, V. Vajnik, and B. Margolis. 1994. A region in Shh distinct from the SH2 domain can bind tyrosine-phosphorylated growth factor receptors. J. Biol. Chem. 269:23011-23015.

Bottaro, D.P., J.S. Rubin, D.L. Faletto, A.M. Chan, T.E. Kmiecik, G.F. Vande Woude, and S.A. Aaronson. 1991. Identification of the hepatocyte growth factor receptor as the c-met proto-oncogene product. Science (Wash. DC). 251:802-804.

Brinkmann, V., H. Forouzan, M. Sachs, K.M. Weidner, and W. Birchmeier. 1995. Hepatocyte growth factor/scatter factor induces a variety of tissue-specific morphogenic programs in epithelial cells. J. Cell Biol. 131:1573-1586.

Bussolino, F., M.F. Di Renzo, M. Ziche, E. Bocchietto, M. Olivero, L. Naldini, G. Gaudio, L. Tamagnone, A. Coffier, and P.M. Comoglio. 1992. Hepatocyte growth factor is a potent angiogenic factor which stimulates endothelial cell motility and growth. J. Cell Biol. 119:629-641.

Carraway, K.L., III, and L.C. Cantley. 1994. A new acquaintance for erbB3 and erbB4: a role for receptor heterodimerization in growth signaling. Cell. 78:5-8.

Cheng, H.J., M. Nakamoto, A.D. Bergemann, and J.G. Flanagan. 1995. Complementary gradients in expression and binding of eif-1 and mid6 in development of the topographic striate projection map, Cell 81:231-241.

Cooper, C.S., M. Park, D.G. Blair, M.A. Tsaknis, K. Huebner, C.M. Croce, and G.F. Vande Woude. 1984. Molecular cloning of a new transforming gene from a chemically transformed human cell line. Nature (Lond.). 313:29-33.

Dike, J., J. Schlessinger, and A.D. Forman. 1984. Phorbol ester-inducing the insulin receptor undergo insulin-dependent neuronal differentiation. Curr. Biol. 4:702-708.

Drechsler, U., C. Kremoser, C. Handwerker, J. Loschinger, M. Noda, and F. Bonhoeffer. 1995. In vitro guidance of retinal ganglion cell axons by RAGS, a 25 kDa tectal protein related to ligands for eph receptor tyrosine kinases. Cell. 82:359-370.

Egan, S.E., B.W. Giddings, M.W. Brooks, L. Buday, A.M. Szeizland, and R.A. Weinberg. 1993. Association of Ras Sos with GRB2 is implicated in tyrosine kinase signal transduction and transformation. Nature (Lond.). 363:45-51.

Falk, D.L., K.M. Rosen, G. Corfas, W.S. Lane, and G.D. Fischbach. 1993. ARIP, a protein that stimulates acetylcholine receptor synthesis, is a member of the new ligand family. Cell. 72:801-815.

Farb, W.J., J.A. Escobedo, G.A. Martina, C.W. Turk, M. del Rosario, F. McCormick, and L.T. Williams. 1992. Distinct phosphorylators on a growth factor receptor bind to specific molecules that mediate different signaling pathways. Cell. 69:413-423.

Fimm, S.A., M.A. Naukosis, G.A. Rodrigues, M.F. Moran, and P. Park. 1995. Efficient cell transformation by the Tpr-Met oncoprotein is dependent upon the ret transducer 489 in the carboxy-terminus. Oncogene. 10:237-249.

Gassmann, M., F. Casagranda, D. Orioli, H. Simon, C. Lai, R. Klein, and G. Lemcke. 1993. Aberrant neural and cardiac development in mice lacking the ErbB4 neuregulin receptor. Nature (Lond.). 378:390-394.

Graham, E.L., and A.J. van der Eb. 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology. 52:456-467.

Grant, D.S., H.K. Kleinman, J.D. Goldberg, M.M. Bhargava, B.J. Nickoloff, J.L. Kinsella, P. Polverini, and E.M. Rosen. 1993. Scatter factor induces human lens cell proliferation in vivo. Proc. Natl. Acad. Sci. USA. 90:1937-1941.

Gustafson, T.A., W.M. He, A. Krasapoto, C.D. Schaub, and T.J. Oncel. 1995. Phosphotyrosine-dependent interaction of shc and insulin receptor substrate 1 with the NPFF1 motif of the insulin receptor via a novel non-SH2 domain. Mol. Cell. Biol. 15:2500-2508.

Hartmann, G., L. Naldini, K.M. Weidner, M. Sachs, E. Vigna, P.M. Comoglio, and W. Birchmeier. 1992. A functional domain in the heavy chain of scatter factor/hepatocyte growth factor binds the c-met receptor and induces cell fusion but does not mitogenesis. Proc. Natl. Acad. Sci. USA. 89:11574-11578.

Hartmann, G.K.M. Weidner, H. Schwarz, and W. Birchmeier. 1994. The motility signal of scatter factor/hepatocyte growth factor mediated through the receptor tyrosine kinase met requires intracellular action of Ras. J. Biol. Chem. 269:21936-21939.

Holmes, W.E., M.S. Slivskowski, R.W. Akita, W.J. Henzel, J. Lee, J.W. Park, D. Yamasu, N. Abadi, H. Raab, G.D. Lewis et al. 1992. Identification of hepatitis C virus core protein as a specific activator of p38 MAP kinase. Science (Wash. DC). 256:1205-1210.

Isakoff, S.I., Y.Y. Su, Y.-C. Su, P. Blakie, V. Vajnik, E. Rose, K.M. Weidner, M. Sachs, B. Margolis, and E.Y. Skolnik. 1996. Interaction between the phosphorytory kinase binding domain of the insulin and the receptor is required for SF6 phosphorylation by insulin in vivo. J. Biol. Chem. 271:3955-3962.

Johnson, J.D., C.J. Edman, and W.J. Rutter. 1993. A receptor tyrosine kinase found in breast carcinoma cells has an extracellular discoidin I-like domain. Proc. Natl. Acad. Sci. USA. 90:5677-5681.

Karanagias, D., E. Tzahor, R.R. Beehl, M. Chen, D. Graupstra, B.J. Ratz-
The Journal of Cell Biology, Volume 133, 1996

Ponzetto, C., A. Bardelli, Z. Zhen, F. Maina, P. Dallazonca, S. Giordano, A. Pelicci, G., S. Giordano, Z. Zhen, A.E. Salcini, L. Lanfrancone, A. Bardelli, G. Peters, K., S. Werner, X. Liao, S. Weft, J. Whitsett, and L. Williams. 1994. Tar Peles, E., S.S. Bacus, R.A. Koski, H.S. Lu, D. Wen, S.G. Ogden, R. Ben-Levy, Naldini, L., K.M. Weidner, E. Vigna, G. Gaudino, A. Bardelli, C. Ponzetto, Montesano, R., G. Schaller, and L. Orci. 1991b. Induction of epithelial tubular morphogenesis and epithelial differentiation of the mouse lung. J. Cell Biol. 110:251-337.

Obermeier, A., R.A. Bradshaw, K. Seedorf, H. Paterson, C.J. Marshall, P. Cohen, and A. Ullrich. 1993. Identification of Trk binding sites for SCF and PLCγ1 to mediate NGF responses. Mol. Cell. Biol. 13:3400-443.

Nakamura, T., T. Nishizawa, M. Hagiya, T. Seki, M. Shimoshima, A. Sugimura, K. Tashiro, and S. Shimosi. 1989. Molecular cloning and expression of human hepatocyte growth factor. Nature (Lond.) 342:440-443.

Naidini, L., K.M. Weidner, E. Vigna, G. Gaudino, A. Bardelli, C. Porozetto, R.P. Narsimhan, G. Hartmann, R. Zarnegar, G.K. Michalopoulos et al. 1991. Scatter factor and hepatocyte growth factor are indistinguishable ligands for the MET receptor. EMBO (Eur. Mol. Biol. Organ.) 7:379-386.

Nakamura, T., T. Nishizawa, M. Hagiya, T. Seki, M. Shimoshima, A. Sugimura, K. Tashiro, and S. Shimosi. 1989. Molecular cloning and expression of human hepatocyte growth factor. Nature (Lond.) 342:440-443.

Naito, H., A. Kikkawa, H. Kishimoto, S. Uehara, Y., O. Minowa, C. Moil, K. Shiota, J. Kuno, T. Noda, and N. Kitamura. 1991. Expression eDNA cloning of the KGF receptor by creation of a trans-acting mutant. Genes & Dev. 5:699-702.

Paszek, M., and I. Levenstein. 1991. Expression of the proto-oncogene c-erbB2 in normal and malignant human breast tissue. Cancer Res. 51:3083-3088.

Paszek, M., and I. Levenstein. 1991. Expression of the proto-oncogene c-erbB2 in normal and malignant human breast tissue. Cancer Res. 51:3083-3088.

Paszek, M., and I. Levenstein. 1991. Expression of the proto-oncogene c-erbB2 in normal and malignant human breast tissue. Cancer Res. 51:3083-3088.

Paszek, M., and I. Levenstein. 1991. Expression of the proto-oncogene c-erbB2 in normal and malignant human breast tissue. Cancer Res. 51:3083-3088.

Paszek, M., and I. Levenstein. 1991. Expression of the proto-oncogene c-erbB2 in normal and malignant human breast tissue. Cancer Res. 51:3083-3088.

Paszek, M., and I. Levenstein. 1991. Expression of the proto-oncogene c-erbB2 in normal and malignant human breast tissue. Cancer Res. 51:3083-3088.

Paszek, M., and I. Levenstein. 1991. Expression of the proto-oncogene c-erbB2 in normal and malignant human breast tissue. Cancer Res. 51:3083-3088.

Paszek, M., and I. Levenstein. 1991. Expression of the proto-oncogene c-erbB2 in normal and malignant human breast tissue. Cancer Res. 51:3083-3088.

Paszek, M., and I. Levenstein. 1991. Expression of the proto-oncogene c-erbB2 in normal and malignant human breast tissue. Cancer Res. 51:3083-3088.

Paszek, M., and I. Levenstein. 1991. Expression of the proto-oncogene c-erbB2 in normal and malignant human breast tissue. Cancer Res. 51:3083-3088.

Paszek, M., and I. Levenstein. 1991. Expression of the proto-oncogene c-erbB2 in normal and malignant human breast tissue. Cancer Res. 51:3083-3088.

Paszek, M., and I. Levenstein. 1991. Expression of the proto-oncogene c-erbB2 in normal and malignant human breast tissue. Cancer Res. 51:3083-3088.

Paszek, M., and I. Levenstein. 1991. Expression of the proto-oncogene c-erbB2 in normal and malignant human breast tissue. Cancer Res. 51:3083-3088.

Paszek, M., and I. Levenstein. 1991. Expression of the proto-oncogene c-erbB2 in normal and malignant human breast tissue. Cancer Res. 51:3083-3088.

Paszek, M., and I. Levenstein. 1991. Expression of the proto-oncogene c-erbB2 in normal and malignant human breast tissue. Cancer Res. 51:3083-3088.

Paszek, M., and I. Levenstein. 1991. Expression of the proto-oncogene c-erbB2 in normal and malignant human breast tissue. Cancer Res. 51:3083-3088.

Paszek, M., and I. Levenstein. 1991. Expression of the proto-oncogene c-erbB2 in normal and malignant human breast tissue. Cancer Res. 51:3083-3088.

Paszek, M., and I. Levenstein. 1991. Expression of the proto-oncogene c-erbB2 in normal and malignant human breast tissue. Cancer Res. 51:3083-3088.
Weidner, K.M., M. Sachs, and W. Birchmeier. 1993. The Met receptor tyrosine kinase transduces motility, proliferation, and morphogenic signals of scatter factor/hepatocyte growth factor in epithelial cells. *J. Cell Biol.* 111:2097-2108.

Weidner, K.M., M. Sachs, D. Rietheimacher, and W. Birchmeier. 1995. Mutation of juxtamembrane tyrosine residue 1001 suppresses loss-of-function mutations of the met receptor in epithelial cells. *Proc. Natl. Acad. Sci. USA.* 92: 2597-2601.

Wen, D., E. Peles, R. Cupples, S.V. Suggs, S.S. Bacus, Y. Luo, G. Trail, S. Hu, S.M. Silbiger, R. Ben-Levy et al. 1992. Neu differentiation factor: a transmembrane glycoprotein containing an EGF domain and an immunoglobulin homology unit. *Cell.* 69:559-572.

Wennström, S., A. Siegbahn, K. Yokote, A.K. Arvidsson, C.H. Heldin, S. Mori, and L. Claesson-Welsh. 1994. Membrane ruffling and chemotaxis transduced by the PDGF beta-receptor require the binding site for phosphatidylinositol 3’ kinase. *Oncogene.* 9:651-660.

Werner, S., W. Weinberg, X. Liao, K.G. Peters, M. Blessing, S.H. Yuspa, R.L. Weiner, and L.T. Williams. 1993. Targeted expression of a dominant-negative FGF receptor mutant in the epidermis of transgenic mice reveals a role of FGF in keratinocyte organization and differentiation. *EMBO (Eur. Mol. Biol. Organ.)* J. 12:2635-2643.

Zarnegar, R., and G. Michalopoulos. 1989. Purification and biological characterization of human hepatopoietin A, a polypeptide growth factor for hepatocytes. *Cancer Res.* 49:3314-3320.

Zhou, M.-M., K.S. Ravichandran, E.T. Olejniczak, A.M. Petsos, R.P. Meadows, M. Sattler, J.E. Harlan, W.S. Wade, S.J. Burakoff, and S.W. Festk. 1995. Structure and ligand recognition of the phosphotyrosine binding domain of Shc. *Nature (Lond.)* 378:584-592.

Zhu, H., M.A. Naujokas, and M. Park. 1994. Receptor chimeras indicate that the met tyrosine kinase mediates the motility and morphogenic responses of hepatocyte growth/scatter factor. *Cell Growth & Differ.* 5:359-366.