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MEMBRANE DYNAMICS DURING SCATTER FACTOR/HEPATOCYTE GROWTH FACTOR-STIMULATED EPITHELIAL MORPHOGENESIS

by

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This thesis is dedicated to my father, Herbert Murray Pollack, whose memory I treasure and whose vigor for life I carry within me

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MEMBRANE DYNAMICS DURING SCATTER FACTOR/HEPATOCYTE GROWTH FACTOR-STIMULATED EPITHELIAL MORPHOGENESIS

ANNE LESLIE POLLACK

ABSTRACT

Epithelial morphogenesis is a process that is important in both organ development and tissue repair. Epithelial/mesenchymal interactions regulate the specific alterations in cell adhesion, migration, division, and polarization that are necessary for morphogenesis of complex epithelial structures. In this thesis I examine epithelial cell-cell adhesion and cell polarity during scatter factor/hepatocyte growth factor (SF/HGF)-induced tubulogenesis and morphogenesis of pseudostratified layers to gain insight into the role of these parameters in epithelial morphogenesis. Models of tubulogenesis have predicted that loss of cell-cell adhesion and transient cell-cell dissociation are steps in the formation of tubules. I specifically address the question of whether cell-cell adhesion is retained during SF/HGF-induced morphogenesis. The results of both morphological analysis and functional tests of paracellular transport indicate that apical and basolateral membrane subdomains are significantly altered but cells remain in contact during morphogenesis. The data suggests that SF/HGF modulates desmosomal and adherens junctional adhesion but tight junction functional integrity is retained during both tubulogenesis and formation of pseudostratified layers. I conclude from these results that SF/HGF can differentially regulate different adhesive mechanisms and that maintenance of cell-cell contacts is important for the organization of complex epithelial structures.

SF/HGF has been shown to have multiple effects on epithelial cells, inducing mitogenesis, morphogenesis, motogenesis, and invasiveness. The response to SF/HGF

depends on both cell type and environmental context. Direct activation of the SF/HGF receptor, the c-met protooncogene (c-met), has been shown to be the mechanism by which all of these effects are transduced. I demonstrate that SF/HGF induces an increase in transepithelial resistance that is not stimulated by direct activation of c-met. These data demonstrate that plasma membrane subdomains rearrange while tight junctions remain functionally intact during SF/HGF-induced epithelial morphogenesis and provide the first functional evidence for a novel SF/HGF signalling mechanism.

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ABBREVIATIONS

AJ, adherens junction

CEA, carcinoembryonic antigen

c-met, c-met protooncogene

DS, desmosome

dpI/II, desmoplakins I and II

HGF, hepatocyte growth factor

HSPG, heparan sulfate proteoglycan

MDCK, Madin Darby canine kidney

NGF, nerve growth factor

pIgR, polymeric immunoglobulin receptor

ppI, propidium iodide

rhHGF, recombinant human hepatocyte growth factor

SF, scatter factor

SF/HGF, scatter factor/hepatocyte growth factor

TER, transepithelial resistance

TJ, tight junction

trk/met receptor, nerve growth factor receptor/c-met protooncogene chimeric receptor

INTRODUCTION

INTRODUCTION

Many organ systems in the body, including lung, mammary gland, salivary gland, pancreas and kidney, are composed of organized networks of epithelial tubes. The function of the epithelial cells within these tubes is to maintain localized transport and barrier systems that are critical for sustaining environmental differences between the inside of an organism and the outside world. To accomplish this function epithelial cell membranes are polarized, containing distinct apical and basolateral epithelial cell membrane domains that are separated by tight junctions (Simons and Fuller, 1985; Gumbiner, 1987; Rodriguez-Boulan and Nelson, 1989). Basolateral membrane domains face the interior of the body and contain cell-cell and cell-substrate adhesion systems and receptors for growth factors and hormones that regulate responses to internal cues. An important issue in biology is how epithelial cell polarity and adhesion contribute to the development of epithelial organs. The question of how distinct epithelial membrane domains are established and/or altered during the development of complex tubular systems sparked our interest in studying membrane polarity and cell-cell adhesion during epithelial morphogenesis.

Formation of epithelial tubules is dependent on multiple cell processes, including mitogenesis, migration into extracellular matrix, cell-cell and cell-substrate adhesion, differentiation, and polarization. Balancing the level of activity of each of these processes at each stage of development is crucial for formation of correct tissue architecture. This is accomplished through highly localized expression and action of many factors, including growth factors; growth factor activators, inhibitors and receptors; cell-cell and cell-extracellular matrix adhesion molecules; and matrix-degrading proteases and inhibitors. These components regulate, for example, the development of nephrons in the kidney, stimulating the sequential stages of mesenchymal induction, proliferation, condensation of cell aggregates, epithelialization, and subsequent tubule formation (Grobstein, 1956;

Ekblom, 1984; Saxen et al., 1986; Saxen, 1987a). Individual components can regulate different steps of tubulogenesis. For example, adhesion of cells to the extracellular matrix molecule laminin (Klein et al., 1988), possibly through $\alpha 6\beta 1$ integrins (Sorokin et al., 1990), is important for development of polarity during the epithelialization stage of kidney tubulogenesis.

Epithelial-mesenchymal interactions are important in the development of many parenchymal tissues (Saxen et al., 1976a; Sawyer and Fallon, 1983; Bernfield et al., 1984). Scatter factor/hepatocyte growth factor (SF/HGF) is a soluble mesenchymally-derived growth factor that has been suggested to act as a paracrine inducer of morphogenesis of both kidney and mammary epithelial tubules (Montesano et al., 1991a; Montesano et al., 1991b; Sonnenberg et al., 1993a; Sonnenberg et al., 1993b; Santos et al., 1994; Soriano et al., 1995; Woolf et al., 1995). An in vitro cell culture system to study SF/HGF-induced tubulogenesis was developed by Montesano, et al. (Montesano et al., 1991a; Montesano et al., 1991b). In this culture system, single cell suspensions of Madin Darby canine kidney (MDCK) epithelial cells are grown into polarized cysts in type I collagen gels and respond to SF/HGF by forming tubules. This morphogenetic response to SF/HGF was surprising in light of earlier results that had shown that MDCK cells scatter in response to SF/HGF (Stoker et al., 1987b). However, since scattering of MDCK cells was stimulated when cells were grown under different culture conditions (plated at low density on plastic), the tubulogenesis assay demonstrated that this single growth factor could have multiple effects and that the specific response was dependent on the environmental milieu.

Several studies have demonstrated that MDCK cell cysts grown in collagen gels are polarized, with basolateral membrane facing the collagen substrate and tight junctions sealing a fluid-filled central lumen (Wang et al., 1990; Montesano et al., 1991a; Santos et al., 1993a). The responsiveness of polarized MDCK cell cysts to SF/HGF suggested that the receptor for SF/HGF, the c-met protooncogene (c-met), was localized on the basolateral

cell surface, where it would have the opportunity to interact with its ligand. In vivo, basolateral membranes of polarized epithelial cells are in contact with basement membrane where they are available to serum components. Studies of liver injury in vivo have shown that liver damage or disease induces an increase in serum levels of SF/HGF (Gohda et al., 1988; Kinoshita et al., 1991). In addition, heparin-binding properties of SF/HGF have suggested that this growth factor can bind extracellular matrix molecules and may be stored within the basement membrane (Masumoto and Yamamoto, 1993). However, studies of mammary carcinoma cells and embryonic gut suggested that c-met is localized on apical plasma membranes (Tsarfaty et al., 1992). It is possible that the embryonic and carcinoma cells used in the study by Tsarfaty et al. were not fully polarized. We hypothesized that in polarized epithelial cells c-met would be found preferentially at the basolateral plasma membrane. MDCK cells grown on Transwell filters provide an excellent model system for studying epithelial cell polarity. In chapter 1 of this thesis I present the results of biochemical and morphological analysis of the targeting of newly synthesized c-met to the cell surface of polarized MDCK epithelial cells. Steady state cell surface distribution of c-met was also determined. In addition, the polarity of c-met in sections from human colon is shown and c-met localization in other tissues is discussed.

Morphological detection and antibody perturbation of constituents of basement membrane and cell adhesion in organ explant culture systems have provided some insight into the function of these molecules during kidney development (reviewed in Ekblom et al., 1986; Ekblom, 1989). In addition, insight into the regulation of tubulogenesis by extracellular matrix molecules, matrix degrading enzymes, growth factors, and modulators of intracellular signalling has been obtained using collagen gel cultures of renal cell lines (Montesano et al., 1991b; Santos et al., 1993a; Santos and Nigam, 1993b; Cantley et al., 1994). However, a detailed understanding of the role of cell-cell adhesion and membrane polarity during tubulogenesis is lacking. One model for tubulogenesis, based on the ability of SF/HGF to induce both scattering and morphogenesis, suggests that scattering activity

of SF/HGF is integral to the formation of tubules. This "cell dissociation" model proposes that polarized epithelial cells initially lose both cell polarity and cell-cell adhesion, migrate as individual cells, and subsequently reassociate into tubules (Thiery and Boyer, 1992). An alternative model, the "fixed cortex hypothesis", was first suggested to explain epithelial migration after epithelial to mesenchymal transformation, and could apply to tubulogenesis. In this model epithelial cells retain some cell polarity and adhesive contacts as they initiate migration by synthesizing new cortical and plasma membrane elements and inserting them preferentially at the leading edge (Bilozur and Hay, 1989; Hay, 1989). A third hypothesis derives from the observation that, in transitional stages of tubulogenesis, cells appear morphologically to have extensive cell-cell contacts, suggesting that highly localized regulation of cell-cell adhesion and polarity is important for "adherent migration" of epithelial cells into new and highly organized structures. In chapter 2 of this thesis I present confocal and electron microscopic analysis of epithelial morphogenesis to assess the role of membrane polarity and cell-cell adhesion during tubulogenesis.

All presently known pleiotrophic effects of SF/HGF, including stimulation of cell motility, invasiveness, proliferation and morphogenesis of tubules have been demonstrated to be mediated through direct activation of c-met (Weidner et al., 1993b). One hypothesis to explain the ability of SF/HGF to induce multiple effects is that SF/HGF activates multiple regions of the c-met cytoplasmic tail which in turn stimulates multiple second messenger signalling pathways (Longati et al., 1994; Ponzetto et al., 1994; Weidner et al., 1995). Mutational analysis has shown that specific regions of the cytoplasmic tail of c-met are important for transducing signals for different responses, ie. scattering versus morphogenesis (Weidner et al., 1995). However, an issue that has never been thoroughly explained or examined is how activation of different regions of c-met is regulated. Are there cofactors/receptors that modify responses (of c-met) to SF/HGF?

Naturally occurring variants of both c-met and SF/HGF have been identified, which are generated by alternative splicing or posttranslational proteolysis (Chan et al., 1991; Prat

et al., 1991a; Rodrigues et al., 1991; Weidner et al., 1993a; Lee and Yamada, 1995). Expression of multiple receptor isoforms or availability of particular variants in different cell types may explain why, for example, SF/HGF stimulates mitogenesis of hepatocytes but not MDCK cells when these cells are cultured on plastic. However, the fact that SF/HGF does stimulate mitogenesis of MDCK cells when these cells are cultured in collagen gels suggests that both cell type and environmental context affect the response of cells to SF/HGF.

An alternative hypothesis to explain the multiple actions of SF/HGF is that direct activation of c-met is not sufficient to induce all responses to SF/HGF. Multiple components may regulate SF/HGF activity and may be more or less available under different culture conditions or in different cell types. For example, urokinase (uPA), an enzyme that has been shown in vivo and in vitro to cleave proHGF to its mature active form (Naldini et al., 1992; Naldini et al., 1995), has been shown to associate with SF/HGF and c-met at cell surfaces (Naldini et al., 1995). Alternatively, responses to SF/HGF may be affected by availability of specific extracellular matrix components and receptors, matrix degrading enzymes, cell-cell adhesion molecules, and cell surface molecules such as heparan sulfate proteoglycans (HSPG). Low affinity SF/HGF binding sites at the cell surface have been identified. Analysis of binding to HSPGs or sulfoglycolipids found endogenously on kidney and liver, and skin cell surfaces has identified structural components of sulfated oligosaccharides and lipids that are important for SF/HGF interactions. In the course of studying morphogenetic alterations that are induced by SF/HGF (described in chapter 2), I discovered a unique effect of SF/HGF on the transepithelial resistance of polarized MDCK cell monolayers. The potential that this effect is transduced by a novel SF/HGF signalling mechanism that may involve interaction with low affinity cell surface binding sites is explored in chapter 3.

CHAPTER ONE

TARGETING OF THE SF/HGF RECEPTOR TO THE BASOLATERAL DOMAIN OF POLARIZED EPITHELIAL CELLS

Chapter One

The work presented in this chapter was completed and published in collaboration with Tiziana Crepaldi, Maria Prat and Anna Zborek, members of Paolo Comoglio's laboratory at the University of Torino in Torino, Italy. Tiziana Crepaldi and I shared first authorship of the paper. The text of this chapter is reproduced from **The Journal of Cell Biology, 1994, 125(2): 313-320** (Crepaldi et al., 1994) by copyright permission of The Rockefeller University Press. The experiments to detect steady state localization and intracellular targeting of the SF/HGF receptor using cell surface biotinylation were designed and carried out by me. Solubility properties of the SF/HGF receptor were tested in both laboratories. I contributed the specific comparison of the solubility of the polymeric immunoglobulin receptor to that of E-cadherin and c-met.

ABSTRACT

Scatter Factor, also known as Hepatocyte Growth Factor (SF/HGF), has pleiotropic functions including direct control of cell-cell and cell-substrate adhesion in epithelia. The subcellular localization of the SF/HGF receptor is controversial. In this work, the cell surface distribution of the SF/HGF receptor was studied in vivo in epithelial tissues and in vitro in polarized MDCK monolayers. A panel of monoclonal antibodies against the β chain of the SF/HGF receptor stained the basolateral but not the apical surface of epithelia lining the lumen of human organs. Radiolabeled or fluorescent-tagged anti-receptor antibodies selectively bound the basolateral cell surface of MDCK cells, which form a polarized monolayer sealed by intercellular junctions, when grown on polycarbonate filters in a two-chamber culture system. The receptor was concentrated around the cell-cell

contact zone, showing a distribution pattern overlapping with that of the cell adhesion molecule E-cadherin. The basolateral localization of the SF/HGF receptor was confirmed by immunoprecipitation after domain selective cell surface biotinylation. When cells were fully polarized the SF/HGF receptor became resistant to non-ionic detergents, indicating interaction with insoluble component(s). In pulse-chase labeling and surface biotinylation experiments, the newly synthesized receptor was found exclusively at the basolateral surface. We conclude that the SF/HGF receptor is selectively exposed at the basolateral plasma membrane domain of polarized epithelial cells and is targeted after synthesis to that surface by direct delivery from the trans-Golgi network.

INTRODUCTION

Scatter Factor (SF) and Hepatocyte Growth Factor (HGF) are identical glycoproteins controlling motility, mitogenesis, and morphogenesis in epithelial cells (Naldini et al., 1991b; Weidner et al., 1991). SF/HGF was originally described as a secretory product of fibroblasts which dissociates epithelial cells, increasing their motility and invasiveness (Stoker et al., 1987b; Weidner et al., 1990). The factor was reported to have chemotactic properties (Gherardi et al., 1989) and to promote the progression of carcinoma cells toward malignant invasive phenotypes (Weidner et al., 1990); the factor regulates the level of ECM degradation by enhancing the synthesis of enzymes involved in ECM proteolysis (Pepper et al., 1992). SF/HGF is a powerful mitogen for hepatocytes in primary cultures (Miyazawa et al., 1989; Nakamura et al., 1989; Zarnegar and Michalopoulos, 1989). It also stimulates the growth of other epithelial tissues, such as kidney tubular epithelium and keratinocytes (Kan et al., 1991), melanocytes (Rubin et al., 1991), and endothelial cells (Bussolino et al., 1992; Grant et al., 1993). SF/HGF is a mediator of kidney and liver regeneration (Michalopoulos, 1990; Higuchi and Nakamura, 1991; Nagaike et al., 1991). It acts as a morphogen in the chick embryo (Stern et al., 1990) and induces the three-dimensional organization of MDCK epithelial cells in vitro (Montesano et al., 1991a) and blood vessels in vivo (Bussolino et al., 1992; Grant et al., 1993). These features indicate that SF/HGF plays a major role in the differentiation and morphogenetic events which lead to the formation of branching tubules.

The receptor for SF/HGF is the tyrosine kinase encoded by the *MET* proto-oncogene (Bottaro et al., 1991; Naldini et al., 1991b; Naldini et al., 1991a). The receptor is a 190-kD heterodimer of a 50-kD α subunit, covalently linked to a 145-kD β subunit (Giordano et al., 1989b). The α subunit is extracellular; the β subunit bears an extracellular portion involved in ligand binding, a membrane-spanning segment, and a cytoplasmic tyrosine kinase domain (Tempest et al., 1986; Gonzatti-Haces et al., 1988).

Both subunits originate from glycosylation and proteolytic cleavage of a common precursor of 170 kD (Giordano et al., 1989a). The SF/HGF receptor is widely expressed in epithelial tissues lining the lumen of several organs including intestine and kidney (Di Renzo et al., 1991; Prat et al., 1991b). The receptor is expressed in the early stages during development of epithelial organs, while the ligand is expressed in the surrounding mesenchyme (Sonnenberg et al., 1993a; Sonnenberg et al., 1993b). SF/HGF and its tyrosine kinase receptor are thought to control mesenchymal/epithelial interactions during development. The mechanisms by which epithelial cells receive and transduce the signal have been analyzed in detail (Bardelli et al., 1992; Graziani et al., 1993; Ponzetto et al., 1993). However, modifications in cell-cell and cell-substrate interactions, leading to dissociation, migration, and remodeling of epithelial monolayers, have remained poorly understood.

The subcellular localization of growth factor receptors expressed by epithelial structures, particularly in polarized layers or tubules, is largely unknown. In a recent report the SF/HGF receptor was found to be associated with the apical microvilli of cells bordering mammary ducts and the intestinal lumen (Tsarfaty et al., 1992). This is somewhat unexpected, because the SF/HGF is present in the circulatory system (Nakamura et al., 1986; Zarnegar and Michalopoulos, 1989) and is stored in the pericellular matrix, mainly as a biologically inactive single-chain precursor (Masumoto and Yamamoto, 1991; Naldini et al., 1992). In this work, the subcellular distribution of SF/HGF receptor was studied in vivo in epithelial tissues stained by a panel of monoclonal antibodies, and in vitro, in polarized MDCK cell monolayers, by domain selective biotinylation and pulse-chase experiments. The receptor was found to be localized at the basolateral cell surface. Newly synthesized SF/HGF receptor reaches this plasma membrane domain by targeting and direct delivery from the trans-Golgi network.

MATERIALS AND METHODS

Cells and Antibodies

MDCK type II cells were grown in DMEM supplemented with 5 % FCS. Cells grown on Transwells (Costar, Cambridge, MA) were seeded at 4.2 x 10^5 cells/cm^2. Cell monolayers were used for experiments on the fourth day of culture. Polarization of the monolayer was assessed by measuring electrical resistance with the Millicell-ERS instrument (Millipore Continental Water Systems, Bedford, MA). In some experiments cells were grown as small colonies, at a final density of 4.2 x 10^3 cells/cm^2. The murine mAbs, D0-24 and DN-30, directed against the extracellular domain of the SF/HGF receptor, were obtained after immunization with living cells from the human gastric carcinoma cell line GTL-16 (Prat et al., 1991a). mAb DQ-13, raised against a peptide corresponding to nineteen COOH- terminal amino acids (from Ser^{1372} to Ser^{1390}) of the human MET sequence, was also used. Other mAbs and their source were: DECMA-1 to canine E-cadherin (Sigma Chem. Co., St. Louis, MO); 4B4 to the integrin β1 chain (Coulter Immunology, Hialeah, FL). Antiserum against fimbrin was obtained from K. Weber, Max-Planck-Institut fur Biophysikalische Chemie, Goningen, Germany. Antisera against carcinoembryonic antigen (CEA) and rhodamine-labeled secondary antibodies were purchased from Dakopatts (Glostrup, Denmark).

Immunohistochemical Staining

Normal human tissues were obtained from surgical samples. The tissues were mounted in OCT 4583 embedding compound (Miles Scientific. Naperville, IL) and immediately frozen in liquid nitrogen. Cryostat sections (6-μm thick) cut in a Reichert cryomicrotome were transferred onto microscope slides coated with poly-L-lysine (Sigma Chem. Co.), air-dried, and stored at room temperature overnight. The samples were fixed in a chloroform acetone mixture (1:1), air-dried, and incubated for 10 min in PBS

supplemented with 1% serum of the same species as the secondary antibody. Sections were overlaid with 50 µl of undiluted supernatant or ascitic fluid at 1:400 dilution and incubated at room temperature for 30 min in a moist chamber. After a thorough wash in PBS, the sections were incubated with biotinylated secondary antibodies and processed for the ABC method (avidin-biotin-peroxidase complex) using the Vectastain ABC kit (Vector Labs. Inc., Burlingame, CA). After several washes, 100 µl of substrate were added for 5 min, prepared as follows: 5 mg 3-amino 9-ethylcarbazole (Sigma Chem. Co.) were dissolved in 1 ml N,N-dimethylformamide (Merck, Darmstadt, Germany) supplemented with 9 ml 100 mM sodium acetate pH 5.2 and 100 µl of 12% H_2O_2. All samples were counterstained with Mayer's haemalum solution, mounted in Kaiser's glycerol gelatin (Merck) and examined with a Zeiss Axiophot photomicroscope equipped with planapochromatic lenses.

For indirect immunofluorescence microscopy, MDCK cells grown on 6.5-mm Transwells were fixed on ice for 5 min with freshly prepared 3% paraformaldehyde, 2% sucrose and incubated at 37°C for 30 min with antibodies. An appropriate dilution of mAbs was added to either the apical or the basolateral compartment of the Transwell unit. Filters were then washed with four changes of PBS-0.2 % BSA and incubated with rhodamine-labeled secondary antibodies at 37°C for 30 min. After four washings with PBS-0.2% BSA, filters were cut, mounted on glass slides in a solution of Mowial (Calbiochem-Behring Corp., La Jolla, CA) and viewed with Zeiss epifluorescence optics.

Binding Assays

MDCK cells grown on 6.5-mm Transwells were starved for 30 min in serum-free DMEM and cooled on ice, and 2 µg/ml ^{125}I-labeled D0-24 or DN-30 mAbs (10×10^6 cpm/µg, in PBS) was added to the apical or basolateral compartment of the Transwell unit. Binding was carried out for 3 h on ice. Intactness of monolayers was determined by counting the media from both compartments in a gamma counter; generally, less than 1% of

the initially added label diffused to the opposite side. Filters were washed with five changes of cold PBS containing 1 mM $CaCl_2$, and 0.5 mM $MgCl_2$ and cut. Bound radioactivity was then counted. Assays were performed in duplicate. Nonspecific binding, determined in the presence of a 100-fold excess of cold mAb, was subtracted and represented 15-20% of the total.

Domain-Selective Cell Surface Biotinylation and Immunoprecipitation

MDCK cells grown on 24.5-mm Transwells were washed three times at 4°C with Hank's Balanced Salts Biotinylation Buffer, pH 7.4 (HBB) consisting of 1.3 mM $CaCl_2$, 0.4 mM $MgSO_4$, 5 mM KCl, 138 mM NaCl, 5.6 mM D-glucose and 25 mM Hepes, pH 7.4, and then cooled for 10 min in HBB at 4°C. Sulfosuccinimidobiotin (sulfo-NHS-biotin Pierce Chem. Co., Rockford, IL) was made 0.5 mg/ml in HBB and applied to either the apical (0.5 ml) or basolateral (1 ml) side of the monolayer with HBB on the opposite surface. After 15 min, the biotin solution was removed and replaced with fresh biotin solution for another 15 min. The reaction was stopped by removing the biotin solution, washing four times quickly and incubating 10 min with Minimal Essential Media containing Hank's Balanced salts, 0.6% BSA, 20 mM Hepes, pH 7.3 (MEM/BSA) at 4°C. Filters were cut from the support with a scalpel and cells were lysed by placing the filter into 0.8 ml of RIPA lysis buffer (20 mM Tris-HCl pH 7.4. 150 mM NaCl, 0.1% SDS, 1% TX-100, 1% DOC, 5 mM EDTA), containing inhibitors of proteases (2 mM PMSF [Sigma], 50 µg/ml pepstatin, 50 µg/ml chymostatin and 10 µg ml antipain [Chemicon, CA]) for 15 min on ice. In some experiments cells on filters were lysed in DIM buffer (5 mM Pipes, pH 7.6, 100 mM NaCl, 5 mM $MgCl_2$, 300 mM sucrose, 5 mM EGTA) containing 1% Triton X-100 and the protease inhibitors described above. Cell extracts were precleared with protein-A Sepharose and rotated 2.5 h at 4°C with anti-SF/HGF receptor mAbs (DN-30 or D0-24). Immunocomplexes were collected with affinity purified rabbit anti-mouse IgG (Jackson Immuno Research Labs., Inc., West Grove, PA) coupled to Protein-A

Sepharose Beads which were washed three times with lysis buffer. Samples were eluted by boiling in Laemmli buffer containing 100 mM dithiothreitol. Immunoprecipitates were electrophoresed on 8% SDS-PAGE. Samples were transferred to nitrocellulose, probed with streptavidin-HRP, and visualized on Kodak XOMAT AR film (Rochester, NY) with an enhanced Chemiluminescence kit (ECL, Amersham, UK). Quantification of biotinylated protein was achieved with a laser scanning Personal Densitometer (Molecular Dynamics, Sunnyvale, CA) using multiple exposures. Care was taken to scan exposures within the linear range of the film.

Analysis of Protein Targeting to the Cell Surface

MDCK cells grown on 24.5-mm Transwells were starved for 15 min at 37°C in MEM lacking cysteine and methionine and supplemented with 5% dialyzed FCS, then pulse labeled for 15 min in the same medium supplemented with [^{35}S] Translabel (100 µCi/filter, New England Nuclear, Boston, MA). Chases were performed in MEM/BSA at 37°C. Cells were then biotinylated and immunoprecipitated as described above. Immunoprecipitated proteins were eluted from Protein-A beads by boiling 5 min in 10% SDS, diluted in 1 ml of 2.5% Triton Dilution buffer (2.5% Triton X-100, 100 mM NaCl, 5 mM EDTA, 100U/ml Trasylol, 0.02% NaN3, 100 mM triethanolamine-HCl, pH 8.6), and samples were precleared twice with Sepharose CL-2B. Biotinylated proteins were precipitated by rotating overnight with Streptavidin-agarose (Sigma). Beads were washed three times with mixed micelle buffer and one time with final wash buffer before analysis of samples by electrophoresis and either autoradiography or phosphorimaging. A Molecular Dynamics Phosphorimager was used to quantify newly synthesized proteins on the apical or basolateral cell surface.

RESULTS

Immunolocalization of the SF/HGF Receptor in Human Tissues

To analyze the subcellular localization of the SF/HGF receptor, immunoperoxidase staining was performed on cryostat sections of human tissues. A panel of monoclonal antibodies (mAbs) directed against the SF/HGF receptor was used: two mAbs recognized different epitopes on the extracellular domain (D0-24 and DN-30) and one mAb reacted against the COOH-terminal tail (DQ-13). In the human colon, the whole panel of anti-SF/HGF receptor mAbs selectively stained the basolateral plasma membranes of epithelial cells: staining with D0-24 mAb is depicted in Fig. 1.1 a. The pattern of reactivity perfectly matches that obtained with the antibody against the β_1 integrin, known to be selectively expressed at basolateral cell sides (Fig. 1.1 b). The SF/HGF receptor was absent from the apical membrane, where fimbrin, a structural marker of the apical brush border, was detected as a positive control (Fig. 1.1 c). Immunohistochemical analysis on other tubular epithelia, including those lining distinct segments of the gastrointestinal tract, bile ducts, respiratory tract, mammary gland ducts and kidney tubules, confirmed that the receptor is exposed at the basolateral, but not apical rims (not shown; cfr. (Prat et al., 1991b)).

Immunolocalization of the SF/HGF Receptor in MDCK Cells Polarized In Vitro

MDCK cells form epithelial polarized monolayers when grown in culture under appropriate conditions. The apical cell surface is covered with microvilli and faces the culture medium. The basal cell surface adheres to the substratum; the lateral surface is in close contact with adjacent cells in the monolayer. The basolateral surface domain is separated from the apical domain by a junctional complex including tight junctions (Rodriguez-Boulan and Sabatini, 1978; Simons and Fuller, 1985). Monolayers grown on polycarbonate permeable filters (Transwell chambers) expose separately the apical and the basal surfaces to experimental probes (Lisanti et al., 1988). MDCK cells grown on

Transwells were fixed without permeabilization and stained with DN-30 or D0-24, two of the above anti-receptor mAbs that are cross-species reactive. Immunofluorescence staining occurred only when antibodies were presented to the basolateral side of MDCK cells (Fig. 1.2, compare a with b). The antibodies stained the basolateral surface at regions of cell-cell contact, with a pattern indistinguishable from the staining with anti-E-cadherin mAb (Fig. 1.2, compare a with c). The cell adhesion molecule E-cadherin is known to be selectively localized at the basolateral cell surface domain (Behrens et al., 1985; Le Bivic et al., 1990). In the same experiment, the CEA, analyzed as positive control, was both apical and basolateral, with preferential localization at the apical cell surface covered with microvilli (Fig. 1.2, compare e with f).

To quantify the expression of the SF/HGF receptor to the different plasma membrane domains, the binding of ^{125}I-labeled monoclonal antibodies was evaluated following presentation of the mAb either to the apical or to the basolateral surfaces of MDCK cells, by addition to the upper or to the lower Transwell chamber. As shown in Fig. 1.3, more than 90% binding occurred at the basolateral surface. A radiolabeled monoclonal antibody, directed against the intracellular COOH-terminal tail of the SF/HGF receptor, used as a negative control, did not bind either of the cell surface domains (data not shown).

Domain Selective Surface Biotinylation of the SF/HGF Receptor

The presence of SF/HGF receptors on the basal but not apical surface of MDCK cells was confirmed by domain selective surface biotinylation and immunoprecipitation. MDCK cells grown on Transwell filters were biotinylated with sulfo NHS-biotin added either to the apical or to the basolateral surfaces, as described in Materials and Methods. The cells were then lysed, the SF/HGF receptor immunoprecipitated, separated by SDS-PAGE, and transferred to nitrocellulose. The extent of biotinylation was assessed by probing nitrocellulose blots with HRP-conjugated streptavidin and densitometric analysis.

More than 95 % of the SF/HGF receptor labeled by the biotin was located on the basolateral membrane (Fig. 1.4, *left panels).* Under identical conditions, ~95 % of the cell adhesion molecule E-cadherin, studied as a control, was labeled from the basolateral side (Fig. 1.4, *right panel).* The additional band of 70 kD associated with immunocomplexes formed by the anti-SF/HGF receptor and anti-E-cadherin antibodies is a non-specific contaminant, likely to be albumin from FCS. Biotin labeled the β (150 kD) and the α (55 kD) chains of the heterodimeric SF/HGF receptor, showing that both are exposed at the basolateral cell surface. The low level of biotinylation of the α chain likely reflects the relative abundance of lysine residues in the α and β chains. The α and β chains of canine SF/HGF receptor displayed a slightly higher molecular weight than the α and β chains of the human receptor (Giordano et al., 1989b). These differences are likely due to species differences in protein glycosylation.

Solubility Properties of SF/HGF Receptor Exposed at the Cell Surface of Polarized or Non-Polarized MDCK Cells

MDCK cells grown on Transwell filters, under conditions of polarization, were biotinylated, and lysed with buffers containing either ionic (SDS) or non-ionic (Triton X-100) detergents. The recovery of surface-labeled receptor was quantified by immunoprecipitation and Western blot with HRP-conjugated streptavidin. The SF/HGF receptor was relatively insoluble in non ionic-detergent (Fig. 1.5, *left panel*). Similar results were obtained when the solubility of E-cadherin was studied (Fig. 1.5, *left panel*). It is known that the relative insolubility in non-ionic detergent of E-cadherin is due to formation of non-covalent complexes with components of the cytoskeleton (Hirano et al., 1987; Nelson et al., 1990; Tsukita et al., 1992). In a parallel experiment the polymeric immunoglobulin receptor, expressed in polarized MDCK cells, was slightly better solubilized in Triton X-100 than SDS (not shown). We then dealt with the question of whether polarization could influence detergent solubility properties of the SF/HGF

receptor. Steady-state biotinylation followed by immunoprecipitation and probing with HRP-streptavidin was performed on MDCK cells grown in small colonies (non-polarized). Fig. 1.5 *(right panels)* shows that, unlike polarized cells, a significant fraction of the receptor exposed at the surface of non-polarized cells was soluble in Triton X-100.

Pulse-Chase Analysis of SF/HGF Receptor Biosynthetic Delivery

The cell-surface delivery of newly synthesized SF/HGF receptor in polarized MDCK cells was examined by the widely used approach of combining pulse-chase and domain-selective biotinylation (Le Bivic et al., 1990). MDCK cells grown on Transwell filters were pulse-labeled with [^{35}S] Translabel for 15 min and biotinylated after various periods of chase on either the apical or the basolateral surface. The SF/HGF receptor was immunoprecipitated, solubilized, reprecipitated with streptavidin-agarose, and revealed by fluorography after SDS-PAGE (Fig. 1.6). The amount of SF/HGF receptor on the apical or basolateral surface was quantified by phosphorimaging. The SF/HGF receptor appeared at the plasma membrane surface after 20 min of chase as a mature heterodimer composed of a 150-kD (β) and a 55-kD (α) chains. More than 90% of the newly synthesized receptor exposed at the cell surface was directly delivered to the basolateral membrane domain.

DISCUSSION

In this work we show that the SF/HGF receptor is selectively localized at the basolateral surface of the polarized epithelia in vivo as well as in experimental conditions in vitro. This conclusion is supported by three independent types of evidence: (1) immunohistochemical staining pattern obtained with different monoclonal antibodies on frozen sections; (2) binding of the specific antibodies to the basolateral side of MDCK cells polarized in vitro; and (3) selective biotinylation of proteins exposed at the basolateral surface. A similar basolateral distribution of the EGF receptor has been previously demonstrated by measuring the binding of radiolabeled ligand to MDCK cells polarized in Transwell chambers (Maratos-Flier et al., 1987). Previous work has reported the association of the SF/HGF receptor with the apical microvilli of cells bordering mammary ducts and intestinal lumen (Tsarfaty et al., 1992). The basolateral localization of the SF/HGF receptor is consistent with the site of action of the ligand, which is present in the circulatory system (Nakamura et al., 1986; Zarnegar and Michalopoulos, 1989) and is stored in the pericellular matrix (Masumoto and Yamamoto, 1991; Naldini et al., 1992).

Interestingly, the staining of SF/HGF receptor is intense at the lateral surfaces of the cell in the region of cell-cell contact, and displays a pattern similar to that elicited by antibodies against the cell adhesion molecule E-cadherin (Behrens et al., 1985; Vestweber and Kemler, 1985; Gumbiner et al., 1988). A junctional localization of the SF/HGF tyrosine kinase receptor is potentially significant. A role of tyrosine kinases in the control of junction formation is suggested for tyrosine kinases of the c-Src family: elevated levels of tyrosine phosphorylation induced by the *c-Src* family kinases have been found in isolated adherens junctions (Tsukita et al., 1991; Volberg et al., 1992). Transformation with *v-Src* causes loss of epithelial differentiation and gain of invasiveness, which correlate with tyrosine phosphorylation of the E-cadherin/β-catenin complex (Behrens et al., 1993). SF/HGF, which disrupts cell-cell adhesion, does not appear to influence the steady-state

synthesis, the down-modulation and the phosphorylation of E-cadherins (Weidner et al., 1990), while it causes rapid internalization of desmosomal proteins (Bhargava et al., 1991). It has been recently claimed that β-catenin is tyrosine-phosphorylated in response to cell stimulation with SF/HGF (Shibamoto et al., 1994). Whether the SF/HGF receptor phosphorylates β-catenin directly or through other associated tyrosine kinases has yet to be investigated. In this context, it is worth mentioning that activated SF/HGF receptor associates with the c-Src tyrosine kinase (Ponzetto et al., 1994).

The SF/HGF receptor is highly insoluble in non-ionic detergents when cells are fully polarized. The detergent insolubility of the receptor correlates with that of E-cadherin, known to form complexes with insoluble cytoskeletal components upon extensive cell-cell contact (Hirano et al., 1987; McNeill et al., 1990; Nelson et al., 1990; Ozawa et al., 1990; Wollner et al., 1992). On the contrary, the polymeric immunoglobulin receptor, known not to associate with cytoskeleton, is even better solubilized in Triton X-100 (not shown). Polarization in epithelial cells is regulated through different mechanisms, which include selective retention and vectorial delivery at one surface. The first mechanism is mediated by interaction with other proteins, such as the cytoskeleton, extracellular matrix, or adhesion proteins on adjacent cells (for review see (Nelson et al., 1992)). A number of basolateral proteins form complexes with detergent insoluble cytoskeletal proteins (Nelson and Veshnock, 1987; McNeill et al., 1990; Wollner et al., 1992). In the case of the Na+/K+ ATPase the basolateral cell surface distribution is determined by stabilization and accumulation on the basolateral membrane (Hammerton et al., 1991). In the case of E-cadherin, both mechanisms are operative (Wollner et al., 1992). The data reported in this paper suggest that the basolateral distribution of the SF/HGF receptor might be mediated by stabilization through association with detergent insoluble intracellular components. However, the hypothesis that the detergent insolubility of the receptor results from its localization in caveolae, where other cellular kinases have been recently found (Sargiacomo et al., 1993), has to be taken into consideration.

Vectorial delivery of proteins to either the apical or the basolateral cell surfaces is another mechanism operating for maintaining polarization in epithelial cells (for review see (Mostov et al., 1992)). This paper shows that the newly synthesized SF/HGF receptor is directly targeted to the basolateral surface from the trans-Golgi network. MDCK cells, that have already established a polarized distribution of membrane proteins, sort newly synthesized proteins in the Golgi complex and deliver them either to the apical or to the basolateral plasma membrane upon recognition of specific sorting signals (Hopkins, 1991). Computer analysis of the SF/HGF receptor amino acid sequence failed to identify any homologies to the signals for basolateral sorting that have been found in the LDL receptor (Matter et al., 1992) and the polymeric IgA receptor (Casanova et al., 1991). Cytoplasmic domains involved in selective sorting have also been found in the transferrin and immunoglobulin FcRII receptors (Hunziker et al., 1991). We should thus assume that the SF/HGF receptor contains a yet unidentified sorting signal for basolateral targeting. This will be unveiled by mutagenesis studies.

Figure 1.1. Localization of SF/HGF receptor in human colon epithelium. Cryostat sections were fixed in cold absolute acetone and stained by immunoperoxidase with (a) mAb D0-24 directed against the extracellular domain of the SF/HGF receptor; (b) mAb 4B4 recognizing β1 integrin; (c) antiserum against fimbrin. The SF/HGF receptor was localized to the basolateral plasma membrane of epithelial cells, with a pattern of staining indistinguishable from that observed for the β_1 integrin. The receptor was absent from the apical side, where fimbrin, a structural marker of the apical brush border, was located. Bar represents 3 μm.

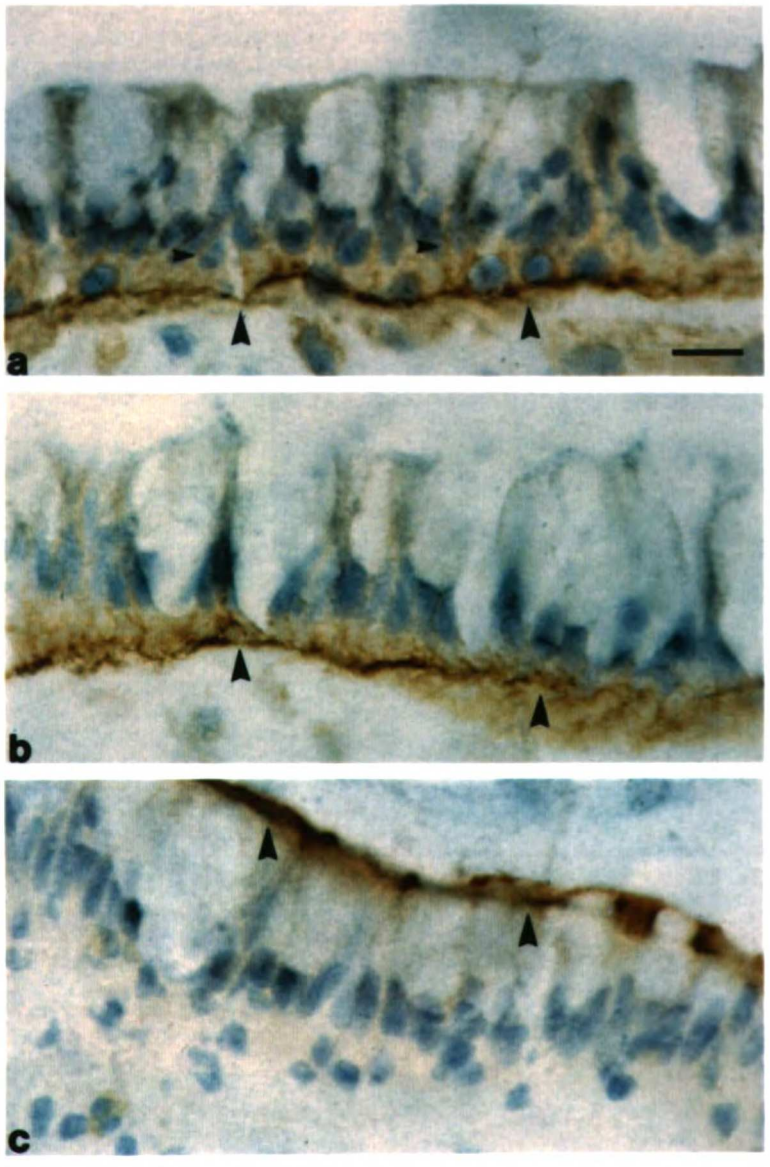

a
b
c

Figure 1.2. Localization of SF/ HGF receptor in polarized MDCK cells. MDCK confluent cells were grown on Transwell filters and fixed with 3% paraformaldehyde. Immunofluorescence was performed with anti-SF/HGF receptor rnAb *(a* and *b),* anti-E-cadherin rnAb (*c* and *d),* and anti-CEA antiserum *(e* and *f).* Antibodies were presented to either the basolateral *(a, c,* and *e)* or the apical *(b, d,* and *f)* surface of MDCK cells. The SF/HGF receptor is exclusively basolateral and shows a pattern of staining overlapping that of E-cadherin. The CEA antigen is expressed also on the apical surface covered with microvilli. Bar represents 5 μm.

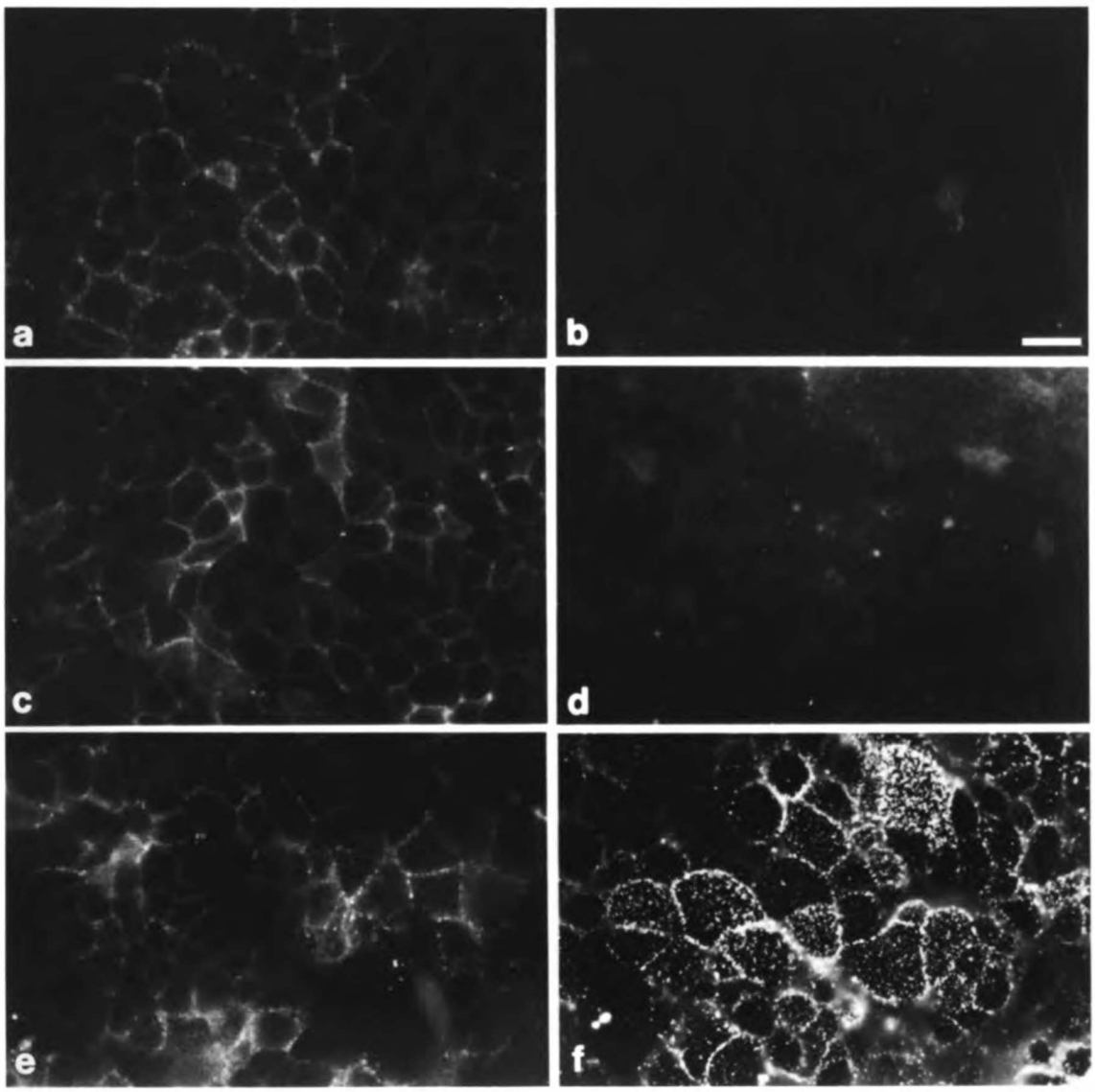

a
b
c
d
e
f

Figure 1.3. Cell surface distribution of SF/HGF receptor in MDCK cells. The ^{125}I-labeled D0-24 and DN-30 mAbs were presented to either the upper or the lower surface of MDCK cells grown on Transwells. Given are mean values ± SD of three independent experiments performed in duplicate. Total values were between 8,000 and 15,000 cpm. More than 90% binding occurred at the basolateral surface.

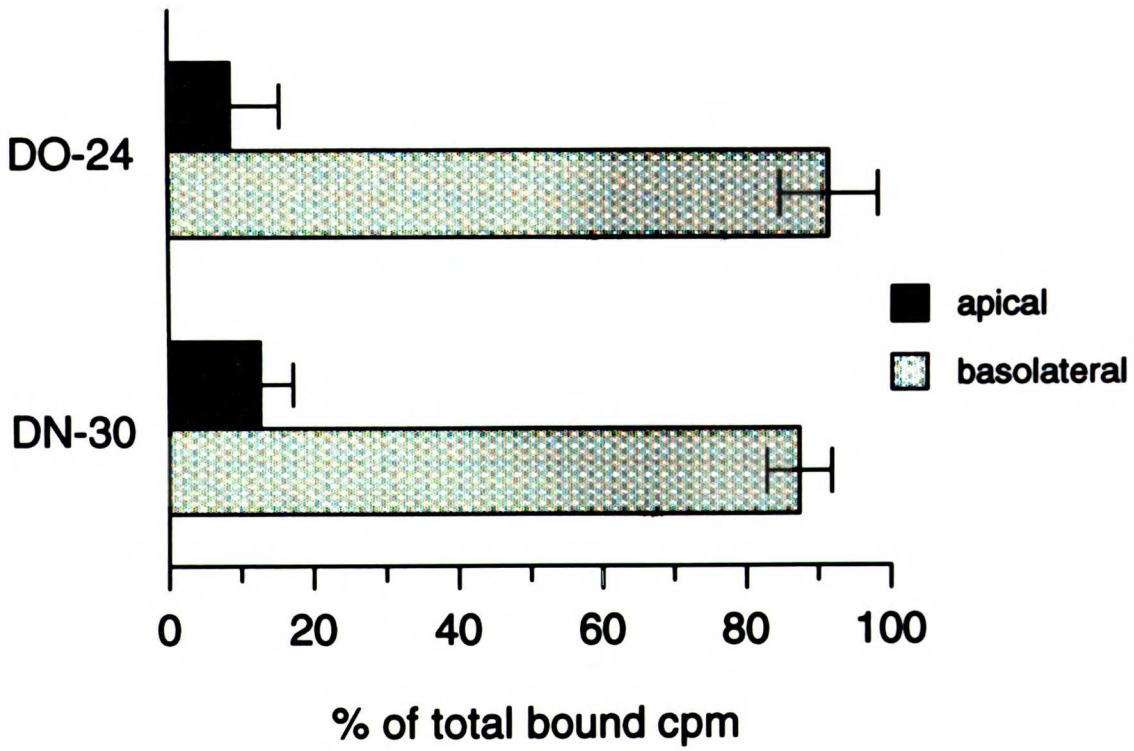

DO-24
DN-30
apical
basolateral
0
20
40
60
80
100
% of total bound cpm

Figure 1.4. Steady state distribution of the SF/HGF receptor on the plasma membrane of MDCK cells. MDCK cells, grown on Transwell filters, were biotinylated on either their apical *(ap)* or basolateral *(bl)* surface. Biotinylated SF/HGF receptor and E-cadherin were immunoprecipitated with D0-24 and DECMA-I mAbs, respectively. Samples were run on SDS-PAGE under reducing *(R)* and non-reducing *(NR)* conditions, transferred to nitrocellulose, and probed with streptavidin-HRP. Apical or basolateral proteins were visualized by ECL. The SF/HGF receptor in MDCK cells is exposed exclusively at the basolateral surface, as a 190-kD heterodimer of a SS-kD α subunit, covalently linked to a 150-kD β subunit.

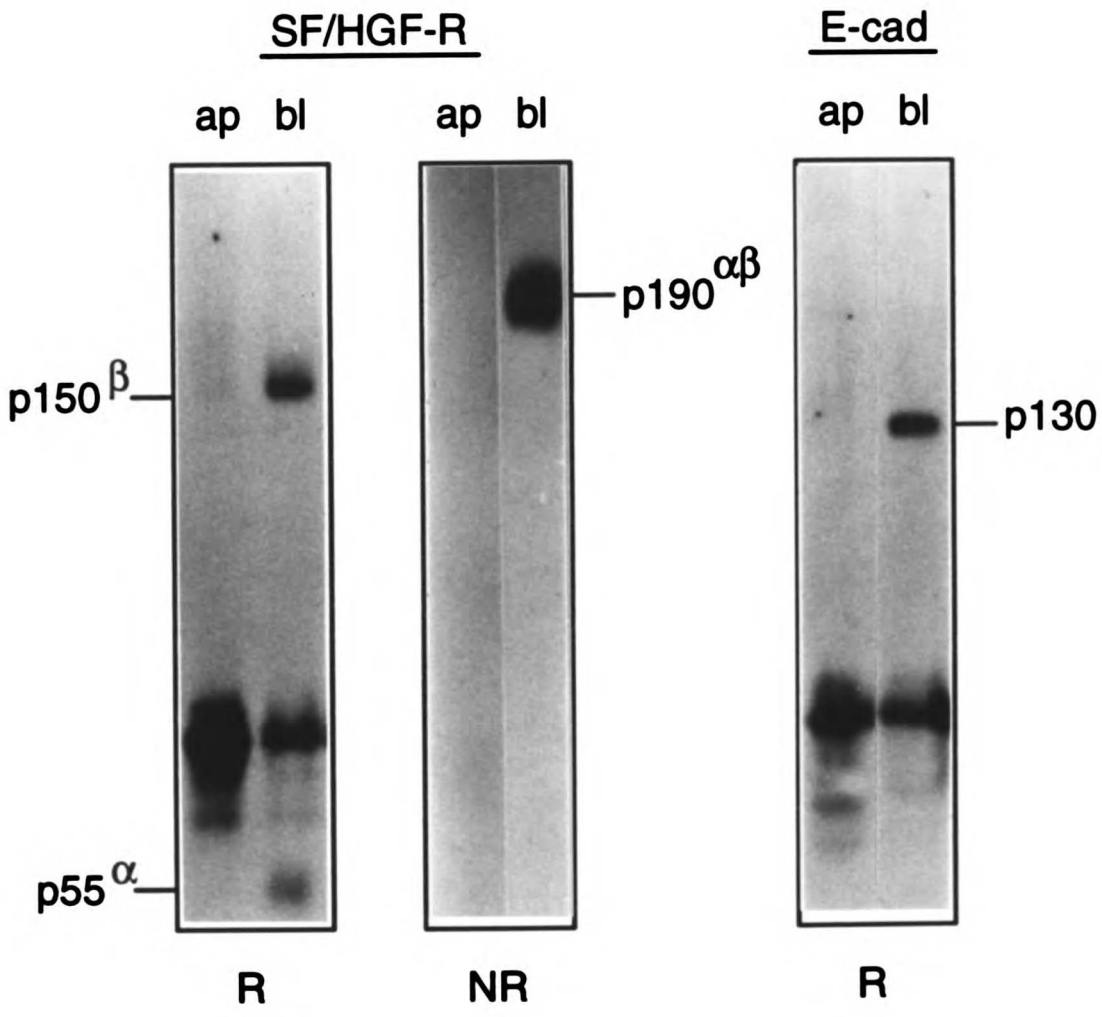

SF/HGF-R
E-cad
ap
bl
ap
bl
ap
bl
p150β
p55α
p190αβ
p130
R
NR
R

Figure 1.5. Solubility properties of the SF/HGF receptor in polarized and non-polarized MDCK cells. MDCK cells were grown on Transwell filters at final cell density of 4.2 x 10^5 cells/cm^2 (polarized) and 4.2 x 10^3 cells/cm^2 (non-polarized). Biotinylated SF/HGF receptor and E-cadherin were immunoprecipitated from cells extracted with buffers containing SDS (S) or Triton X-100 (T), as detergents. Reduced samples were SDS-gel electrophoresed, transferred to nitrocellulose, and blotted with streptavidin-HRP. If cells are polarized, more than 95% of the receptor exposed at the cell surface is insoluble in non-ionic detergent.

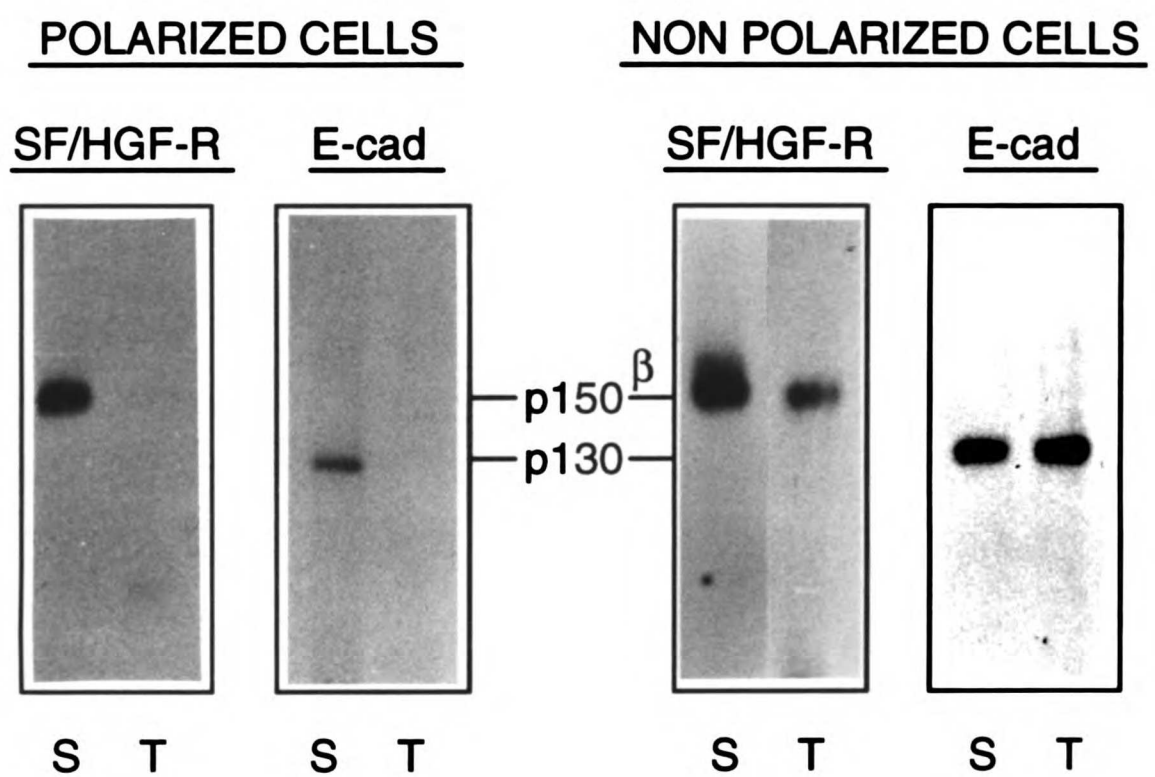
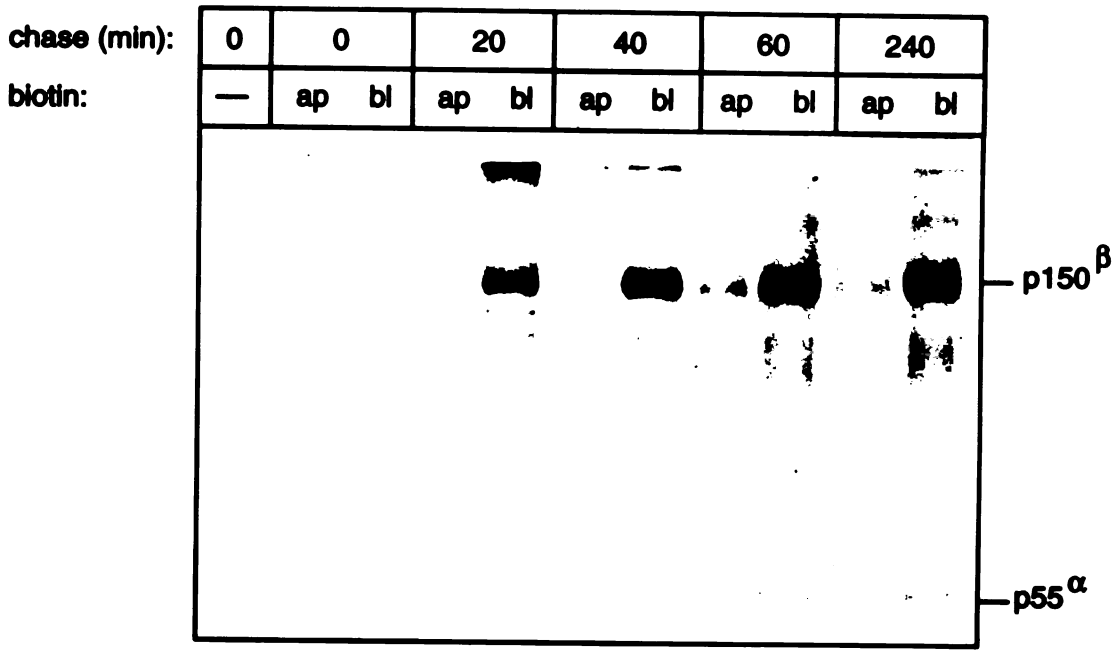

POLARIZED CELLS
NON POLARIZED CELLS
SF/HGF-R
E-cad
SF/HGF-R
E-cad
p150β
p130
S T
S T
S T
S T

Figure 1.6. Targeting of the SF/HGF receptor to the basolateral plasma membrane of MDCK cells. MDCK cells on Transwell filters were metabolically pulse labeled with [^{35}S]-Translabel for 15 min and biotinylated at the apical or basolateral cell surface after various periods of chase. The SF/HGF receptor was immunoprecipitated, solubilized, reprecipitated with streptavidin-agarose, and detected by autoradiography. At the earliest timepoints at which the SF/HGF receptor is detected at the cell surface it is found almost exclusively basolateral. These results are representative of four experiments using the mAbs DN-30 or D0-24 for immunoprecipitation. All experiments gave similar results.


chase (min):
0
0
20
40
60
240
biotin:
—
ap
bl
ap
bl
ap
bl
ap
bl
ap
bl
p150β
p55α

CHAPTER TWO

SCATTER FACTOR/HEPATOCYTE GROWTH FACTOR INDUCES REARRANGEMENT OF MDCK CELL MEMBRANE SUBDOMAINS WITHOUT LOSS OF TIGHT JUNCTION FUNCTIONAL INTEGRITY

ABSTRACT

Scatter factor/hepatocyte growth factor (SF/HGF) has multiple effects on epithelial cells, inducing morphogenesis, mitogenesis and motogenesis. The ability of SF/HGF to increase cell motility and cause scattering of MDCK epithelial cells suggested a mechanism for tubule morphogenesis from MDCK cell cysts in which SF/HGF stimulates a loss of cell-cell contacts, an increase in cell migration and a reorganization of individual cells into new structures. In contrast, we have found that during SF/HGF-induced morphogenesis of tubules from MDCK cell cysts, cells remain in contact while markers for membrane polarity and cell-cell junctional proteins undergo profound rearrangements. E-cadherin, which was distributed basolaterally in polarized cells, completely surrounded cells in a developing tubule. Localization of gp135, an apical membrane marker, showed that apical membrane polarity was lost during early stages of tubule development and regained as tubule lumens form. Desmoplakins, which appeared as punctate spots at lateral surfaces of polarized cells, accumulated intracellularly within cells of a developing tubule. The tight junction component ZO-1 remained localized at points of cell-cell contact at all stages of tubule development. When Transwell filter-grown MDCK cell monolayers were treated with SF/HGF they formed pseudostratified layers. During the transition from a monolayer to a pseudostratified layer SF/HGF caused morphological rearrangements of markers for membrane polarity and cell-cell junctions similar to those observed during tubule development. Tight junctions remained functionally intact during pseudostratified layer formation. Therefore, we propose that cell-cell contacts are maintained while cell junctions and membrane polarity are rearranged during SF/HGF-induced epithelial morphogenesis.

INTRODUCTION

Tubulogenesis is a developmental process common to the formation of many organs, including lung, salivary gland, mammary gland, pancreas, and kidney. In each of these organ systems tubulogenesis involves regulation of growth, invasion, migration and differentiation that results in the development of branched networks of epithelial tubes. How this morphogenetic process is accomplished is an important question in modern biology. To organize into new structures cells must alter cell-cell adhesive contacts and polarity. These modifications are controlled by many factors, one of which is scatter factor/hepatocyte growth factor (SF/HGF). In order to establish how tubulogenesis occurs we have examined markers of MDCK cell polarity and cell-cell adhesive contacts during SF/HGF-induced epithelial morphogenesis.

Interactions between epithelia and closely apposed mesenchymal cells have been shown to be important in regulating epithelial morphogenesis either through direct cell-cell contacts (Cutler and Chaudhry, 1973; Wartiovaara et al., 1974; Lehtonen, 1975a; Lehtonen et al., 1975b; Bluemink et al., 1976; Saxen et al., 1976b) or through the paracrine action of soluble molecules (Grobstein, 1967; Cunha et al., 1983; Cunha et al., 1995; Saxen, 1987a; Saxen and Sariola, 1987b; Ekblom, 1991; Ekblom et al., 1994). SF/HGF is a soluble factor that has been suggested to have an important role as a paracrine mediator of epithelial/mesenchymal interactions during development. For example, in embryonic kidney, at the time when the ureteric bud undergoes branching morphogenesis in contact with the nephrogenic mesenchyme, the expression patterns of SF/HGF and c-met suggest that a paracrine and/or autocrine interaction between SF/HGF and c-met is important for morphogenesis of kidney collecting and renal tubules (Sonnenberg et al., 1993a; Sonnenberg et al., 1993b; Woolf et al., 1995).

SF was identified as a factor in fibroblast-conditioned medium that, when added to Madin-Darby canine kidney (MDCK) cells grown as small islands on an impermeable

support, causes the cells to scatter (Stoker and Gherardi, 1987b; Stoker et al., 1987a). HGF was defined by its ability to induce mitogenesis of cultured hepatocytes (Miyazawa et al., 1989; Nakamura et al., 1989). It was later discovered that these two factors are identical (Naldini et al., 1991b; Weidner et al., 1991; Bhargava et al., 1992) and that SF/HGF is a ligand for c-met (Bottaro et al., 1991; Naldini et al., 1991b), a receptor tyrosine kinase (Cooper et al., 1984; Park et al., 1987; Giordano et al., 1989b; Naldini et al., 1991a). In addition to its scattering and mitogenic activities, SF/HGF induces morphogenesis of branched tubules from MDCK cell cysts grown in type I collagen gels (Montesano et al., 1991; Montesano et al., 1991). This provides a model system with which to study MDCK cell polarity and cell-cell interactions during tubulogenesis.

When grown as a monolayer on a permeable filter support, MDCK cells form a well polarized epithelial monolayer, exhibiting apical and basolateral plasma membrane domains with unique compositions (Leighton et al., 1970; Rindler et al., 1979), and well-defined cell-cell junctional complexes containing tight junctions (TJ), adherens junctions (AJ) and desmosomes (DS) (Misfeldt et al., 1976; Cereijido et al., 1978). The apical membrane contains particular structural features such as microvilli, and proteins such as the glycoprotein, gp135 (Ojakian and Schwimmer, 1988). The TJ, located at the apical-most aspect of the lateral membrane, separates the apical and basolateral membrane domains and forms a selective permeability barrier to paracellular transport (Gumbiner, 1987; Rodriguez-Boulan and Nelson, 1989). The TJ cytoplasmic plaque component, ZO-1, is found exclusively at the tight junction of polarized epithelial cells (Stevenson et al., 1986; Siliciano and Goodenough, 1988; Stevenson et al., 1988). During embryonic development, the timing and localization of the expression of ZO-1 suggests that it has a role in de novo TJ formation (Fleming et al., 1989; Fleming et al., 1993). The basolateral membrane contains growth factor receptors, such as c-met (Crepaldi et al., 1994), and is divided into subdomains containing cell-cell or cell-substrate adhesion molecules. For example, the lateral membrane contains the AJ in the region immediately subjacent to the TJ

and DS at cell-cell borders below the AJ. The cell-cell adhesion molecule, E-cadherin is a basolateral membrane protein (Behrens et al., 1985; Vestweber and Kemler, 1985; Gumbiner and Simons, 1986; Gumbiner et al., 1988; Le Bivic et al., 1990; Shore and Nelson, 1991) that concentrates in the region of the apical junctional complex of MDCK cells (Nathke et al., 1994) and maintains cell-cell contacts through calcium-dependent homotypic interactions with adjacent cells (Takeichi, 1991). Multiple DS are located along the lateral membrane and function to maintain lateral cell-cell contacts. The DS contain unique members of the cadherin superfamily and cytoplasmic plaque components, desmoplakins I and II (dpI/II) (Pasdar and Nelson, 1988b; Pasdar et al., 1991; Garrod, 1993).

The morphogenesis of branched tubules observed when MDCK cell cysts are treated with SF/HGF provides an excellent opportunity to study the mechanisms of tubulogenesis. Like MDCK cells grown on filters, MDCK cells in both cysts and lumen-containing tubules are well polarized, (Wang et al., 1990; Santos et al., 1993a), with microvilli on lumenal membranes and junctional complexes containing tight junctions and desmosomes at the apical aspect of lateral cell-cell borders (Montesano et al., 1991). Given the ability of SF/HGF to cause scattering of MDCK cells grown as islands on plastic, a two stage model of tubule formation in response to SF/HGF has been proposed (Thiery and Boyer, 1992). In the first stage, MDCK cells would detach from the cyst, lose their polarity, and migrate as single cells out from the cyst and into the surrounding collagen gel. In the second stage, the migrating single cells would coalesce and reassemble into multicellular structures that would form tubules of polarized cells.

In this paper we have examined the mechanisms of tubulogenesis, both to test this particular model and more generally to examine the spatiotemporal distribution of markers for apical and basolateral polarity and cell-cell junctions during tubule formation. We find that during the transition from a cyst to a tubule there is an initial loss of cell polarity, which is re-established as lumen formation proceeds. We also find that lumen formation occurs

de novo at sites of cell-cell contact, rather than by extrusion of the cyst lumen. To study the response of MDCK cells to SF/HGF in a system where the function of tight junctions could be assessed, we also treated filter-grown monolayers of MDCK cells with SF/HGF. These cells responded by forming a pseudostratified layer that maintained tight junctions. This result, coupled with the distinct patterns of relocalization of cell junction proteins during morphogenesis of both tubules and pseudostratified layers suggests that cell-cell adhesion is maintained throughout morphogenesis. We propose that, during SF/HGF-induced morphogenesis, cell-cell adhesion is modulated while the polarity and specialization of plasma membrane subdomains reorganize, enabling cells to remain in contact as they rearrange into new structures.

MATERIALS AND METHODS

Cell culture

MDCK type II cells were maintained in MEM containing Earle's balanced salt solution (MEM-EBSS; Cellgro, Mediatech, Inc., Washington, D. C.) supplemented with 5% FCS (Hyclone, Logan, UT), 100 U/ml penicillin, and 100 mg/ml streptomycin in 5% CO_2/95% air. Cells were seeded at confluent density onto Transwells (cat. #3401, polycarbonate membrane, 12 mm diameter, 0.4 μm pore size; Costar, Cambridge, MA) and grown for four to six days, with daily medium changes, prior to use. For coculture experiments, MDCK cells expressing the wild type rabbit polymeric immunoglobulin receptor (pIgR) (Mostov and Deitcher, 1986) were mixed with untransfected MDCK cells prior to plating onto Transwells, so that 10% of the cells plated contained the pIgR. For growth of cells in three dimensional collagen gels, MDCK cells were triturated at room temperature into a single cell suspension of $5x10^4$ cells/ml in a type I collagen solution containing 66% Vitrogen 100 (3 mg/ml, Celtrix, Palo Alto, CA), 1X MEM, 2.35 mg/ml $NaHCO_3$, 0.02 M Hepes, pH7.6, and 12% dH_2O. Cells in suspension were then plated onto Nunc filters (cat# 162243, 10 mm, 0.02-0.2 μm pore size, Applied Scientific, San Francisco, CA). The type I collagen solution was allowed to gel by incubation at 37°C, 95% air/5% CO_2 prior to addition of medium. Cultures were fed every 3-4 days and grown for 10-12 days, until MDCK cell cysts with lumen were formed.

Antibodies/Reagents

Hybridoma cells secreting mouse anti-E-cadherin mAb (rr1; (Gumbiner and Simons, 1986)) were a kind gift from B. Gumbiner (Sloan Kettering, NY). Rat mAb R40.76 against ZO-1, a tight junction peripheral membrane protein (Stevenson et al., 1986; Anderson et al., 1988) was obtained from B. Stevenson or Chemicon (Chemicon International, Temecula, CA). Rabbit polyclonal Ab against the desmosomal proteins

desmoplakins I and II (dpI/II; Pasdar and Nelson, 1988a) was generously provided by M. Pasdar (University of Alberta, Edmonton) and W. J. Nelson (Stanford University, Stanford, CA). Mouse mAb supernatant against gp135, a MDCK apical membrane glycoprotein (Ojakian and Schwimmer, 1988) was kindly provided by W. J. Nelson (Stanford University, Stanford, CA) and G. Ojakian (SUNY Health Science Center, Brooklyn, NY). Affinity purified guinea pig polyclonal antibody against rabbit polymeric immunoglobulin receptor (pIgR) secretory component has been described previously (Breitfeld et al., 1989a; Breitfeld et al., 1989b). Secondary antibodies for immunofluorescence were goat anti-mouse-FITC, goat anti-rat-FITC, goat anti-guinea pig-LRSC and goat anti-rabbit-FITC from Jackson Immunoresearch Laboratories (WestGrove, PA). Propidium iodide (ppI) was purchased from Sigma Chemical Co. (St. Louis, MO). Recombinant human HGF (rhHGF) was generously provided by R. Schwall (Genentech, South San Francisco).

Production of fibroblast conditioned media containing SF/HGF

To obtain conditioned medium containing SF/HGF, MRC5 human lung fibroblasts (ATCC #CCL171) were grown to confluence in DME (DMEH21, obtained from the UCSF cell culture facility) containing 10% FBS (Hyclone, Logan, UT), 100 U/ml penicillin, and 100 mg/ml streptomycin in T75 tissue culture flasks (Corning, Corning, NY) or, for 2X conditioned medium, in T150 ridged tissue culture flasks (Corning, Corning, NY). The medium was replaced with 30 ml of fresh medium and cell culture was continued for 3 days. Conditioned medium was collected, centrifuged to remove cell debris, and frozen at -20°C until use. Control conditioned medium was obtained by growing D550 human foreskin fibroblasts (a kind gift from Ward D. Peterson, Detroit Medical Center, Detroit) under the same culture conditions as for MRC5 cells, but in MEM-EBSS supplemented with 0.1 mM nonessential amino acids ((100X stock, UCSF cell culture facility), 0.1%

lactalbumin hydrolysate (Sigma, St. Louis, MO), 0.1 mg/ml sodium pyruvate (100X stock, UCSF cell culture facility) and 10% FBS.

Treatment of MDCK monolayers and cysts with SF/HGF-containing media

MDCK cells plated at confluent density and grown for 4-6 days on 12 mm Costar polycarbonate Transwells to form electrically tight, polarized monolayers were treated for 20-24 hours with 100 ng/ml rhHGF and assayed as described below. Three dimensional cultures of MDCK cells grown to form cysts were treated for various numbers of days with D550 or MRC5 fibroblast conditioned media diluted 1:1 with MEM-EBSS, 5% FBS. Cultures were refed daily with freshly diluted conditioned medium.

Immunofluorescence and Confocal Microscopy

Cells on Transwell filters or in collagen gel cultures were processed for immunofluorescence at room temperature as follows: Cultures were rinsed with PBS, pH 7.4, containing 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$ (PBS^+), fixed for 30 minutes with 4% paraformaldehyde in PBS^+, permeabilized for 30 minutes with 0.025% saponin in PBS^+, rinsed with PBS^+ and quenched for 10 minutes with 75 mM NH_4Cl, 20 mM glycine in PBS^+, pH 8.0. For Transwell filter-grown cells, nonspecific binding sites were blocked by rocking for 10 minutes in PBS+, 0.025% saponin, 0.7% fish skin gelatin (block buffer) followed by 10 minutes in block buffer + 0.1 mg/ml boiled RNAase A. Filters were incubated with primary antibody diluted in block buffer, either for 60 minutes in a humidified chamber at 37°C or overnight at 4°C. Primary antibody concentrations were as follows: rr1 mAb supernatant, 3:1; R40.76 anti ZO-1 ascites, 1:150; R40.76 anti ZO-1 mAb supernatant, 3:1; rabbit anti-dpI/II, 1:100; gp anti-SC, 1:130; gp135 mAb supernatant, 3:1. After extensive washing with PBS+/saponin and blocking buffer, cells were incubated for 30 minutes at 37°C in a humidified chamber in a block buffer solution containing fluorophore-conjugated secondary antibodies, diluted 1:100 and ppI, diluted

1:1000 from a 3-4 mg/ml stock. Samples were then washed extensively, postfixed with 4% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.4 and mounted in vectashield (Vector Labs, Burlingame, CA).

Nonspecific binding sites in collagen gel cultured cells were blocked by a 30 minute incubation in blocking buffer followed by 10 minutes in blocking buffer + 0.1 mg/ml boiled RNAase A. Samples were then rocked 1-3 days at 4°C in primary antibodies diluted into blocking buffer containing 0.02% azide, washed extensively with PBS+/saponin and blocking buffer, incubated overnight at 4°C in FITC-conjugated secondary antibodies + ppI diluted into blocking buffer containing 0.02% azide, and washed extensively prior to postfixing and mounting as above. Specific staining was clearly detected in confocal images of collagen gel samples. However trapping of secondary antibody aggregates within the collagen gel sometimes resulted in bright spots of staining in the collagen gel surrounding the cell cultures.

Confocal images were collected using a krypton-argon laser with K1 and K2 filter sets coupled to a Biorad MRC600 confocal head and an Optiphot II Nikon microscope with a Plan Apo 60X 1.4 NA objective. Transwell filter samples were imaged either in the x-y plane or in the x-z plane with a motor step size of 0.5 or 1 μm, Kalman filtering with 5 frames/image, diaphragm set at 1/3 open. Collagen gel cultures were imaged as above in the x-y plane of the sample. Individual x-y sections were selected from sets of serial sections collected in the x-y plane that completely spanned through control cysts or tubule-containing structures. The data was analyzed using Comos software. Images were converted to TIFF format and the contrast levels of the images adjusted using Adobe Photoshop (Adobe Co., Mountain View, CA) on a MacIntosh 7100/66 (Apple, Cupertino, CA). Composites of contrast-corrected images were prepared in Freehand (Aldus Corporation, Seattle, WA).

Electron Microscopy

For ultrastructural analysis, MDCK cells on Transwell filters were fixed for 60 minutes on ice with 2% gluteraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4 containing 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$, postfixed for 3 h at room temperature with 1% OsO_4, counterstained overnight with 0.5% uranyl acetate, dehydrated in a graded series of ethanol and embedded in epon (LX-112; Ladd Res. Inds., Inc. Burlington, VT). Alternatively, cells were fixed 60 minutes on ice with 1.3% gluteraldehyde, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.05% ruthenium red, 67 mM sodium cacodylate, pH 7.4 on the apical side of the Transwell and 67 mM cacodylate, pH 7.4 on the opposing side. These samples were then postfixed for 3 h at room temperature in 1.7% OsO4, 0.05% ruthenium red, 67 mM sodium cacodylate, pH 7.4, counterstained overnight with 0.5% uranyl acetate, dehydrated in ethanol, and embedded in epon. Thin sections were cut in the x-z plane of the Transwell with a diamond knife (Diatome, Fort Washington, PA) and were observed at 80 kV in a Zeiss EM-10 electron microscope (West Germany).

Inulin Diffusion Measurements

To quantitatively measure the functional integrity of the tight junctions we determined apical to basolateral ^{14}C-inulin leakage across MDCK cell cultures grown on 12 mm Costar Transwells. MDCK cell cultures were plated at confluent density and grown 4-5 days in MEM, 5% FBS followed by 24 hours in either MEM, 5% FBS alone or MEM, 5% FBS + 100 ng/ml rhHGF. To measure inulin diffusion, cultures were washed 1X with MEM containing Hank's Balanced salts, 0.6% BSA, 20 mM Hepes, pH 7.3 (MEM/BSA) at 37°C and then 0.5 ml of MEM/BSA containing 1.25 x 10^5 cpm of ^{14}C-inulin was placed in the apical compartment and 1 ml of MEM/BSA in the basal well. These volumes were chosen because they result in matching fluid levels across the filter. Cultures were maintained at 37°C and aliquots were collected, 20 µl from the apical side and 40 µl from the basal side at 1, 2, 4 and 8 hours after addition of ^{14}C-inulin. The controls used were

both blank filters and MDCK cultures, grown as above in MEM, 5% FBS, that were transferred 12 hours prior to the diffusion assay to MEM suspension medium (S-MEM, GibcoBRL, Gaithersburg, MD) containing 5% dialyzed FBS and 2 μM $CaCl_2$ ("minus Ca^{+2}" cultures). The inulin diffusion assay for "minus Ca^{+2}" cultures was carried out in MEM/BSA containing 2 μM $CaCl_2$. Aliquots were counted in a liquid scintillation counter (Beckman Instruments, Irvine, CA).

RESULTS

To assess the effect of SF/HGF on epithelial cell surface domains during morphogenesis we studied the distribution of markers for apical, basolateral and junctional membrane specializations in two MDCK cell model systems. In the first model system we studied the development of tubules from MDCK cell cysts grown in collagen. In the second model system we studied the formation of pseudostratified layers from MDCK cell monolayers grown on filters.

Development of lumen-containing tubules from MDCK cell cysts occurs in four distinct stages

Previous studies have shown that MDCK cells plated in single cell suspension in type I collagen gels grow to form cysts and that stimulation of MDCK cell cysts with SF/HGF induces the formation of branching tubules (Montesano et al., 1991; Montesano et al., 1991). To investigate further the morphology of structures that develop during branching tubule morphogenesis we analyzed individual confocal sections and projections of serial confocal sections from collagen gel cultures of MDCK cell cysts before and after various amounts of time in the presence of SF/HGF. Confocal images of cultures stained with ppI to detect nuclei show that cysts are composed of a monolayer of MDCK cells that seals off a fluid filled central lumen (Figures 2.1 A, right image). Similar cysts are seen in Figures 2.2A, 2.3A, and 2.4A. While some cysts are spherical, others are irregularly shaped and/or contain cells within the cyst that project into or connect across the lumen. We stimulated tubule development by treating MDCK cell cysts for 1 to 7 days with SF/HGF-containing conditioned medium from the human lung fibroblast cell line, MRC5, or conditioned medium that does not contain SF/HGF from the human foreskin fibroblast cell line, D550. Although the morphogenesis of lumen-containing tubules occurs as a continuous process, it is useful to divide the sequence into four stages. We have defined

these stages with the following terms: extensions form after 1 day, chains within 1-3 days, cords between 3-5 days and lumen-containing tubules after 7 days of stimulation. The development of tubules in this system is not completely synchronous and tubules in several stages of development may be found emanating from a single cyst. Therefore, we present our data in terms of morphological stages rather than absolute time after addition of SF/HGF-containing medium.

Polarity of MDCK membrane domains is lost in cells that extend from a cyst to form a branching chain and is regained once a tubule lumen is formed

To analyze the polarity of MDCK cells during SF/HGF-induced tubule morphogenesis we used confocal immunofluorescence imaging to detect well known markers for basolateral and apical membranes. Double-labeled images were collected that contained staining for either E-cadherin, as a marker for the basolateral membrane, or gp135, as a marker for the apical membrane, and ppI to denote the localization of nuclei relative to these membrane markers. First we wanted to identify where markers of polarized membrane domains are localized in MDCK cell cysts grown in the absence of SF/HGF stimulation. In individual confocal sections, with images split to distinguish membrane from nuclear staining, we find that E-cadherin is localized predominantly at lateral membranes but is also found on membranes that are in contact with the extracellular matrix (Figure 2.1 A, arrows). E-cadherin is absent from membranes facing the lumen of the cyst. Gp135 staining is localized towards the interior of the cyst relative to ppI stained nuclei, delineating the lumenal membrane of MDCK cell cysts (Figure 2.2 A, arrows). Some gp135 appears to be within the cyst lumen, but most likely represents staining of apical microvilli. Gp135 is clearly absent from lateral cell-cell borders and cell-substrate contacts in MDCK cell cysts grown in the absence of SF/HGF. These results confirm that MDCK cell cysts grown in three dimensional collagen gel cultures are polarized with

markers of the basolateral surface facing the extracellular matrix and markers of the apical surface at the membrane facing the lumen of the cyst.

To investigate the effect of SF/HGF-containing conditioned medium on membrane polarity during morphogenesis we followed the localization of E-cadherin and gp135 during the development of branching tubules. In the first stage of SF/HGF-induced tubule formation, the development of extensions, individual cells of a cyst are stimulated to extend into the surrounding collagen matrix while remaining attached to the cyst. E-cadherin staining surrounds the cellular extensions, localizing at both cell-substrate and cell-cell borders (Figure 2.1 B, arrows, left image). The filled arrowhead in Figure 2.1B points to the remaining lumenal surface of the extending cell and indicates that this surface is devoid of E-cadherin. The open arrowhead in Figure 2.1B is at the mouth of a narrow lumenal space that is between two cells that adjoin the extending cell and project under it. These results suggest that during initial stages of morphogenesis, apical and basolateral domains are altered but remain distinct. As tubulogenesis proceeds into the second step, chain formation, single-file chains of cells develop that are either linear (Figure 2.1 C) or branching (data not shown). E-cadherin circumscribes each cell of the chain, indicating that the distinct apical versus basolateral localization of E-cadherin is lost in cells of the chain (Figure 2.1 C, arrowhead). In contrast, basolateral polarity of E-cadherin is maintained in cells of the cyst wall that are not undergoing morphogenesis (Figure 2.1 C, arrows). In the final stages of tubule formation, chains of cells thicken into cords, followed by development of tubules via de novo formation of lumens within the cord at discrete sites of cell-cell contact. This is most clearly seen in a confocal image showing the transition from a cord (Figure 2.1 D, arrowhead) to a tubule (Figure 2.1 D, arrows). E-cadherin completely surrounds each cell in a cord, but in lumen-containing regions of developing tubules the basolateral polarity of E-cadherin is restored.

Rearrangements in apical membrane polarity are also induced by SF/HGF-stimulated morphogenesis, as indicated by gp135 relocalization during the development of

branching tubules. As mentioned in the previous paragraph, during the first stage of tubule formation, the extending cell retains a small lumenal membrane, which is in contact with the lumen of the cyst. Gp135 is localized at this remaining lumenal membrane, establishing that at this stage of tubule development distinct apical membranes are maintained (Figure 2.2 B, arrows). As morphogenesis continues gp135 staining disappears from cells that are connected in short chains (Figure 2.2 C, arrowheads) or localizes in intensely stained intracellular patches (data not shown), indicating that apical membrane polarity is lost. During the development of cords gp135 staining is concentrated at or near cell-cell borders (Figure 2.2 D, arrows). These sites might be described as "protolumens," since they may indicate the location of future lumen formation. It is possible that minute lumens that are below the resolution of the confocal microscope exist at these sites. Some of the gp135 may also be associated with intracellular vesicles (Vega-Salas et al., 1988). The distinct localization of gp135 at membranes that are not in contact with the extracellular matrix at this stage of tubule development and the subsequent formation of discrete lumens in these regions, suggests that cell-substrate interactions act as primary determinants of apical membrane polarity. As tubule formation proceeds, and morphologically distinguishable lumens form, gp135 is localized at 'free' cell surfaces facing the tubule lumens (Figure 2.2 E, arrows). These results suggest that apical cell surfaces rearrange during tubulogenesis and that apical membrane polarity is restored during de novo lumen formation.

HGF-induced branching morphogenesis causes rearrangements in the location of cell junction proteins

Our results above show that membrane domains containing the cell-cell adhesion molecule, E-cadherin, rearrange during tubule morphogenesis. Since E-cadherin is concentrated at the lateral membranes of polarized MDCK cells where it functions to maintain cell-cell contact, the alterations in E-cadherin distribution that occur during

morphogenesis suggest that SF/HGF induces changes in cell-cell adhesion during tubule formation.

To assess the effect of SF/HGF on other components of cell-cell adhesion during morphogenesis we followed the localization of markers for DS and TJ at different stages of tubule formation. We first established the localization of DS and TJ proteins in individual confocal sections of MDCK cell cysts, prior to addition of SF/HGF. Markers for the desmosomal complex, desmoplakins I and II, appeared as discrete spots along basolateral plasma membranes of cells in polarized MDCK cell cysts (Figure 2.3 A, arrows). ZO-1, a marker for tight junctions, is localized at the apical-most aspect of lateral cell-cell borders of polarized MDCK cell cysts (Figure 2.4 A, arrows). By summing multiple confocal sections through a cyst and creating a projection of the images we find that ZO-1 forms an apical "net" facing the lumen of the cyst (Figure 2.4 B).

The treatment of MDCK cell cysts with SF/HGF causes disparate changes in the distribution of desmoplakins and ZO-1. To study SF/HGF-induced alterations in the localization of dpI/II and ZO-1, individual confocal sections of SF/HGF-treated MDCK cell cysts were examined during early stages of tubule formation. We found that during the formation of chains dpI/II were localized in large intracellular pools with a concomitant loss of organized arrays at cell-cell borders (Figure 2.3 B, arrows), suggesting that adhesion through desmosomes is decreased during tubule formation.

The localization of membrane domains containing punctate ZO-1 staining is altered by SF/HGF, but ZO-1 remains at sites of cell-cell contact throughout morphogenesis. During the extension stage of SF/HGF-induced morphogenesis, ZO-1 remains at the lumenal aspect of lateral membranes of cells extending from the cyst (data not shown). However, as tubule formation continues, ZO-1 localizes to regions of the membrane that link cells into single-file chains (Figure 2.4 C, arrows), suggesting that cell-cell adhesion is maintained during tubulogenesis by ZO-1-containing junctions. During de novo lumen formation, ZO-1 localizes at cell-cell contact points at the apical-most aspect of lateral

borders between cells surrounding the newly formed lumen (Figure 2.4 D, arrowhead), suggesting that at this stage of tubule development the apical polarization of TJ is re-established. In regions of the developing tubule where lumen formation is more advanced, ZO-1 staining outlines the apical membrane, reminiscent of ZO-1 localization at the apical-most aspect of lateral membranes in polarized cysts (Figure 2.4 D, arrow). EM studies confirm the presence of TJ at the lumenal aspect of lateral cell-cell borders between cells in lumen-containing tubules (Figure 2.4 E, box; Figure 2.4 F, arrow). The localization of ZO-1 throughout tubulogenesis at membranes involved in cell-cell contact suggests that ZO-1-containing junctions maintain adhesion during the transition between a cyst and a tubule. Concomitant with repolarization of the tubule epithelium, ZO-1 demarcates lumenally polarized tight junctions that separate the apical and basolateral plasma membrane domains.

HGF stimulation of tight polarized monolayer cultures of MDCK causes morphogenetic changes similar to the rearrangements seen in early stages of tubule formation

The development of tubules from cysts is a spatially complex process, in which it is difficult to access and dissect out the individual steps involved. For instance, the functional state of tight junctions that might exist at different stages in tubulogenesis cannot be directly assessed. The other major in vitro system where the effects of SF/HGF on cell adhesion and motility have been studied is the treatment of MDCK cells grown as islands on an impermeable support with SF/HGF. Under these growth conditions MDCK cells are far from completely polarized (Herzlinger and Ojakian, 1984) and SF/HGF treatment causes loss of cell-cell adhesion and cell scattering (Stoker et al., 1987b). This is quite different from the selective modulation of cell adhesion that we observed with SF/HGF treatment of cysts. Studying the effects of SF/HGF on MDCK cells grown on impermeable supports may provide insight into developmental responses where cell

migration is critical, such as the limb (Bladt et al., 1995; Warn, 1995). However, we reasoned that following the effects of SF/HGF in this environment is less physiologically germane to the process of tubule morphogenesis which, in vivo, involves the development of a polarized epithelium (Saxen et al., 1968; Ekblom et al., 1981; Saxen, 1987a). We wished to develop a system that would retain the physiological polarization of MDCK cells, providing a starting point for assaying morphogenesis that was similar to the model system using polarized MDCK cell cysts grown in collagen gels, but that would also be highly amenable to detailed analysis of the changes induced by SF/HGF.

A very well-established method for growing MDCK cells under conditions where they can fully polarize and closely resemble an in vivo situation is to grow the cells as a tight monolayer on a permeable filter. We therefore treated filter-grown monolayers of MDCK cells with SF/HGF and examined the effects on polarity, morphology, adhesion, and tight junctions. MDCK II cells were grown on Transwell filters in the absence of SF/HGF to form polarized monolayers in which the basolateral and apical plasma membrane subdomains are separated by tight junctions that maintain a resistance of around 100 ohm-cm^2. After a 24 hour treatment of a tight MDCK cell monolayer with MRC5 cell conditioned medium or 100 ng/ml rhHGF we observed in X-Z confocal and EM sections (in Figures 2.5-2.9, described below) that SF/HGF caused some cells to extend over and under adjacent cells. The monolayer assumed the appearance of a pseudostratified layer, although we cannot rule out that in some cases a true multilayer forms, i.e. that some cells might lose all connection with the filter.

To obtain a better picture of how the cells rearrange from a uniform monolayer into a pseudostratified layer, we grew Transwell filter cultures containing 90% untransfected MDCK cells and 10% MDCK cells transfected with the pIgR into a polarized monolayer. The use of a low percentage of cells expressing the pIgR enabled us to mark individual cells and better resolve cell boundaries. (This approach is not feasible with cyst-grown cells, as each cyst grows up from a single cell.) After stimulating for 20 hours with

SF/HGF we collected X-Z confocal sections of untreated and SF/HGF-treated cultures (Figures 2.5 A and B, respectively) stained with ppI to detect nuclei (arrows, right images), for E-cadherin to detect membrane borders (left images) and for pIgR to distinguish individual cell cytoplasms (arrowheads). We find that individual cells extend over each other while maintaining a common cell-cell border. These results suggest that SF/HGF causes MDCK cells within a tight monolayer to crawl over each other and restructure into a pseudostratified layer without cell dissociation.

We also used Transwell filter-grown MDCK cells to investigate the effect of SF/HGF on the localization of markers for MDCK cell polarity and cell-cell adhesion. In polarized MDCK cell monolayers, over 90% of newly synthesized E-cadherin is targeted directly to the basolateral membrane (Le Bivic et al., 1990) and, at steady state, is found localized at lateral subdomains (Behrens et al., 1985; Le Bivic et al., 1990; Crepaldi et al., 1994; Nathke et al., 1994). Using X-Z confocal sections we find that, prior to treatment of MDCK monolayers with SF/HGF, E-cadherin is localized mostly at lateral membranes (Figure 2.6 A, arrowhead). However, after stimulation with SF/HGF, E-cadherin completely surrounds cells that are enveloped in the multilayer (Figure 2.6 B, arrowheads).

In polarized MDCK cell monolayers, the apical marker protein, gp135, is restricted to the "free" cell surface facing the apical medium (Figure 2.6 C, arrow). After SF/HGF-induced formation of pseudostratified layers, we detect gp135 on apical membranes and at lateral borders between cells in confocal X-Z sections (Figure 2.6 D, arrows). Gp135 is always located above the level of the tight junction, suggesting that the mechanism responsible for apical localization of gp135 is still functional. Therefore, during formation of both pseudostratified layers and tubules, gp135 localizes to apical surfaces or lateral membranes in regions of cell-cell contact, but not to membranes that are in contact with extracellular matrix.

Changes in the localization of desmosomal proteins are also caused by SF/HGF-stimulated morphogenesis of pseudostratified layers. In confocal X-Z sections dpI/II are

primarily on the lateral membranes of polarized monolayers of MDCK cells (Figure 2.7 A, arrow). After SF/HGF treatment of monolayers, dpI/II are localized in large intracellular pools in cells that extend out of the monolayer (Figure 2.7 B, arrows). Our results from studies of both SF/HGF-stimulated morphogenesis of pseudostratified layers and assays of tubule formation from cysts suggest that SF/HGF induces changes in epithelial cell polarity and cell-cell adhesive junctions during morphogenesis that enable cells to rearrange without completely dissociating.

We studied ZO-1 localization with confocal microscopy to obtain images in both X-Z and X-Y sections through control and SF/HGF-treated Transwell filter-grown MDCK cell cultures. In X-Z cross-sections through MDCK monolayers that have not been treated with SF/HGF ZO-1 is localized at the apical-most aspect of lateral cell-cell borders (Figure 2.8 A, arrows). After SF/HGF-induced morphogenesis ZO-1 appears in X-Z confocal images as punctate spots at sites of cell-cell contact at multiple levels through the pseudostratified layer (Figure 2.8 B). To determine more clearly the localization of ZO-1 we collected X-Y confocal sections through basal, central and apical planes of untreated and SF/HGF-treated MDCK Transwell cultures. ZO-1 staining appears within a discrete apical plane in untreated MDCK cell monolayers where it forms a bright ring that outlines the plasma membrane of each cell-cell border (Figures 2.8 C, E, and G). After SF/HGF-induced formation of pseudostratified layers, X-Y confocal images show that ZO-1 is found in all planes of the culture -- basal, central and apical (Figures 2.8 D, F, and H). ZO-1 staining in individual X-Y planes of HGF-treated pseudostratified layers appears discontinuous. However, by examining projections of confocal images, obtained by summing all X-Y confocal sections through a sample, we find that there are complete rings of ZO-1 around each cell in both monolayers and pseudostratified layers (Figures 2.8 I and J). This indicates that, although SF/HGF induces changes in the localization of ZO-1, staining of ZO-1 in pseudostratified layers depicts morphologically intact tight junctional

belts. These results suggest that tight junctions remain functionally intact during morphogenesis.

HGF-induced morphogenesis occurs in the presence of functionally intact tight junctions

To determine if the morphologically intact rings of ZO-1 represent functionally intact tight junctions we tested the integrity of tight junctions during SF/HGF-induced morphogenesis. Ruthenium red is a heavy metal conjugate that is too large to cross a functionally intact tight junction and can be visualized using electron microscopy (Luft, 1971a; Luft, 1971b; Wanson et al., 1977). Ruthenium red was added to the apical side of either untreated or SF/HGF-treated Transwell filter cultures of MDCK cells during fixation and processing for EM. X-Z sections were prepared of MDCK cell monolayers and pseudostratified layers and images were collected to determine if ruthenium red crossed the cell layers. In electron micrographs shown in figures 9A and B we see that ruthenium red remains at the apical side of both untreated and SF/HGF-treated MDCK cell layers. Therefore, tight junctions remain functionally intact during morphogenesis. In addition, we find that in SF/HGF-treated cultures the tight junctions between cells are no longer localized specifically at the apical aspect of the plasma membrane, but can be found at different levels along the lateral membrane. To further evaluate the functional intactness of tight junctions during morphogenesis, we studied ^{14}C-inulin diffusion across Transwell filter-grown MDCK cell layers during SF/HGF-stimulated rearrangement of a monolayer into a pseudostratified layer. Tight, polarized MDCK cell monolayers were treated for 24 hours in the absence or presence of SF/HGF and treatment was continued as apical to basolateral diffusion was measured for various times by including ^{14}C-inulin in the apical medium. We find that there is no significant increase in the amount of apical to basolateral ^{14}C-inulin diffusion within the first 2 hours of measurement. A small increase in the amount of basolateral ^{14}C-inulin is detectable 4 to 8 hours after apical application of ^{14}C-

inulin (Figure 2.10). Furthermore, we find that the electrical resistance of the monolayer actually increases transiently in response to SF/HGF, peaking during the early time periods in which we measured inulin diffusion (Pollack and Mostov, manuscript in preparation). This is in contrast with a study from another group, using somewhat different conditions (Nusrat et al., 1994). Our results indicate that morphologically intact rings of tight junctions that act as functionally intact permeability barriers are maintained during rearrangement of a monolayer into a pseudostratified layer (Figure 2.11).

DISCUSSION

The results of this study show that formation of epithelial tubules involves distinct steps in which epithelial cell membrane subdomains are transiently altered as cells rearrange into new structures, and then apical and basolateral polarity and cell-cell junctions are restored as lumen-containing tubules are formed. Based on our results we have defined 4 stages in the development of tubules from MDCK cell cysts (Figure 2.12). In the first stage of SF/HGF-induced tubule formation, the extension stage, individual cells extend into the surrounding collagen matrix while remaining attached to the cyst. Apical and basolateral membranes are morphologically altered but cell polarity and cell junctions are maintained. The second stage, chain formation, is characterized by the appearance of single-file linear or branched chains of cells in which an apical membrane marker (gp135) is absent or intracellular, a basolateral membrane marker (E-cadherin) is randomly localized, desmosomes (dpI/II) are internalized, and the TJ marker (ZO-1) is highly localized to the small regions of cell-cell contact between cells in the chain. Subsequently, in stage 3 or cord formation, the chains of cells thicken into cords in which apical polarity is again observed while the basolateral marker remains randomly distributed. Small lumens begin to appear de novo in segregated regions along the length of the developing tubule at sites of cell-cell contact where apical membrane markers are located. ZO-1 localizes at apical cell-cell contact points surrounding the nascent lumen. In stage 4, tubule formation, individual lumens enlarge and become continuous with the lumen of the cyst, the cells of the tubule become clearly polarized and the normal arrangement of the cell junctions is completely restored.

The picture that emerges from our studies is quite different from the model proposed by Thiery and Boyer (Thiery and Boyer, 1992), where cells completely dissociate and migrate out into the collagen gel as individual cells, prior to reassociating into tubules. We do in fact see some cells that migrate individually into the collagen gel so

we cannot absolutely exclude a model in which these cells contribute to a developing tubule. However, our data clearly show that (a) tubules initiate from cells that are in contact with the cyst and (b) tubules often develop in the absence of a multiplicity of freely dispersed individual cells in the immediate surroundings. This strongly supports the hypothesis that cells that make up the final tubules arise from cells that never completely lost cell-cell interactions. In addition, our data strongly suggests that tubulogenesis involves migration of cells while they remain in contact.

Effects of SF/HGF on cell-cell polarity and components of cell junctions

We have found that the outgrowth and development of tubules involves a transient alteration in MDCK epithelial cell polarity followed by restoration of apical and basolateral polarity during the process of lumen formation. Our results suggest that during SF/HGF-induced morphogenesis there is an interdependence between cell adhesive contacts and the formation of polarized apical and basolateral membrane domains. For example, during tubulogenesis the localization of gp135 changes from 'free' membranes in extensions to cell-cell borders or intracellular pools in cords and back to 'free' surfaces in tubules, but gp135 is never found at cell-substrate interfaces. Gp135 relocalization to cell-cell borders at the cord stage occurs while E-cadherin remains randomly localized, intermixing with apical proteins in regions of cell-cell contact and circumscribing cell membranes that are in contact with the extracellular matrix. This suggests that during morphogenetic rearrangements cell-substrate contacts act as primary determinants of apical membrane polarity.

Our results from studies of the localization of cell junctional proteins after SF/HGF stimulation provide evidence that SF/HGF affects cell-cell adhesive contacts during morphogenesis. The randomization of E-cadherin distribution during tubule morphogenesis suggests that the function of homotypic cell-cell interactions mediated by E-cadherin is altered by SF/HGF stimulation. The redistribution of the desmosomal proteins,

desmoplakins I and II, to large intracellular pools during early stages of SF/HGF-induced tubule morphogenesis is consistent with a cytoplasmic localization of these proteins found during SF/HGF-stimulated MDCK cell scattering (Stoker and Perryman, 1985). This suggests that adhesion through desmosomes is decreased by SF/HGF treatment. Intriguingly, the tight junctional element, ZO-1, remains localized at points of cell-cell contact during tubule morphogenesis, suggesting that SF/HGF does not eliminate the ability of tight junctional elements to maintain cell-cell contacts during morphogenetic cell movements and rearrangements of plasma membrane subdomains.

Use of filter-grown MDCK cells to analyze effects of SF/HGF

To gain further insight into alterations in epithelial cell polarity and cell-cell adhesion caused by SF/HGF we have established a second model system using Transwell-grown MDCK cell monolayers. Numerous studies have documented that filter-grown MDCK cells are well polarized (Misfeldt et al., 1976; Cereijido et al., 1978; Mostov et al., 1992; Rodriguez-Boulan and Powell, 1992), similarly to MDCK cells in cysts grown in collagen gels (Wang et al., 1990; Montesano et al., 1991b). The filter-grown MDCK cell system is amenable to detailed morphological analysis and allows a direct assessment of tight junction function.

Our results demonstrate that SF/HGF induces morphogenesis of a MDCK cell monolayer into a pseudostratified layer. The alterations in polarity and changes in the localization of cell-cell junction components found in the pseudostratified layer apparently reflect early stages in tubule morphogenesis. For example, we see that gp135 remains mostly at apical membranes but also starts to appear at lateral cell-cell borders whereas E-cadherin appears to have a nonpolar distribution in cells that are enveloped in the pseudostratified layer. SF/HGF treatment of MDCK cell monolayers causes the appearance of dpI/II in intracellular pools inside cells that extend out of the monolayer, similar to what we see in cells extending from a cyst during initial stages of tubule

formation. Using Transwell filter-grown MDCK cell cultures we were able to obtain EM and confocal X-Z sections after SF/HGF stimulation of MDCK cell monolayers in which we were able to see that tight junctions and ZO-1 are found at both apical and lateral cell-cell borders within a pseudostratified layer. In addition, this system enabled us to determine that throughout the morphogenetic process (a) rings of ZO-1 remain morphologically intact and (b) functional seals to paracellular transport are maintained. If TJ remain intact how do apical and basolateral plasma membrane subdomains rearrange during morphogenesis? One possible mechanism by which SF/HGF causes rearrangements of plasma membrane domains is by inducing transcytosis (e.g., of E-cadherin from basolateral to apical domains) or alternatively, by altering the sorting of newly synthesized membrane proteins to the cell surface. Although the filter-grown cells are a useful model for the early events in response to SF/HGF, there is an obvious difference from the collagen cyst model: On filters migration in the direction of their basolateral surface is constrained, due to the barrier of the filter, such that cells migrate under and over each other to a limited degree but cannot migrate into the substratum. The apical migration of filter-grown cells on top of each other may be a compensatory mechanism, although in the cyst model we also see apical migration of cells that neighbor an extending cell at sites of tubule initiation (Figure 1B, open arrowhead).

Possible mechanisms for SF/HGF-induced morphogenesis

Our results suggest that during SF/HGF-induced morphogenesis of both pseudostratified layers and tubules there is selective regulation of cell-cell adhesive contacts in which tight junctions relocalize but remain functionally intact, while E-cadherin and desmosomal-based adhesive contacts are altered. The ability of SF/HGF to induce outgrowth of cohesive structures with functionally intact tight junctions suggests that SF/HGF selectively modulates, rather than disrupts, adhesion during morphogenesis. This selective alteration of distinct cell-cell junctional components contrasts with results from a

previous study in which calcium "switch" or antibody disruption of E-cadherin based adhesion caused coordinate regulation of adhesion through desmosomes, adherens junctions and tight junctions (Gumbiner et al., 1988). Individual components of junctional complexes can be differentially and specifically regulated by phosphorylation. For example, tyrosine-specific protein phosphorylation of MDCK cells downregulates AJ contacts without loss of adhesion through tight junctions and desmosomes (Volberg et al., 1992). Phosphorylation of β catenin was found to be responsible for a decrease in E-cadherin-based adhesion in several transformed cell lines (Matsuyoshi et al., 1992; Behrens et al., 1993). In addition, SF/HGF-induced phosphorylation of β catenin and plakoglobin in several epithelial cell lines correlates with an increase in scattering activity (Shibamoto et al., 1994). Since both β catenin and plakoglobin are associated with E-cadherin in AJ of epithelial cells, these observations suggest that SF/HGF-stimulated phosphorylation modulates adhesiveness of E-cadherin. One possibility is that although E-cadherin is uniformly distributed on the surface of SF/HGF treated cells, the phosphorylation of β catenin and its association with E-cadherin is highly regulated in space and time, allowing the adhesive properties of specific areas of individual cells to be modulated as the cells "let go" and reconnect as they rearrange.

SF/HGF may modulate not only adherens junctions, but also desmosomal adhesion through catenin phosphorylation, since plakoglobin is also present in association with desmosomes. We found that MDCK cells undergoing SF/HGF-induced morphogenetic movements have large intracellular pools of desmoplakins. Internalization of desmosomes has been previously shown to be regulated by calcium (Kartenbeck et al., 1982; Mattey and Garrod, 1986; Kartenbeck et al., 1991) and lability of desmosomes has been suggested to be important for development of kidney tubules (Garrod and Fleming, 1990; Burdett, 1993). SF/HGF has been found to cause loss of desmosomal plaques in MDCK cells during scattering (Stoker and Perryman, 1985) and to disrupt desmosomal contacts in keratinocytes (Watabe et al., 1993). Therefore, we can speculate that, during

morphogenesis, catenin phosphorylation participates in desmosome downregulation by inducing internalization of desmosomal components and causes a decrease in desmosome-based adhesion. However, it is also possible that SF/HGF modulation of desmosomes is indirect, through its effects on AJ. Based on our results we suggest that SF/HGF causes a decrease in adhesion through DS and modulates E-cadherin-based adhesion, but does not disrupt TJ function. This segregation of SF/HGF's effects on AJ and DS adhesive contacts from effects on TJ function may be critical for regulating the formation of lumen-containing branching tubules.

In conclusion, we have examined how cell polarity and cell-cell adhesion undergo highly regulated and specific alterations in response to SF/HGF in order for cells to reorganize into tubules. It seems likely that these types of processes are general features of branching tubule morphogenesis in a variety of systems, and perhaps have a role in other types of morphogenetic movements, including those that are not necessarily induced by SF/HGF. During development, specific modulation of adhesion and polarity may enable the developing system to restructure without having to completely redifferentiate. Regulating the extent to which adhesion and polarity are altered during morphogenesis may prime a developing system to rapidly restore or acquire epithelial function. Furthermore, in other processes, such as cell division (Baker and Garrod, 1993; Reinsch and Karsenti, 1994) and wound repair (Madara, 1990; Bement et al., 1993) it is critical for cell rearrangements in epithelial structures to occur with a minimal disruption of polarity and adhesion in order to maintain the functional intactness and permeability characteristics of the epithelial tissue. Finally, further insight into the interplay between the actions of SF/HGF and components of systems that regulate cell-cell and cell-substrate adhesion and polarity may provide a key to understanding the seemingly disparate abilities of SF/HGF to induce epithelial cells to develop into cohesive structures rather than to scatter or invade.

Figure 2.1. Basolateral membrane polarity is altered during the transition from a cyst to a tubule and is restored as lumens form within the tubules. In *A-D* each panel represents a single confocal section taken from serially sectioned samples that were scanned simultaneously for FITC and ppI. Images are split so that staining for E-cadherin, a marker for the basolateral membrane, is in the left half and nuclear staining is in the right half of each panel. Arrows (*A-D*) depict basolaterally localized E-cadherin. In polarized cysts (*A*) E-cadherin staining is strictly basolateral (arrows). During the formation of cellular extensions (*B*) E-cadherin staining is observed on membranes surrounding the extension in regions of cell-cell and cell-substrate contact (arrows). E-cadherin is excluded from the lumenal membrane (closed arrowhead), indicating that apical/basolateral polarity is retained. Cells neighboring the site of tubule initiation project apically under the extending cell (open arrowhead). Cells in both chains (*C*) and in regions of tubules without lumens (*D*) are completely surrounded by E-cadherin (arrowheads). In lumen-containing regions of tubules shown in *D*, E-cadherin staining (arrows) is basolaterally polarized. L, lumen. Bar, 10 μm.

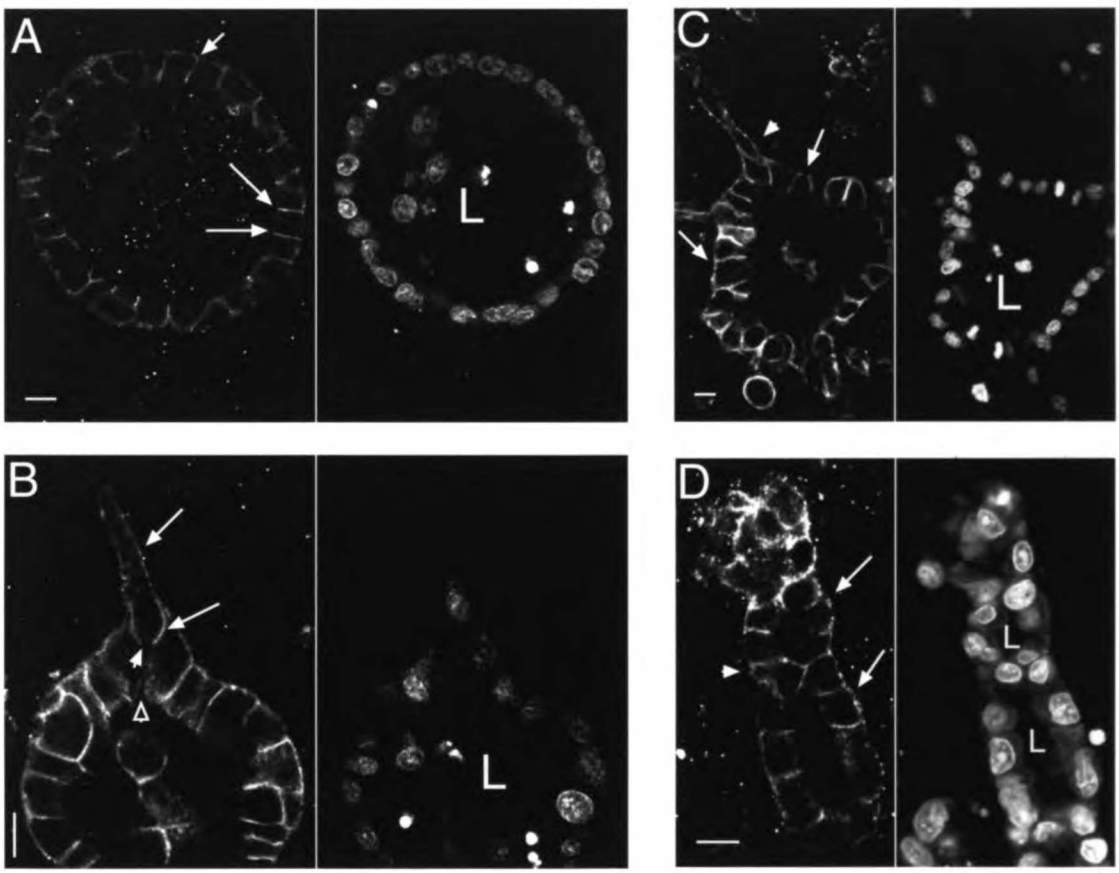

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Figure 2.2. Gp135 staining indicates that apical membrane polarity is lost during tubule morphogenesis and is restored as mature lumen-containing tubules form. In *A-E* each panel is a representative single confocal section taken from serially sectioned samples that were scanned simultaneously for FITC and ppI. Each image is split with gp135 staining on the left side and the nuclear staining on the right side. Arrows in *A*, *B*, *D*, and *E* denote gp135 localization. In *A*, arrows in the left and right image are aligned to demonstrate that in polarized cysts gp135 is strictly localized to the apical membrane facing the cyst lumen. In *B*, gp135 staining indicates that polarity of apical membranes is retained in extensions. In *C*, gp135 staining largely disappears from cells in chains that do not have contact with the cyst lumen (arrowheads). Only background staining remains. During the development of cords (*D*) gp135 staining reappears and is strictly localized to regions of cell-cell contact that represent newly forming lumenal membrane. In lumen-containing tubules (*E*) apical membrane polarity is fully restored. L, lumen. Bar, 10 μm.

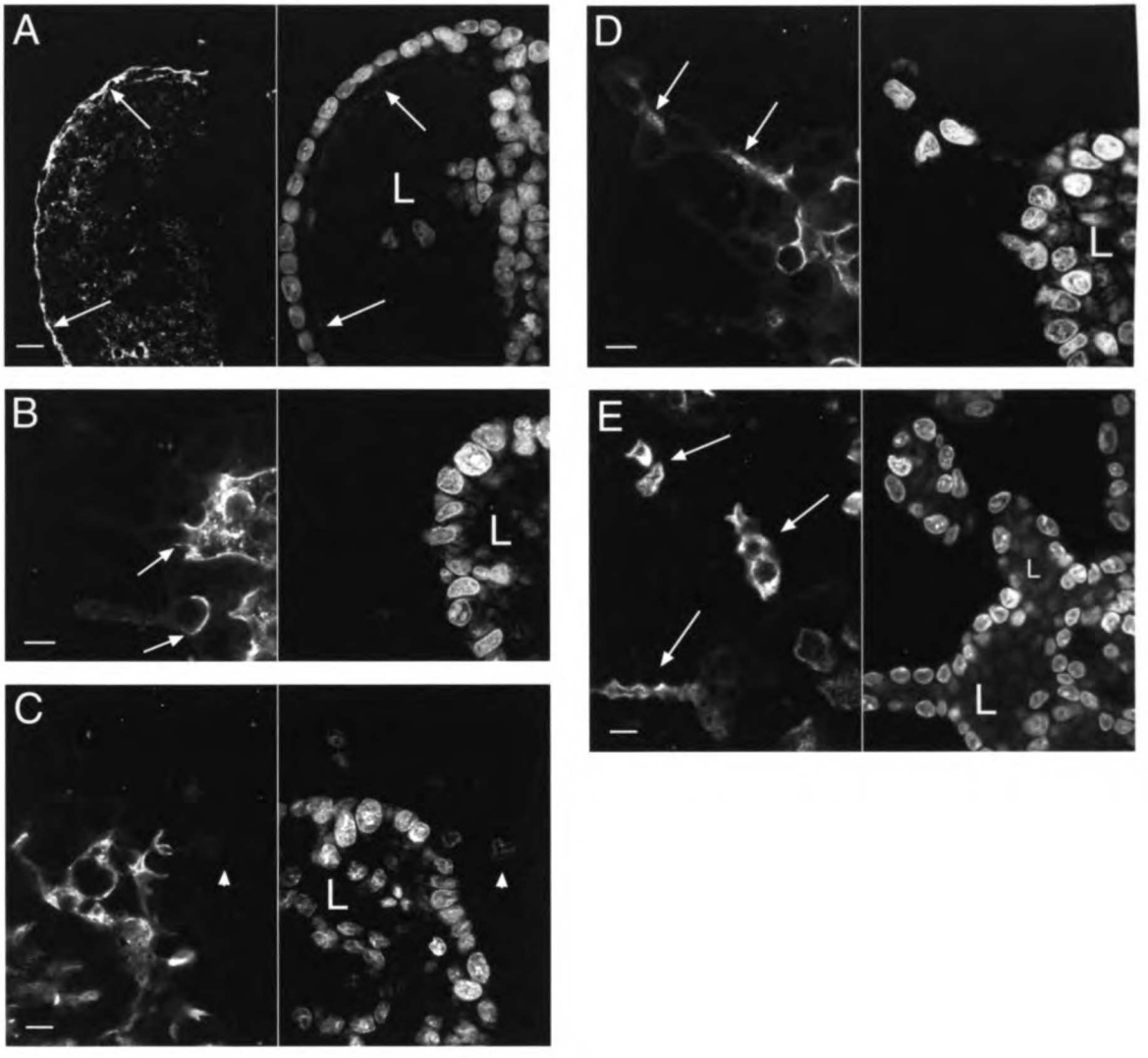
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Figure 2.3. Desmosomal plaque proteins appear in large intracellular pools during early stages of tubule development. Each panel is a representative single confocal section taken from serially sectioned samples that were scanned simultaneously for FITC and ppI. The images are split, with dpI/II shown in the left half and nuclei in the right half. (*A*) In polarized cysts dpI/II are localized to discrete spots along lateral cell membranes (arrows). (*B*) During formation of chains dpI/II appear in intracellular pools (arrows). L, lumen. Bar, 10 μm.

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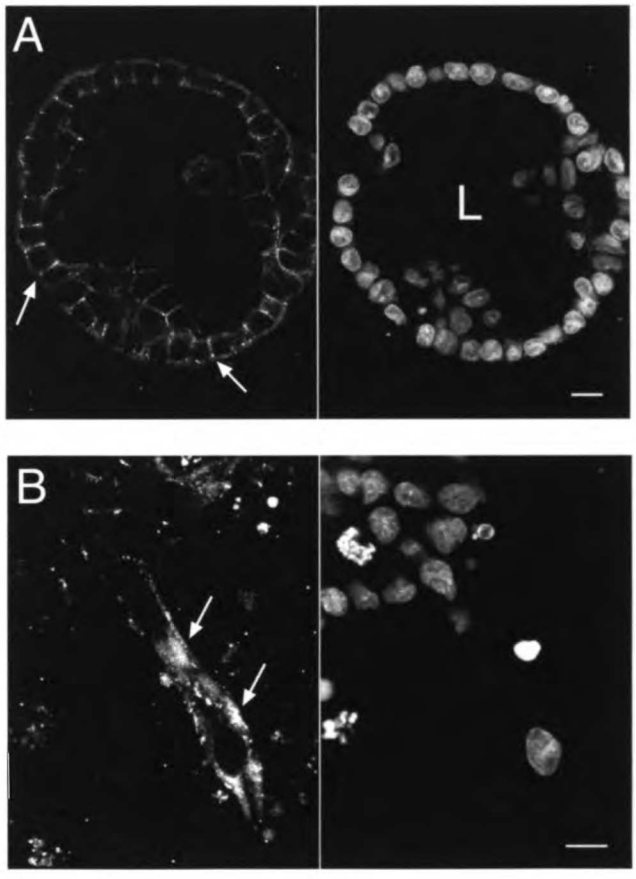
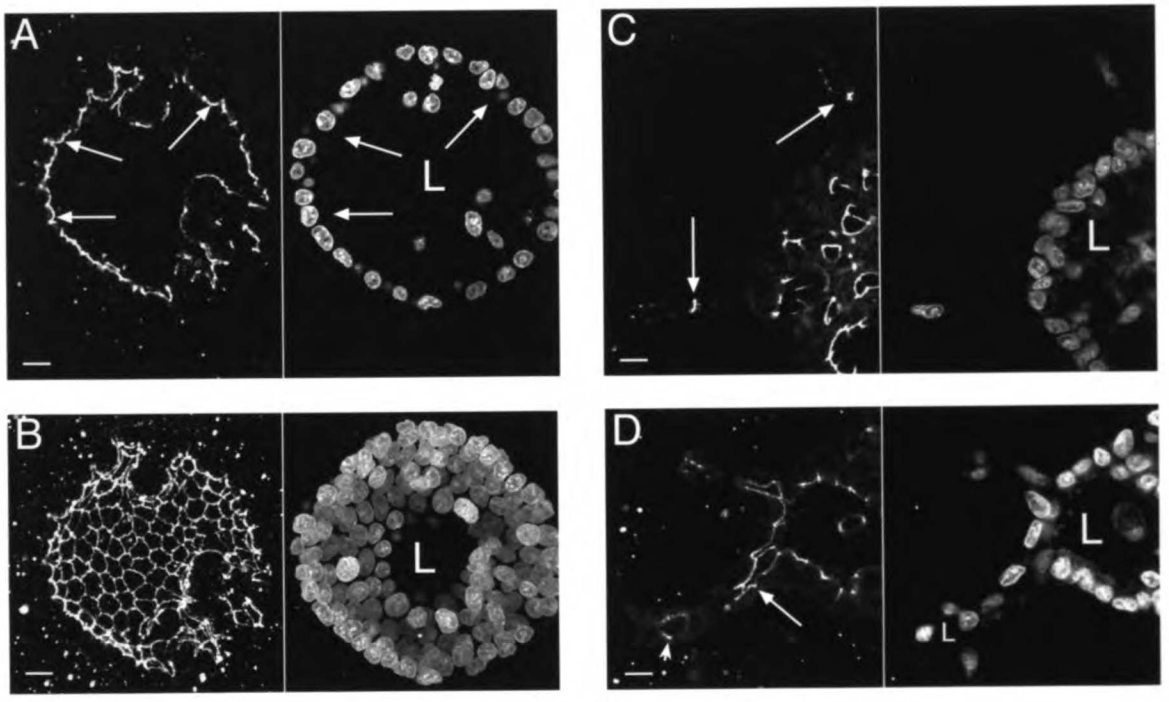


Figure 2.4. The tight junction marker, ZO-1 localizes at sites of cell-cell contact at all stages of tubule formation. In panels *A-D* serial confocal sections were collected from samples at different stages of tubule morphogenesis. FITC and ppI were scanned simultaneously. Each panel depicts a split image with ZO-1 on the left and nuclei on the right. *A*, *C*, and *D* are representative single confocal sections. *B* is a projected sum of thirteen 2 μm serial sections from the same sample as in *A*. In a cross section through a polarized cyst (*A*), arrows in the left and right images are aligned and show that ZO-1 is apically polarized. Sections for the projection in *B* start above the level of the nuclei of cells that are in the center of the image. ZO-1 staining above the level of the nuclei in the projected image demonstrates that ZO-1 is localized to the apical-most aspect of lateral cell-cell borders and forms a ring around each cell of the cyst. During the formation of chains (*C*), ZO-1 is concentrated at cell-cell borders between cells of the chains (arrows). During the development of a lumen-containing tubule (*D*) ZO-1 remains at cell-cell borders and localizes to the apical-most aspect of lateral membranes of both a newly formed lumen (arrowhead) and a more mature lumen (arrow). A cross section of a lumen-containing tubule containing an apical junctional complex is shown in an electron micrograph, *E* (box). The tight junction is clearly distinguishible at higher magnification (*F*, arrow). L, lumen. Bar (*A-D*), 10 μm. Bar (*E*), 2 μm. Bar (*F*), 1 μm.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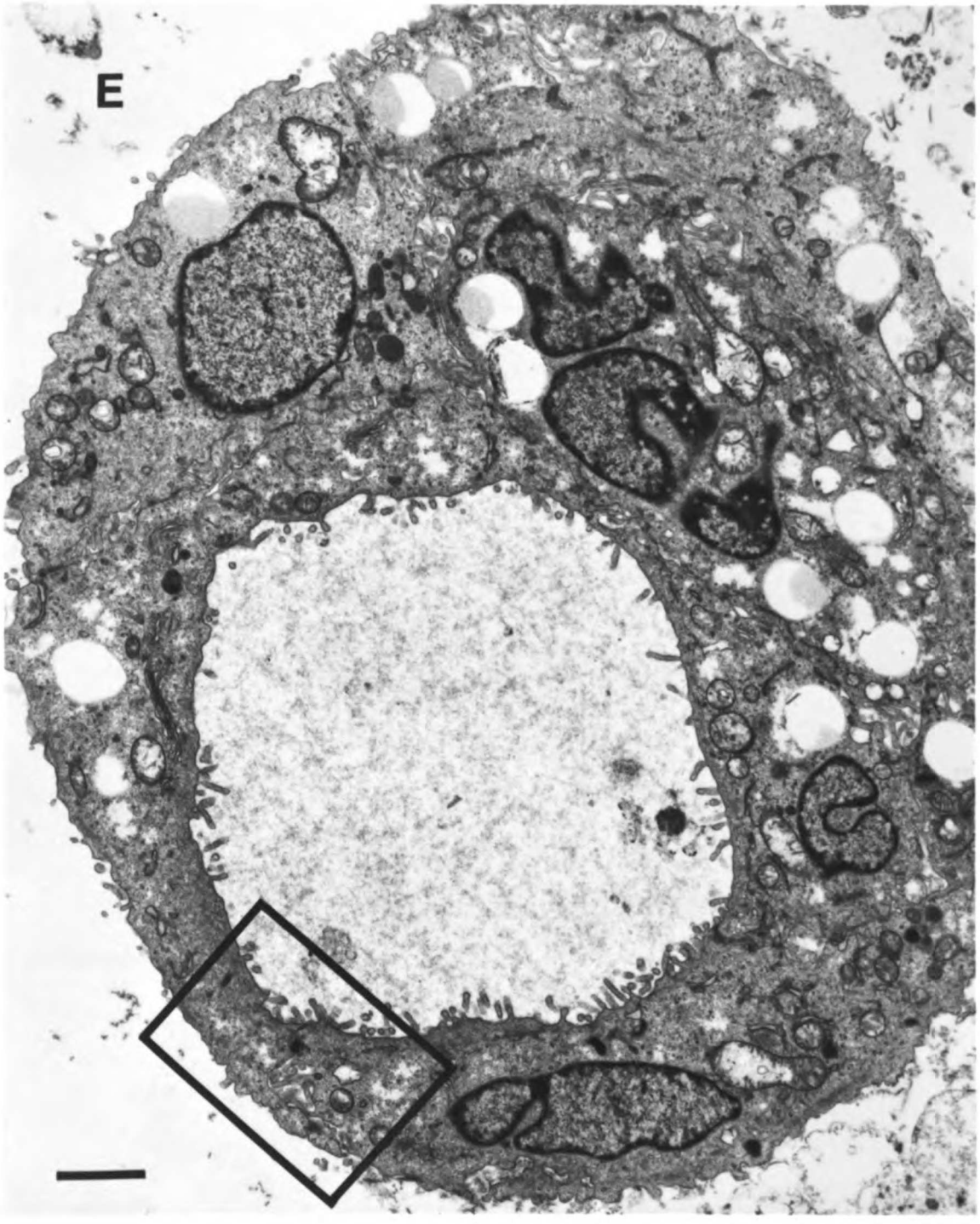
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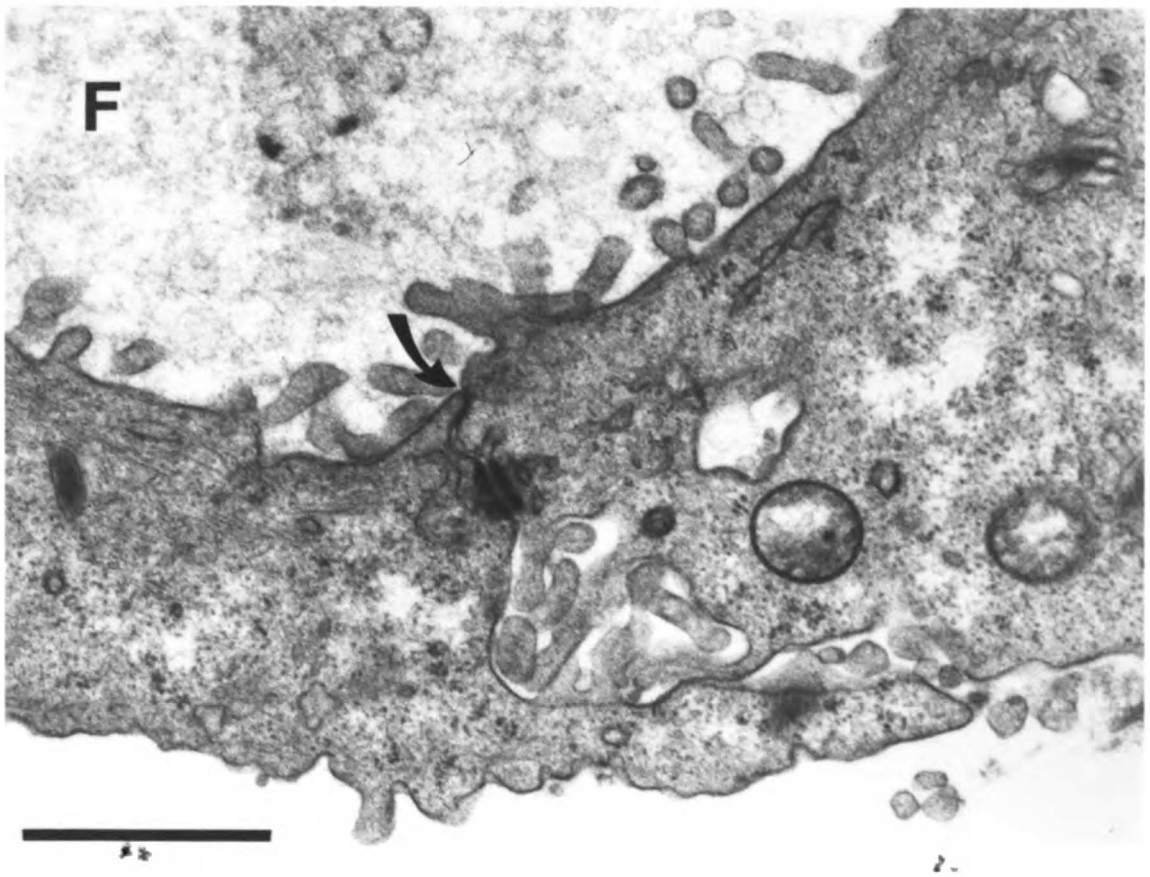
F

Figure 2.5. SF/HGF induces polarized MDCK cells to extend over each other and reshape a monolayer into a pseudostratified layer. Cocultures containing 90% untransfected, 10% pIgR-transfected MDCK cells were grown on transwell filters to form a polarized monolayer prior to 20 hours treatment with SF/HGF. FITC, ppI and TRITC images were simultaneously collected in X-Z confocal sections and represent E-cadherin, nuclei and pIgR, respectively. The images were split so that E-cadherin is on the left and both pIgR and nuclei are shown on the right sides of panels *A* and *B*. Note that pIgR staining fills the cytoplasm of transfected cells (arrowheads) but is distinguishable from the nuclei (arrows). (*A*) In a polarized monolayer, E-cadherin outlines the lateral borders of a transfected cell in which pIgR staining fills the apical cytoplasm. (*B*) After SF/HGF treatment E-cadherin staining at cell-cell borders of a pIgR-transfected cell clarifies that this cell extends over its neighbor, suggesting that SF/HGF causes cells of the monolayer to crawl over each other while retaining cell-cell contacts. Bar, 10 μm.

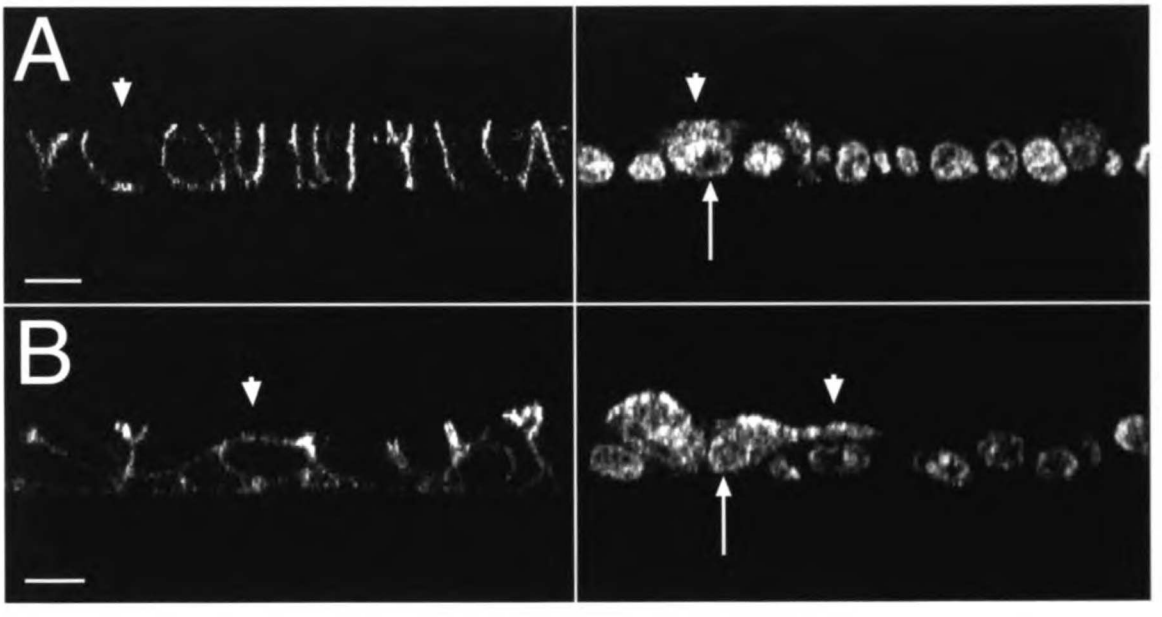
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Figure 2.6. SF/HGF alters the polarity of MDCK cells during pseudostratified layer formation. MDCK cells grown as confluent monolayers on Transwell filters were treated for 24 hours with or without SF/HGF. X-Z confocal sections, simultaneously scanned for FITC and ppI, were collected from monolayers and pseudostratified layers. Representative images for each panel are split so that E-cadherin (*A*, *B*) or gp135 (*C*, *D*) are shown on the left and nuclei (*A-D*) are shown on the right. In polarized monolayers E-cadherin staining is confined to the lateral membrane (*A*, arrowhead) and gp135 is strictly apical (*C*, arrow). After SF/HGF treatment E-cadherin surrounds some cells within the pseudostratified layer (*B*, arrowheads) and gp135 is localized at both apical membranes facing the medium and cell-cell borders (*D*, arrows). Bar, 10 μm.

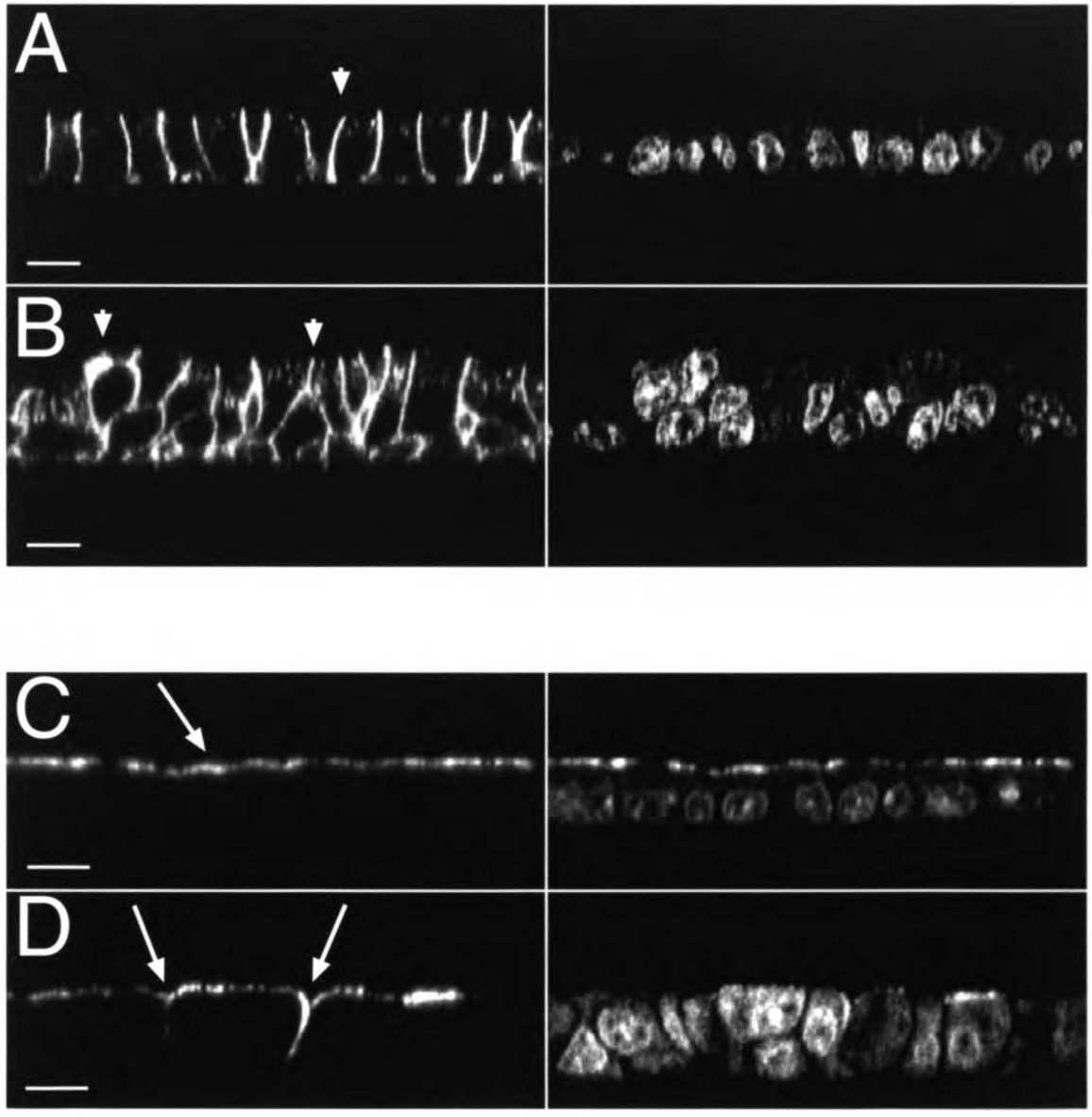
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Figure 2.7. SF/HGF causes intracellular accumulation of desmosomal proteins. X-Z confocal sections, simultaneously scanned for FITC and ppI, were collected from monolayers and pseudostratified layers. Representative images for each panel are split with staining for dpI/II shown on the left and staining for nuclei on the right. In a polarized monolayer punctate spots of dpI/II are observed at lateral cell-cell borders (*A*, arrow). After a 24 hour treatment with SF/HGF dpI/II are observed in large intracellular pools in cells extending from a pseudostratified layer (*B*, arrows). Bar, 10 μm.

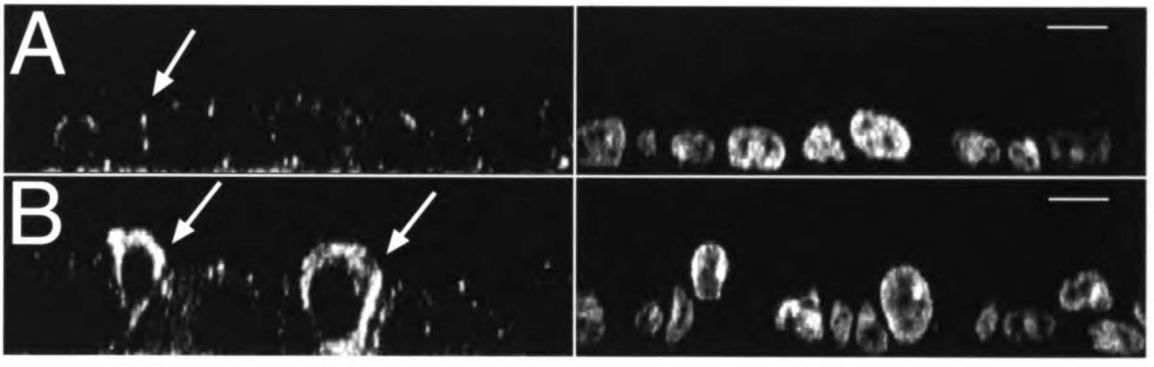
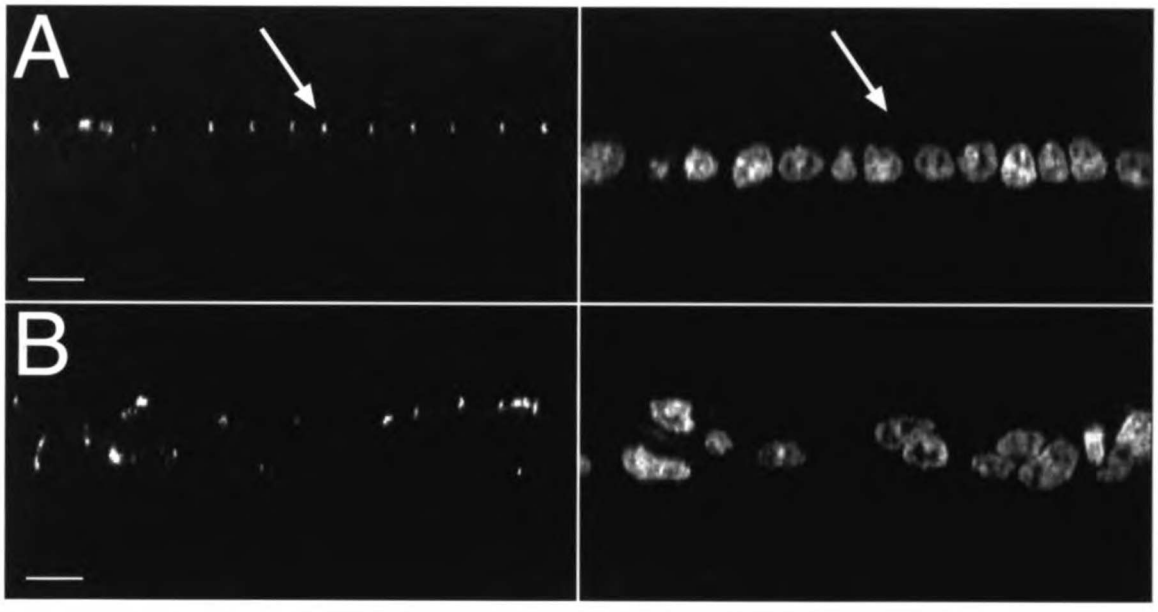

A
B

Figure 2.8. SF/HGF alters ZO-1 localization but intact rings of ZO-1 are maintained in pseudostratified layers. MDCK cells grown as confluent monolayers on Transwell filters were treated for 24 hours with or without SF/HGF. Confocal sections were collected in both the X-Z planes and X-Y planes of each sample. Images were simultaneously scanned for FITC and ppI. Representative images of a monolayer (A, C, E, G, I) and a pseudostratified layer (B, D, F, H, J) are shown. Each panel is split with FITC emission depicting ZO-1 staining on the left and ppI emission showing nuclei on the right. In A, an X-Z section of a polarized monolayer, punctate spots of ZO-1 are localized to the apical-most aspect of lateral cell-cell borders (arrows). Arrows are aligned in the left and right images to clarify the position of ZO-1 relative to the nuclei. In B, an X-Z section through a pseudostratified layer, punctate spots of ZO-1 are detected at cell-cell borders at multiple levels throughout the culture. X-Y sections from a polarized monolayer (C, E, G) and a pseudostratified layer (D, F, H) were collected in basal (C, D), central (E, F) and apical (G, H) regions of the samples. In the polarized monolayer ZO-1 staining is detected only in the apical section (G) and forms rings around individual cells. In a pseudostratified layer discontinuous staining for ZO-1 is observed in all planes of section (D, F, H). Projected images summed from complete series of X-Y sections collected at 0.5 μm increments through each sample are shown in I and J. The resulting image shown in I is almost identical to the single section in G and demonstrates that ZO-1 rings in a polarized monolayer are completely contained within the apical planes of section. In J, the projected image clarifies that ZO-1 staining in the pseudostratified layer is also in intact rings. Bar (*A* and *B*), 10 μm. Bar (*C-J*), 20 μm.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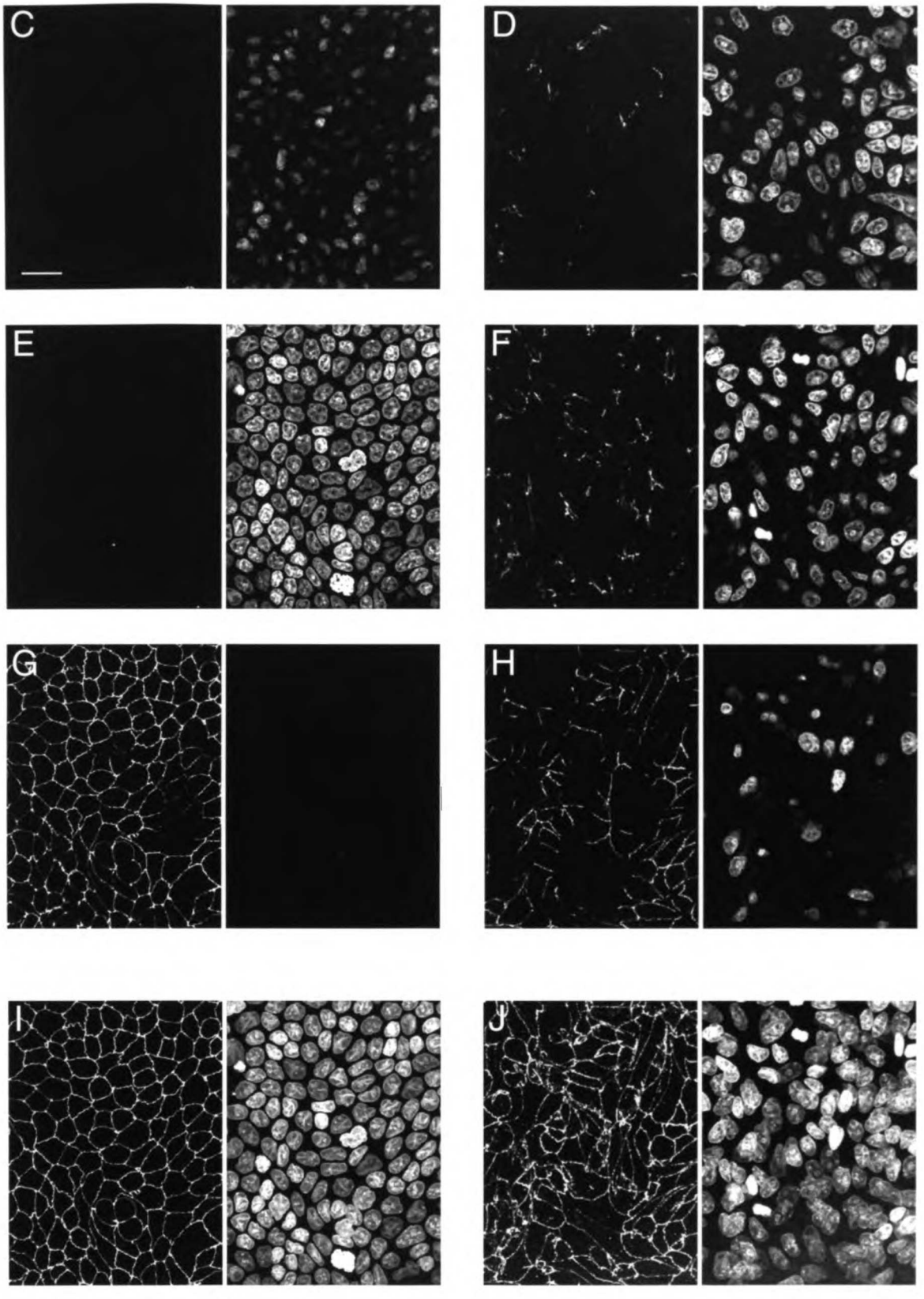
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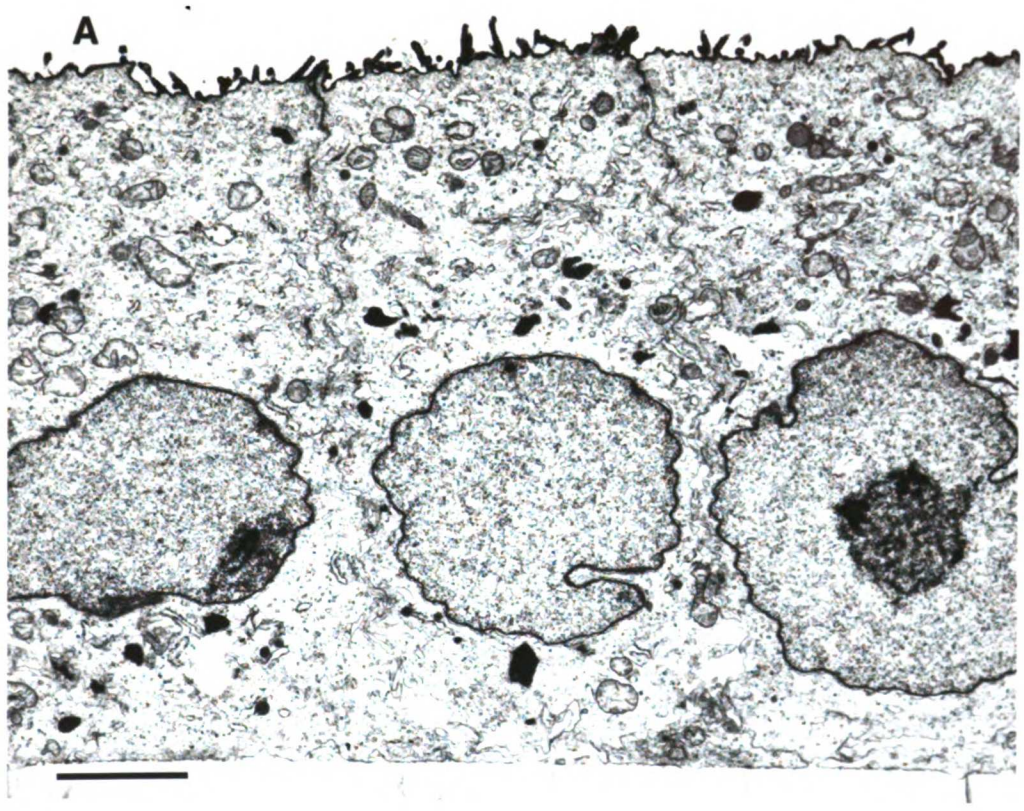
Figure 2.9. Tight junctions of SF/HGF-treated MDCK cell monolayers are impermeable to ruthenium red. MDCK cells grown as confluent monolayers on Transwell filters were treated for 24 hours with or without SF/HGF prior to fixing in the presence of apically applied ruthenium red and processing for electron microscopy. Sections cut in the X-Z plane of the Transwell filter are shown. Electron dense ruthenium red labels the apical side of both untreated (*A*) and SF/HGF-treated (*B*) MDCK cell layers. Ruthenium red is not detected on basal membranes of either untreated or SF/HGF-treated cultures. Bar, 10 μm.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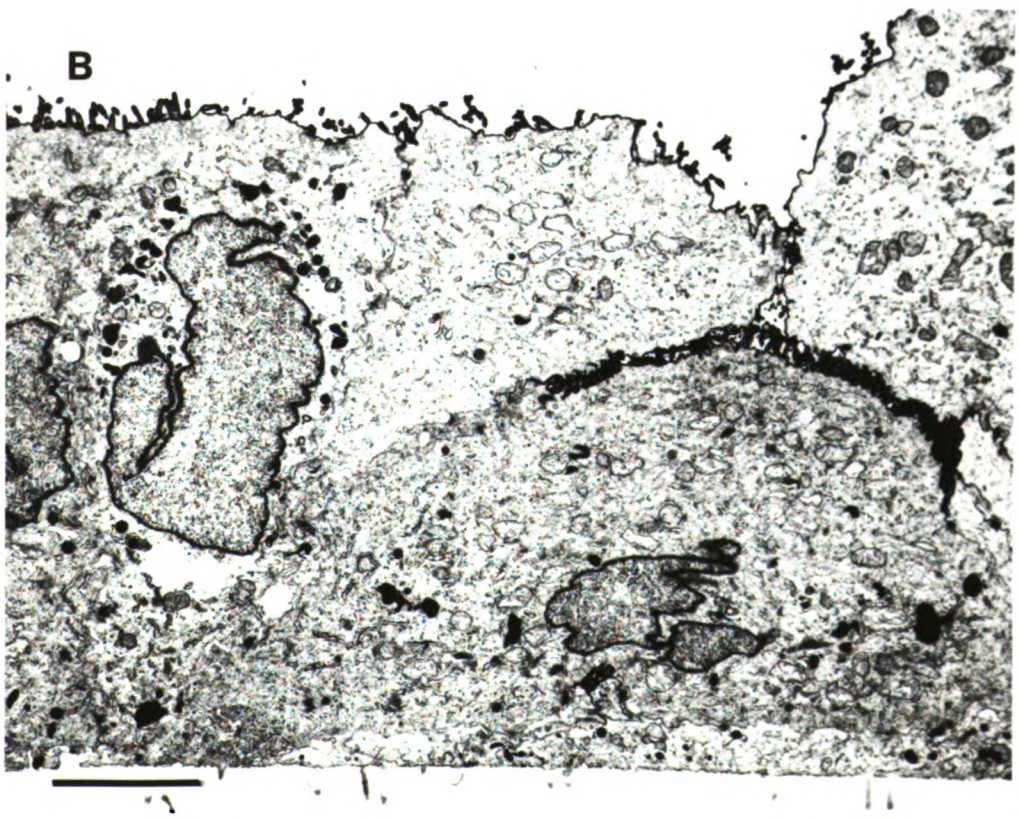
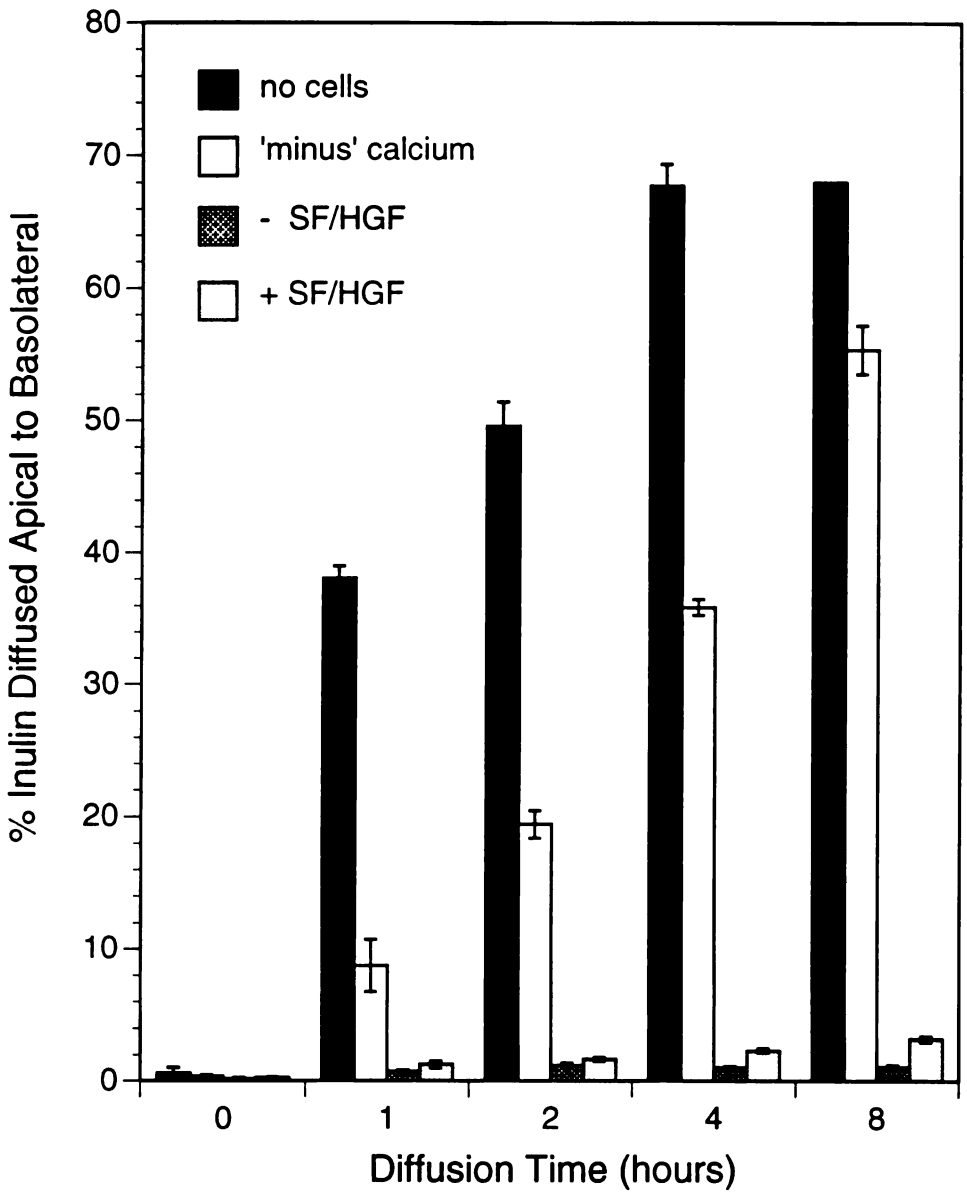

B

Figure 2.10. Analysis of inulin diffusion across Transwell filter-grown MDCK cell monolayers treated -/+ SF/HGF. MDCK cells were cultured for 4 days on Transwell filters to form polarized, confluent monolayers. Culturing was continued for 24 hours in the absence or presence of 100 ng/ml SF/HGF. Both confluent monolayers that were 'switched' into low calcium-containing medium 12 hours prior to the inulin diffusion assay ('minus' calcium) and Transwell filters cultured without cells were used as controls. For the diffusion assay the apical medium of each filter was replaced with ^{14}C-inulin-containing MEM/BSA and the basal medium was replaced with MEM/BSA - /+ SF/HGF. Aliquots were collected and counted after 0, 1, 2, 4, and 8 hours. Each bar represents mean +/- SEM (n=3). ■, no cells; □, 'minus' calcium; ■, - SF/HGF; ▨, + SF/HGF.


80
70
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50
40
30
20
10
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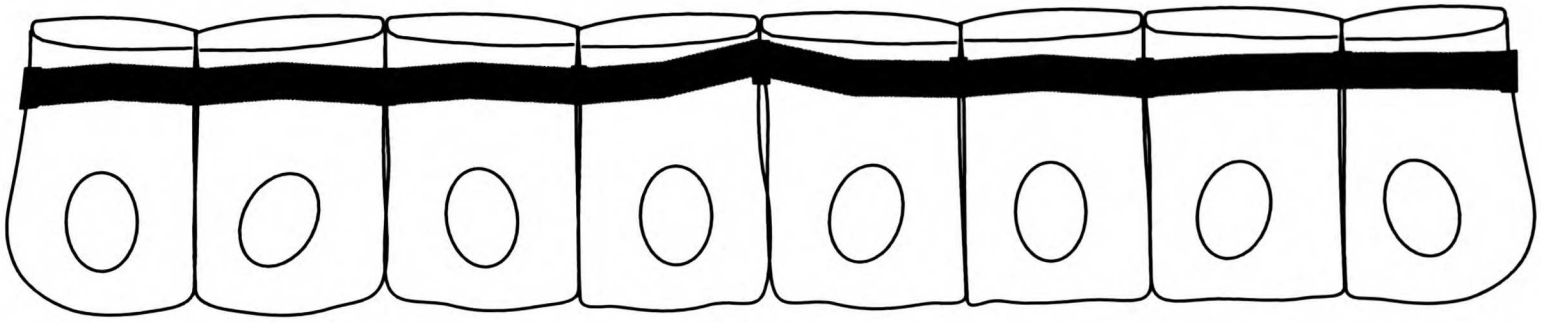
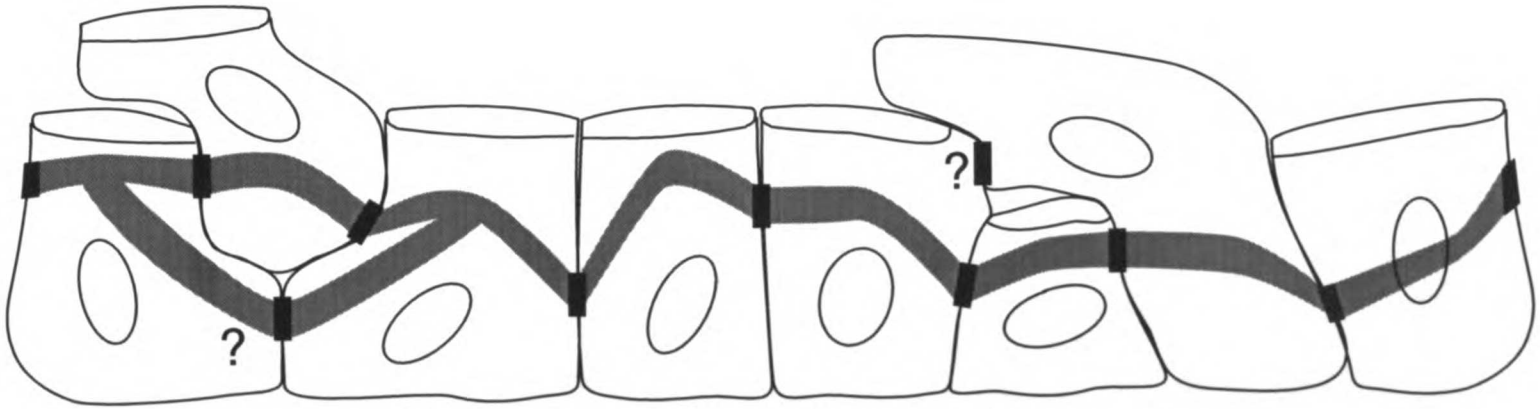
no cells
'minus' calcium
- SF/HGF
+ SF/HGF
% Inulin Diffused Apical to Basolateral
0
1
2
4
8
Diffusion Time (hours)

Figure 2.11. Diagram of tight junctions in monolayer cultures of MDCK cells -/+ treatment with SF/HGF. In polarized MDCK cell monolayers tight junctions (▮) are localized to the apical-most aspect of the lateral cell-cell borders where they form a continuous band around each cell that acts as a permeability barrier and limits membrane protein diffusion. After treatment with SF/HGF tight junctions are localized in multiple regions of the cell layer but retain morphologically intact rings and function to seal cell-cell borders. The (?) indicates that multiple tight junctions may form along the apical to basal stretch of individual lateral cell membranes during the transition from a monolayer to a pseudostratified layer.



Polarized MDCK Cell Monolayer



SF/HGF-treated MDCK Cell Monolayer

Figure 2.12. Model of tubule morphogenesis. Development of tubules from polarized cysts is a continuous process that passes through 4 morphologically distinguishible stages which we have defined as follows: (0) Polarized cysts; (1) Extensions; (2) Chains; (3) Cords; (4) lumen-containing tubules. See text for detailed explanation.

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CHAPTER THREE

SCATTER FACTOR/HEPATOCYTE GROWTH FACTOR INCREASES MDCK CELL TRANSEPITHELIAL RESISTANCE THROUGH A NOVEL MECHANISM

ABSTRACT

Scatter factor/hepatocyte growth factor (SF/HGF) induces mitogenesis, motogenesis, and tubulogenesis of cultured MDCK epithelial cells. In addition to these effects, we have recently reported that SF/HGF stimulates morphogenesis of tight, polarized MDCK cell monolayers into pseudostratified layers. Tight junctional rings remain morphologically intact in pseudostratified layers and act as barriers to diffusion of ruthenium red and inulin. In the present study we tested the effect of SF/HGF on transepithelial resistance (TER) during the morphogenesis of pseudostratified layers. In the absence of SF/HGF treatment MDCK cell monolayer TER was 80-100 ohm-cm^2. We show that, in response to 100 ng/ml SF/HGF, TER increased transiently 2-3-fold during the morphogenetic transition from monolayers to pseudostratified layers and then declined to baseline levels of around 100 ohm-cm^2 once pseudostratified layers formed. To determine if the effect of SF/HGF on TER was induced through direct activation of c-met, we used a system developed by Weidner, et al. (Weidner et al., 1993b), in which NGF activates a trk/met chimeric receptor transfected into MDCK cells and stimulates scattering, mitogenesis and tubulogenesis responses normally induced by SF/HGF. Although both SF/HGF and NGF at concentrations of 2.5 ng/ml induced scattering of MDCK cells containing the trk/met chimera, NGF at concentrations as high as 25 μg/ml had no effect on TER. This suggests that the effect of SF/HGF on TER is mediated through a novel mechanism.

INTRODUCTION

Scatter Factor/Hepatocyte Growth Factor (SF/HGF) is a fibroblast-derived growth factor (Stoker and Perryman, 1985) whose pleiotropic effects are dependent on both the target cell type and environmental context. In vivo, SF/HGF is important for the development of the placenta, liver, limb and kidney (Sonnenberg et al., 1993a; Sonnenberg et al., 1993b; Karp et al., 1994; Bladt et al., 1995; Schmidt et al., 1995; Uehara et al., 1995; Woolf et al., 1995), and induces morphogenesis of blood vessels (Bussolino et al., 1992; Grant et al., 1993; Naidu et al., 1994; Silvagno et al., 1995). In vitro, paracrine actions of SF/HGF cause increases in hepatocyte mitogenesis (Nakamura et al., 1987; Gohda et al., 1988; Miyazawa et al., 1989; Nakamura et al., 1989; Zarnegar and Michalopoulos, 1989), inhibit growth of tumor cells (Tajima et al., 1991; Shiota et al., 1992), stimulate motogenesis and invasiveness of epithelial and endothelial cells (Stoker and Gherardi, 1987a; Gherardi et al., 1989; Rosen et al., 1990; Weidner et al., 1990; Rosen et al., 1991; Weidner et al., 1991; Bussolino et al., 1992; Silvagno et al., 1995) and induce morphogenesis of epithelial tubes (Montesano et al., 1991a; Montesano et al., 1991b; Soriano et al., 1995).

SF/HGF interacts with both high affinity (K_D = 17-200 pM) and low affinity (K_D = 0.26-6.7 nM) binding sites on cell surfaces (Higuchi and Nakamura, 1991; Naldini et al., 1991b; Tajima et al., 1991; Arakaki et al., 1992; Komada et al., 1992; Tajima et al., 1992). The high affinity SF/HGF binding site has been identified as the c-met protooncogene (c-met) (Higuchi et al., 1992; Komada et al., 1992), a tyrosine kinase receptor (Cooper et al., 1984; Park et al., 1987; Giordano et al., 1989b; Naldini et al., 1991a). Multiple effects of SF/HGF have been shown to be induced through direct activation of c-met (Komada and Kitamura, 1993; Weidner et al., 1993b). For example, in MDCK epithelial cells that are transfected with a chimeric receptor containing the ectodomain of the nerve growth factor (NGF) receptor (trk) and the cytodomain of c-met,

direct activation of the trk/met chimeric receptor with nerve growth factor (NGF) stimulates phosphorylation of the c-met cytoplasmic domain, increases cell proliferation, motility and invasiveness, and induces morphogenesis of tubules (Weidner et al., 1993b).

Low affinity binding interactions of SF/HGF with the cell surface can be inhibited or disrupted by the presence of an excess of heparin, suggesting that low affinity SF/HGF binding sites are composed of glycosaminoglycans (GAGs) (Zarnegar et al., 1990; Naldini et al., 1991b; Naka et al., 1993). A distinct function for the interaction of SF/HGF with low affinity cell surface binding sites has not yet been determined. However, studies in which the presence of soluble or substrate-bound heparin altered the mitogenic potential of SF/HGF suggested that interactions with low affinity binding sites regulate SF/HGF activity (Zarnegar and Michalopoulos, 1989; Naka et al., 1993; Kato et al., 1994; Rahimi et al., 1994; Allen et al., 1995; Zioncheck et al., 1995). It is unclear whether these effects are induced by direct activation of low affinity sites or through modulation of the interaction of SF/HGF with c-met. Heparin and other heparan sulfates have been shown to modulate the ability of SF/HGF to bind to or activate c-met. For instance, Naka et al. (Naka et al., 1993) showed that pretreatment of cell cultures with excess heparin or heparatinase decreased binding of SF/HGF to c-met. Zioncheck, et al. (Zioncheck et al., 1995) showed that both heparin and heparan sulfate can induce oligomerization of SF/HGF that correlates with an increase in phosphorylation of c-met and an increase in cell proliferation.

Recent work from this laboratory has shown that SF/HGF induces morphogenesis of tight, polarized monolayers of MDCK epithelial cells into pseudostratified layers. The localization of the tight junctional protein, ZO-1, is altered in pseudostratified layers but tight junctions are functionally intact, as assessed by morphologically continuous rings of ZO-1 and inhibition of paracellular diffusion of inulin. In this study we tested the effects of SF/HGF on transepithelial resistance (TER). We found that during morphogenesis of pseudostratified layers there is a transient increase in TER that returns to baseline levels once pseudostratified layers are formed. We then asked, using MDCK cells transfected

with a trk/met chimeric receptor (Weidner et al., 1993b), whether effects of SF/HGF on electrically tight, polarized monolayers were induced through direct activation of c-met. We found that NGF stimulates MDCK cells containing the trk/met chimeric receptor to scatter but does not affect TER, suggesting that the effects of SF/HGF on TER are mediated through a novel mechanism.

MATERIALS AND METHODS

Cells/Reagents

Recombinant human HGF (SF/HGF) was generously provided by R. Schwall (Genentech, South San Francisco). Rabbit polyclonal anti-HGF antibody was obtained from J. Rubin (NCI, Bethesda, MD). Mouse monoclonal antibody DO24 (mAb DO24) was kindly provided by T., Crepaldi, M. Prat, and P. Comoglio (University of Turin, Turin, Italy). NGF was generously supplied by W. Mobley (UCSF, San Francisco, CA). Porcine intestinal mucosal heparin was purchased from Sigma (St. Louis, MO). MDCK type II cells transfected with pSV2-neo and either a ß-actin promoter expression vector (pBAT) alone (Nagafuchi and Takeichi, 1988) or pBAT vector containing a trk/met hybrid receptor cDNA (WTH2) (Weidner et al., 1993b) were kindly provided by M. Weidner and W. Birchmeier (Max Delbruck Center for Molecular Medicine, Berlin, Germany). MDCK type II cells, either untransfected or expressing the wild type rabbit polymeric immunoglobulin receptor (pIgR) (Mostov and Deitcher, 1986) were maintained in MEM containing Earle's balanced salt solution (MEM-EBSS; Cellgro, Mediatech, Inc., Washington, D. C.) supplemented with 5% FCS (Hyclone, Logan, UT), 100 U/ml penicillin, and 100 mg/ml streptomycin in 5% CO_2/95% air. Identical culturing conditions were used to maintain control transfected MDCK cells expressing neo resistance only and MDCK cells expressing the trk/met chimeric receptor, except that 700 µg/ml G418 was included in the medium.

Production of fibroblast conditioned media containing SF/HGF

To obtain conditioned medium containing SF/HGF, MRC5 human lung fibroblasts (ATCC #CCL171) were grown to confluence in DME (DMEH21, obtained from the UCSF cell culture facility) containing 10% FBS (Hyclone, Logan, UT), 100 U/ml penicillin, and 100 mg/ml streptomycin in T75 tissue culture flasks (Corning, Corning, NY) or, for 2X

conditioned medium, in T150 ridged tissue culture flasks (Corning, Corning, NY). The medium was replaced with 30 ml of fresh medium and cell culture was continued for 3 days. Conditioned medium was collected, centrifuged to remove cell debris, and frozen at -20°C until use. Control conditioned medium was obtained by growing D550 human foreskin fibroblasts (a kind gift from Ward D. Peterson, Detroit Medical Center, Detroit) under the same culture conditions as for MRC5 cells, but in MEM-EBSS supplemented with 0.1 mM nonessential amino acids ((100X stock, UCSF cell culture facility), 0.1% lactalbumin hydrolysate (Sigma, St. Louis, MO), 0.1 mg/ml sodium pyruvate (100X stock, UCSF cell culture facility) and 10% FBS.

Assay for Transepithelial Resistance

Cells were seeded at confluent density onto Transwells (cat. #3401, polycarbonate membrane, 12 mm diameter, 0.4 μm pore size; Costar, Cambridge, MA) and grown in MEM-EBSS, 5% FBS for four to six days, with daily medium changes, prior to use. Confluent, polarized monolayers were treated for 20 hours with 50% D550 or MRC5 conditioned medium (CM) diluted 1:1 in MEM-EBSS, 5% FBS applied to the apical, basal, or apical + basal sides of the Transwell filter. Alternatively, confluent, polarized Transwell cultures were treated basally with either SF/HGF, mAb DO24, or NGF that was diluted into MEM-EBSS, 5% FBS. For antibody inhibition of SF/HGF, rabbit polyclonal anti-HGF antibody was diluted to a final concentration of 27 μg/ml into MEM-EBSS, 5% FBS containing 100 ng/ml SF/HGF and rotated for 2 hours at 4°C prior to use on cell cultures.

To determine the transepithelial resistance (TER) cultures were washed one time quickly and then equilibrated for 10 minutes with MEM containing Hank's Balanced salts, 0.6%BSA, 20 mM Hepes, pH 7.3 (MEM/BSA) at 37°C. A Millicell-ERS instrument (Millipore Continental Water Systems, Bedford, MA) was used to measure transepithelial resistance, being careful to keep distances from the bottom of the well and between the

electrodes standardized. Resistances were calculated after subtracting background values obtained from blank transwells that had been cultured in parallel.

Assay for Scattering

Cells were trypsinized from confluent 10 cm tissue culture plates. After resuspending and counting, 2 mls of cells at a density of 5 X 10^4 cells/ml were plated into each 35 mm well. Cells were cultured for 8-9 hours in a 5% CO_2/95% air incubator to allow cells to attach and grow into small islands containing around 5-20 cells each. Cultures were then treated for 24-28 hours with SF/HGF, mAb DO24, or NGF and photographed using a Nikon camera attached to a Zeiss inverted microscope outfitted with phase and Hoffman Modulation Contrast optics.

RESULTS

To analyze the effect of SF/HGF on transepithelial resistance (TER) MDCK cells were plated at confluent density and grown on Transwell filters to form electrically tight, polarized monolayers and then cultured for an additional 20 hours in the presence or absence of MRC5 fibroblast conditioned medium containing SF/HGF. The TER of MDCK cell cultures treated with SF/HGF-containing medium was increased more than 2-fold compared to untreated cultures. In addition, treatment from the basal side of the filter alone was sufficient to induce the increase in TER (Figure 3.1 A). Similar results were obtained using a clone of MDCK cells transfected with the polymeric immunoglobulin receptor. To test that the effect on TER was due specifically to SF/HGF we treated the basolateral side of filter-grown MDCK cell monolayers with medium containing 100 ng/ml recombinant human SF/HGF or with medium that had been preincubated with both 100 ng/ml SF/HGF and anti-HGF polyclonal antibodies. Figure 3.1 B shows that antibody pretreatment almost completely blocked the SF/HGF-induced increase in TER. These results indicate that SF/HGF activation of a basolaterally localized receptor mechanism causes an increase in MDCK cell monolayer TER. Therefore, all further experiments were carried out by treating monolayers with SF/HGF on the basolateral side only.

Previously, we found that SF/HGF induces filter-grown monolayers of MDCK cells to form pseudostratified layers (see chapter two). To determine if SF/HGF affects TER during the transition from a monolayer to a pseudostratified layer we measured the TER of Transwell filter cultures of MDCK cells after various time periods in the presence and absence of SF/HGF. We found (Figure 3.2) that continuous SF/HGF treatment causes a transient increase in TER, peaking around 24 hours and then declining to baseline by 48 hours. The rise in TER correlates in time with the morphological transition period from a monolayer to a pseudostratified layer, whereas the decline to baseline occurs once a pseudostratified layer has formed.

Our initial studies with MRC5 fibroblast conditioned medium suggested that the concentration of SF/HGF required to cause an increase in TER was much higher than that required to induce scattering (data not shown). Therefore, we tested different concentrations of recombinant human SF/HGF (0-250 ng/ml) for effects on TER versus scattering activity. Figures 3.3 A-C show that MDCK cells plated in small colonies elicit a full scattering response when treated with 2.5 ng/ml SF/HGF (compare Figures 3.3 B and C). In contrast, the dose-response of TER to SF/HGF (Figure 3.3 D) demonstrates that 2.5 ng/ml SF/HGF has no effect on TER and that peak effects are induced by SF/HGF concentrations around 100 ng/ml.

SF/HGF is a heparin-binding growth factor (Nakamura et al., 1987; Gohda et al., 1988; Zarnegar and Michalopoulos, 1989) that can interact with specific residues on heparan sulfate (HS) (Lyon et al., 1994). This suggests that low affinity SF/HGF binding sites are GAGs such as heparin-like molecules or heparan sulfate proteoglycans (HSPG). To determine if interaction with heparin-like molecules affects SF/HGF-induced increases in TER, we measured the TER of Transwell filter-grown monolayers of MDCK cells that were simultaneously treated with SF/HGF (0-100 ng/ml) and heparin (0.1 μg/ml) for 20 hours. TER of cultures treated with both SF/HGF and heparin were compared to that of untreated cultures and monolayer cultures treated singly with either SF/HGF or heparin (Figure 3.4). Heparin potentiated the effect of 25 and 50 ng/ml SF/HGF, doses that had intermediary effects by themselves. Thus, the interaction with heparin-like low affinity binding sites modulates the effect of SF/HGF on TER.

To test whether direct activation of c-met causes an increase in TER similar to that seen with SF/HGF we took the following two approaches: 1) stimulation of MDCK cells with an activating antibody of c-met, DO24 (Prat et al., 1991a; Prat et al., 1991b) and 2) NGF stimulation of transfected MDCK cells expressing a trk/met chimeric receptor containing the ligand binding domain of the nerve growth factor receptor and the transmembrane and tyrosine kinase domains of c-met (Weidner et al., 1993b). The

scattering activity of 2 and 10 nM DO24 is shown in Figures 3.5 A-C. DO24 induced a complete MDCK cell scattering response at a 2 nM concentraton (compare Figures 3.5 B and C). The effect of DO24 on TER was tested at 2, 10, 100, and 200 nM, concentrations that include and far exceed the doses necessary to induce scattering. DO24 induced a small increase in TER that was similar at all concentrations tested, demonstrating that direct antibody activation of c-met produces only a partial effect on TER.

Weidner, et al. (Weidner et al., 1993b) showed previously that all known biological effects of SF/HGF, including cell scattering, invasiveness, morphogenesis, and proliferation are induced by NGF treatment of MDCK cells that are expressing a trk/met chimeric receptor. This demonstrated that direct activation of c-met transduces these cellular responses. The effects of both SF/HGF and NGF could be tested with trk/met transfected MDCK cells because they retain expression of the endogenous c-met receptor. Therefore, we compared the effects of SF/HGF and NGF on the TER of control (pSV2-neo) and trk/met transfected MDCK cells to determine whether the same mechanism, direct activation of c-met, also transduces this response. We first assayed scattering as a functional measure of SF/HGF and NGF activity in our culture system. Similar to the results shown above for pIgR-transfected MDCK cells, 2.5 ng/ml SF/HGF produced a complete scattering response with both control and trk/met clones (Figures 3.6 A-D). Treatment with 2.5 ng/ml NGF has no effect on scattering of control clones (Figures 3.6 A, E and G). However 2.5 ng/ml NGF induces a complete scattering response with trk/met-expressing MDCK cells (Figures 3.6 B, F and H) similar to the effect of 2.5 ng/ml SF/HGF (compare Figures 3.6 D and F). This confirmed that stimulation of the trk/met chimera with NGF fully activated c-met transduction of the scattering response. We then compared the effects of NGF and SF/HGF on TER. Figure 3.7A shows that SF/HGF causes an increase in the TER of monolayer cultures of both control-transfected and trk/met MDCK cells, with peak effects at 100 ng/ml. NGF, at concentrations between 2 and 25,000 ng/ml, does not have any effect on TER of either control or trk/met transfected

MDCK cell cultures (Figure 3.7 B). Therefore direct activation of the trk/met chimeric receptor with NGF is not sufficient to cause an increase in TER. These results suggest that SF/HGF affects MDCK epithelial cell TER through a novel mechanism that may involve interaction of SF/HGF with low affinity binding sites.

DISCUSSION

In this study we have demonstrated that basolateral treatment of polarized MDCK cell monolayers with SF/HGF causes a transient increase in TER. The timing of this response correlates with the transition of a MDCK cell monolayer into a pseudostratified layer (Pollack, et al., see chapter 2), confirming that tight junction functional integrity is maintained during SF/HGF-induced morphogenesis of pseudostratified layers. In addition, we have obtained evidence that the effect of SF/HGF on TER is stimulated through a novel SF/HGF signalling mechanism. This evidence is based on the following observations: 1) the concentration of SF/HGF required to stimulate an increase in TER is much higher than that required to stimulate scattering, indicating a role for low affinity binding sites; and 2) direct activation of the c-met signalling pathway stimulates all of the known effects of SF/HGF on MDCK cells (Weidner et al., 1993b) but not changes in TER.

Concentrations of SF/HGF that are sufficient to stimulate an increase in MDCK cell TER are at saturating levels for the high affinity SF/HGF receptor, c-met, which has a K_D equal to 20 pM (Tajima et al., 1992). However, MDCK cells also contain lower affinity, higher capacity SF/HGF binding sites (Tajima et al., 1992). These sites have an affinity that is ten-fold lower than the high affinity receptor. The concentrations of SF/HGF that induce an increase in TER are close to the K_D of the low affinity MDCK cell surface SF/HGF binding sites. This suggested that interactions of SF/HGF with low affinity sites may be important for mediating the effects on TER.

The ability of sulfated oligosaccharides to enhance SF/HGF activity (Masumoto and Yamamoto, 1993; Kato et al., 1994; Allen et al., 1995; Zioncheck et al., 1995) has suggested that the interaction of SF/HGF with low affinity binding sites has a role in SF/HGF-mediated signalling. This has been further supported by the findings that SF/HGF binds to specific heparan sulfate structures and sulfoglycolipids that are found

endogenously expressed at cell surfaces of various tissues and that these interactions are heparin-sensitive (Kobayashi et al., 1994; Lyon et al., 1994; Ashikari et al., 1995). We have found that low concentrations of heparin potentiate the effect of submaximal doses of SF/HGF on MDCK cell TER. In cells, such as MDCK, which express both the high affinity c-met receptor and low affinity binding sites, the low affinity sites may have a role in sequestering SF/HGF at the cell surface and either present SF/HGF to c-met to enhance c-met signalling or alternatively, form complexes with HGF and c-met which may modulate c-met signalling.

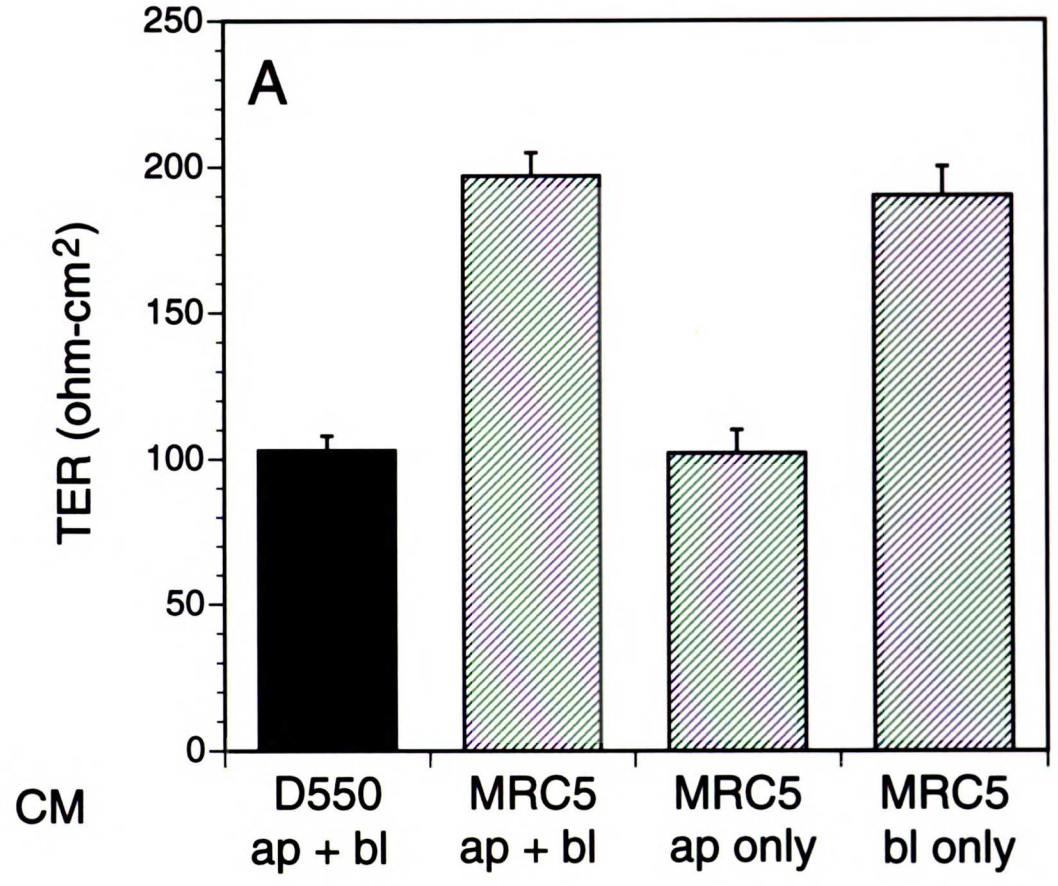
Previous studies have shown that direct activation of the endogenous c-met receptor with mAb DO24 is sufficient to stimulate tyrosine phosphorylation of c-met, tubulogenesis, and scattering of both endothelial cells and MDCK epithelial cells (Silvagno et al., 1995). Direct activation of c-met signalling through NGF stimulation of trk/met chimeric receptors also induces multiple effects on MDCK cells, including activation of the met tyrosine kinase, tubulogenesis, and MDCK cell scattering. However, neither of these agonists were able to stimulate an increase in TER. The ability to dissociate activation of scattering from stimulation of TER indicates that distinct signalling mechanisms are required to affect TER. Interactions of mAb DO24 and NGF with the cell surface are specific for c-met and trk/met, respectively. The absence of interaction of these agents with low affinity sites may preclude the formation of complexes with the receptor that may normally modulate the response to SF/HGF. For example, interaction of SF/HGF with low affinity sites at the cell surface may enhance formation of clusters of met receptors. Since c-met also contains potential GAG binding domains, trimeric complexes may form between cell surface HSPGs or sulfoglycolipids, SF/HGF, and c-met. Alternatively, SF/HGF bound to low affinity sites may be more readily available to interact with urokinase that is bound to its receptor at the cell surface. Urokinase has been shown to cleave pro-SF/HGF to its active two-chain form, both in vitro (Naldini et al., 1992) and at the cell surface (Naldini et al., 1995) and has been identified in cell surface complexes

containing both c-met and SF/HGF (Naldini et al., 1995). We used SF/HGF in our assays of TER that is already greater than 90% activated, two-chain form. Therefore, an increase in the formation of active SF/HGF is unlikely to explain the ability of SF/HGF, but not DO24 or NGF to affect TER. However, we suggest that the formation of complexes at the cell surface, that contain low affinity SF/HGF binding sites, SF/HGF and c-met and/or urokinase and urokinase receptor, modulates signalling through c-met and stimulates an increase in TER.

The increase in TER that we observe in response to SF/HGF contrasts with studies by Nusrat, et al. (Nusrat et al., 1994), in which recombinant human SF/HGF caused a decrease in TER of T84 intestinal cell monolayers and mouse SF/HGF caused a similar decrease with MDCK cells. It is possible that variations in culture environment resulted in this difference in effect. We have tested four different subclones of MDCK cells with recombinant human SF/HGF concentrations ranging from 2.5-500 ng/ml. In our hands, higher doses of SF/HGF can shift the timecourse so that peak responses occur earlier and decline to baseline earlier but treatment of Transwell filter-grown cultures of MDCK cells with SF/HGF for up to 72 hours does not cause a decline of TER below baseline. Culturing MDCK cells on crosslinked or uncrosslinked collagen-coated filters reduced the baseline TER, but fold increases in TER in response to either 100 or 500 ng/ml SF/HGF were similar to that observed with uncoated Transwell filters.

We have demonstrated that SF/HGF induces an increase in MDCK cell TER and provide the first report of an effect of SF/HGF that is not stimulated by direct activation of c-met. This assay system may provide a useful tool to find low affinity SF/HGF binding sites that have functional significance. Once functionally significant molecules are identified, the mechanistic contribution of these molecules to the multiple actions of SF/HGF can be identified.

Figure 3.1. SF/HGF stimulates an increase in the TER of polarized MDCK cell monolayers through a basolaterally localized signalling mechanism. Polarized monolayers of Transwell filter-grown MDCK cells were cultured +/- conditioned medium (CM) or SF/HGF for 20 hours. TER was assayed as described in materials and methods. (*A*) Monolayers were treated apically, basolaterally or both apically and basolaterally with 50% D550 or MRC5-CM; data shown are pooled results of 8-10 monolayers for each treatment. (*B*) Monolayers were treated basally +/- 100 ng/ml SF/HGF or with 100 ng/ml SF/HGF pretreated with 27 µg/ml rabbit anti-human HGF polyclonal antibodies; n=3 for each treatment. Each bar represents mean +/- SEM. ■, control (D550 CM or MEM); ▨, MRC5 CM; ⊠, SF/HGF; ■, SF/HGF + anti-HGF.


A
TER (ohm-cm2)
250
200
150
100
50
0
CM
D550
ap + bl
MRC5
ap + bl
MRC5
ap only
MRC5
bl only

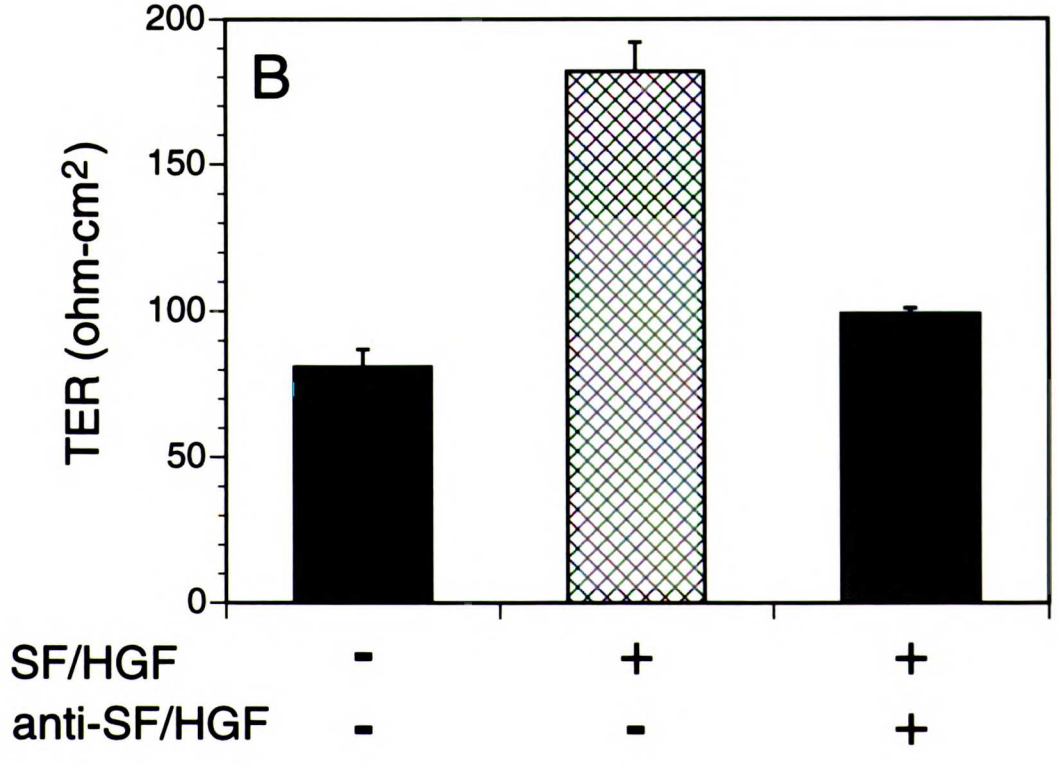

B
TER (ohm-cm2)
200
150
100
50
0
SF/HGF
anti-SF/HGF
-
-
+
-
+
+

Figure 3.2. The effect of SF/HGF on TER is transient. Polarized monolayers of Transwell filter-grown polymeric immunoglobulin receptor-transfected MDCK cells were treated basally +/- 100 ng/ml SF/HGF prior to measurement of TER. Each bar is the average of duplicate samples for each condition from one representative experiment. Similar results were obtained in four separate experiments with either untransfected or pIgR-transfected MDCK cells. □, untreated; ■, SF/HGF.

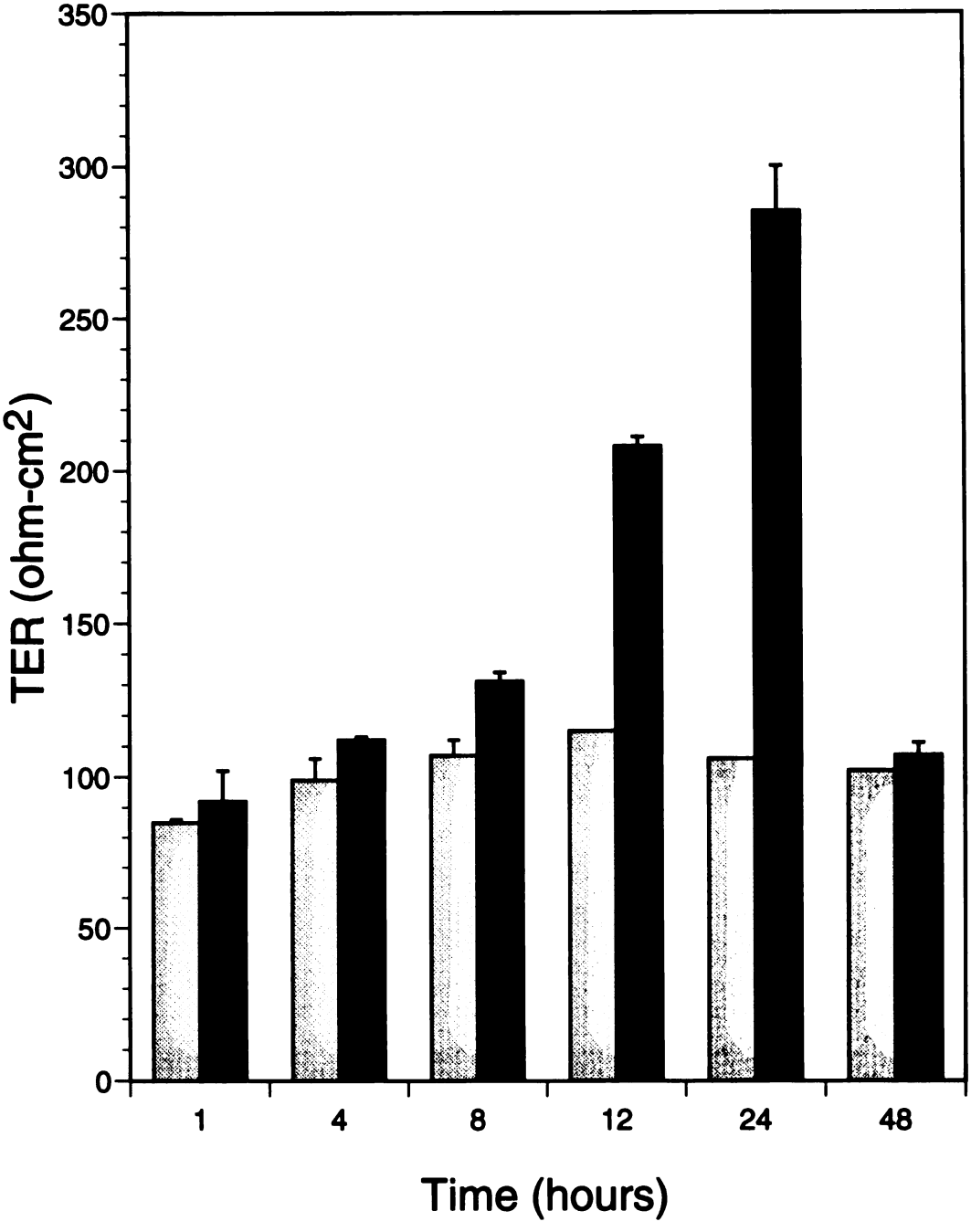
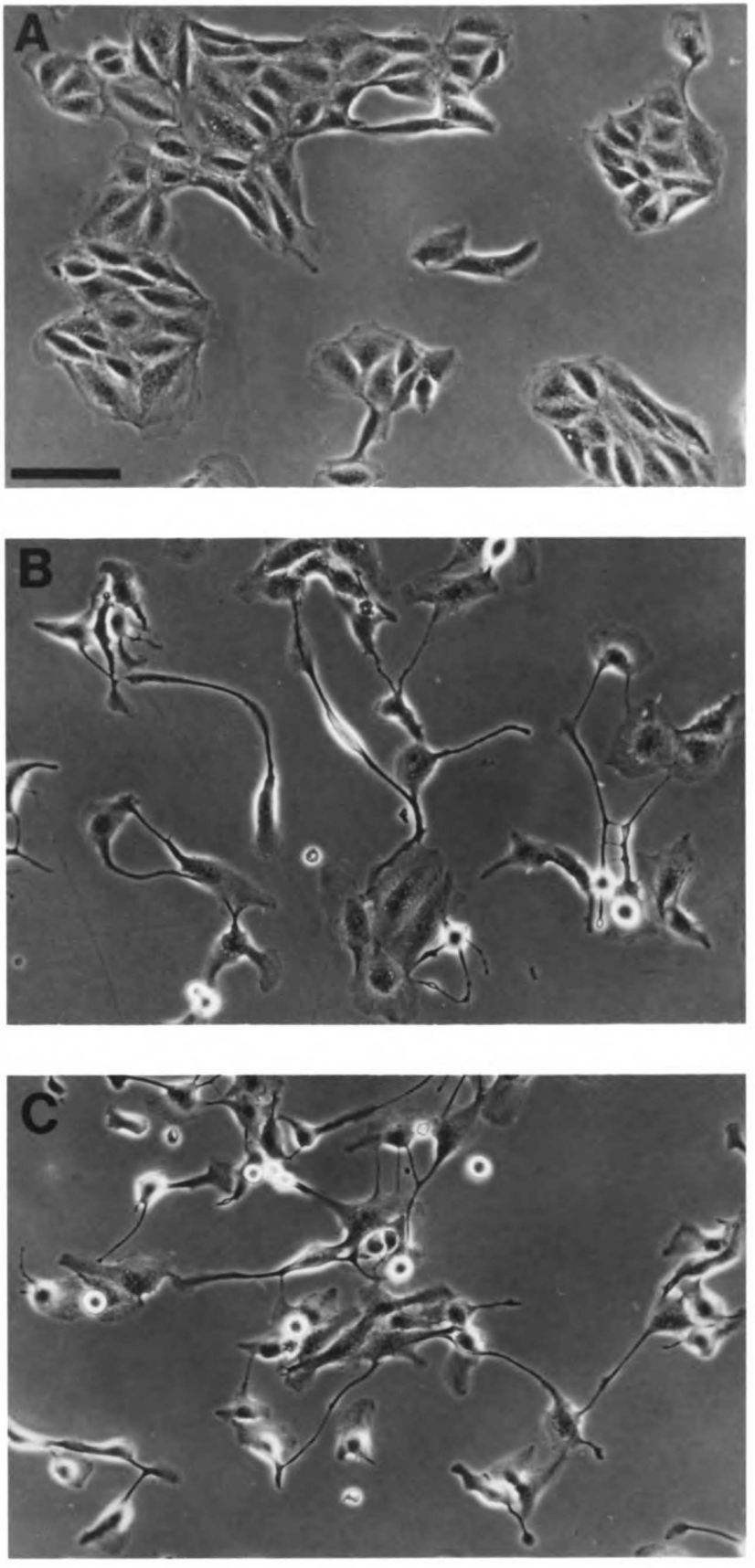

350
300
250
200
150
100
50
0
TER (ohm-cm2)
1
4
8
12
24
48
Time (hours)

Figure 3.3. SF/HGF induces scattering of MDCK cells at much lower concentrations than are needed to stimulate TER. (*A-C*) MDCK cells transfected with polymeric immunoglobulin receptor were plated at low density and cultured for 9 hours to form small islands. Cells were cultured for an additional 26-28 hours in the absence (*A*) or presence of 2.5 ng/ml (*B*) or 100 ng/ml (*C*) SF/HGF prior to photographing. Bar equals 100 μm. (*D*) Polarized monolayers of MDCK cells transfected with the polymeric immunoglobulin receptor were treated basally for 20 hours with different concentrations of SF/HGF. TER was measured as described in materials and methods. Data presented is the mean +/- SEM of triplicate filters for each condition from one representative experiment. Similar results were obtained in four separate experiments and with untransfected MDCK cells.


A
B
C

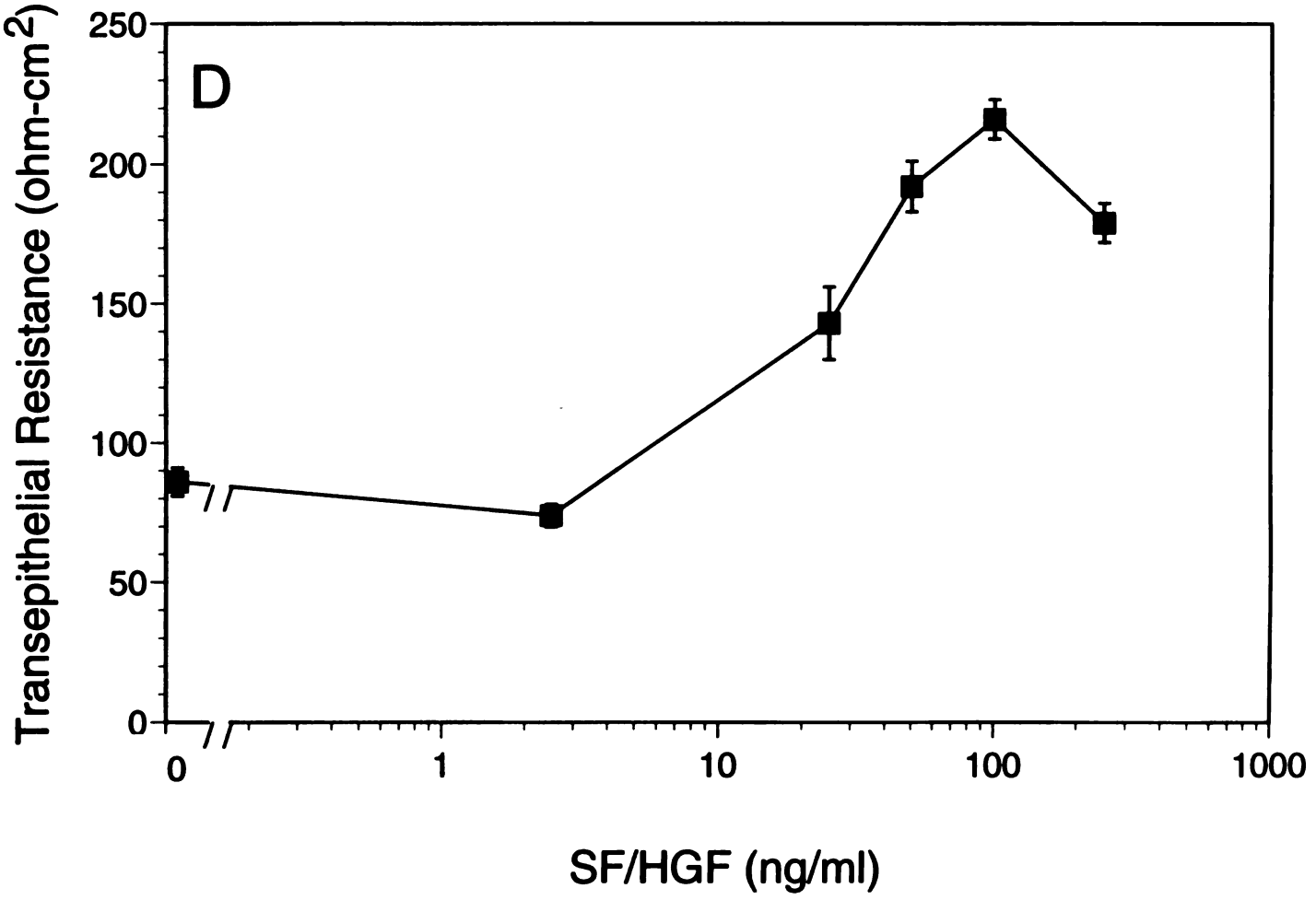

D
Transepithelial Resistance (ohm-cm2)
250
200
150
100
50
0
0
1
10
100
1000
SF/HGF (ng/ml)

Figure 3.4. Increases in TER induced by SF/HGF are modulated by heparin. Polarized monolayers of MDCK cells transfected with the polymeric immunoglobulin receptor were treated for 20 hours with different concentrations of SF/HGF +/- 0.1 μg/ml heparin prior to measurement of TER. Data presented is the mean +/- SEM of triplicate filters for each condition. ■, minus heparin; ●, plus heparin.

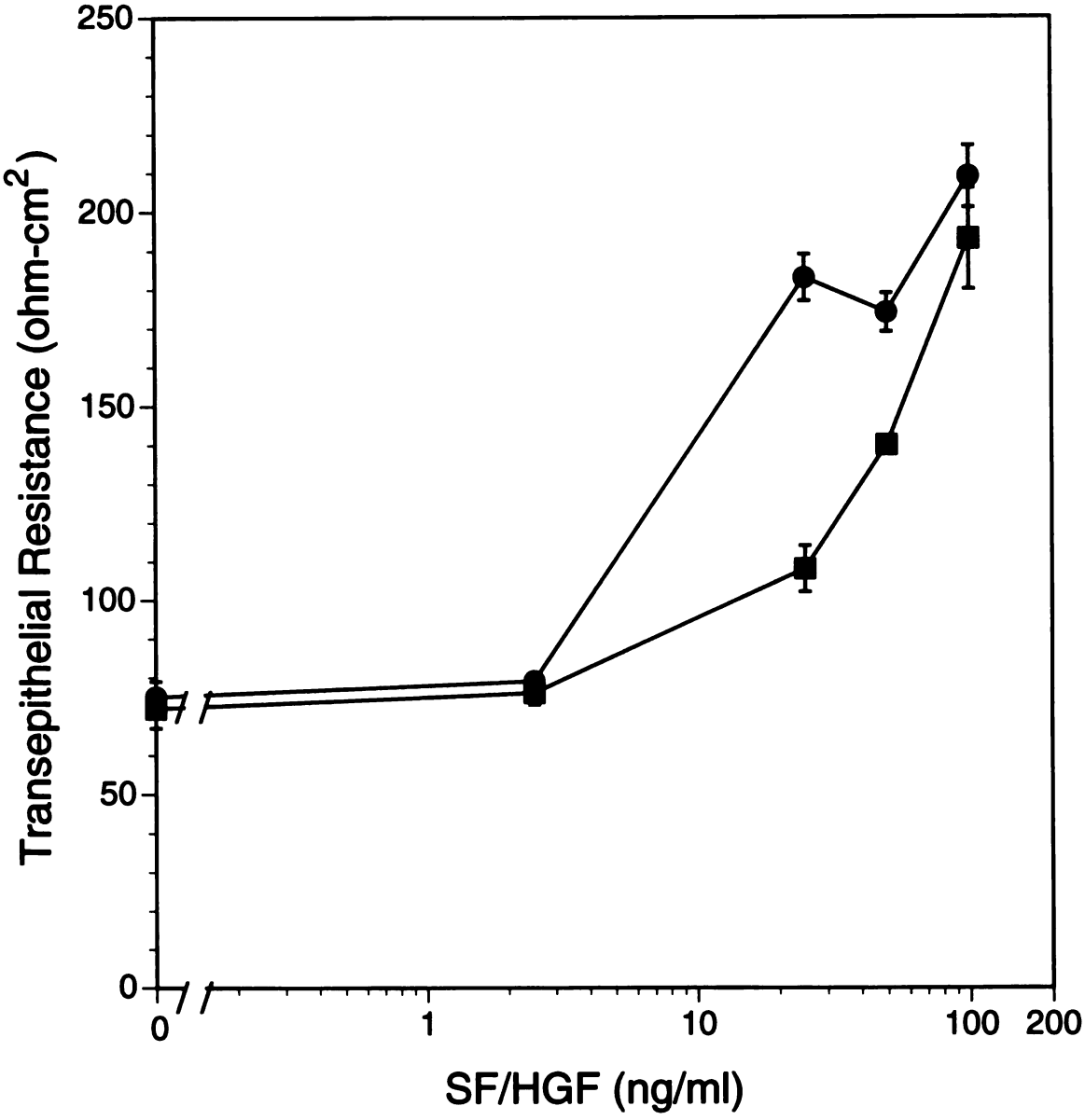
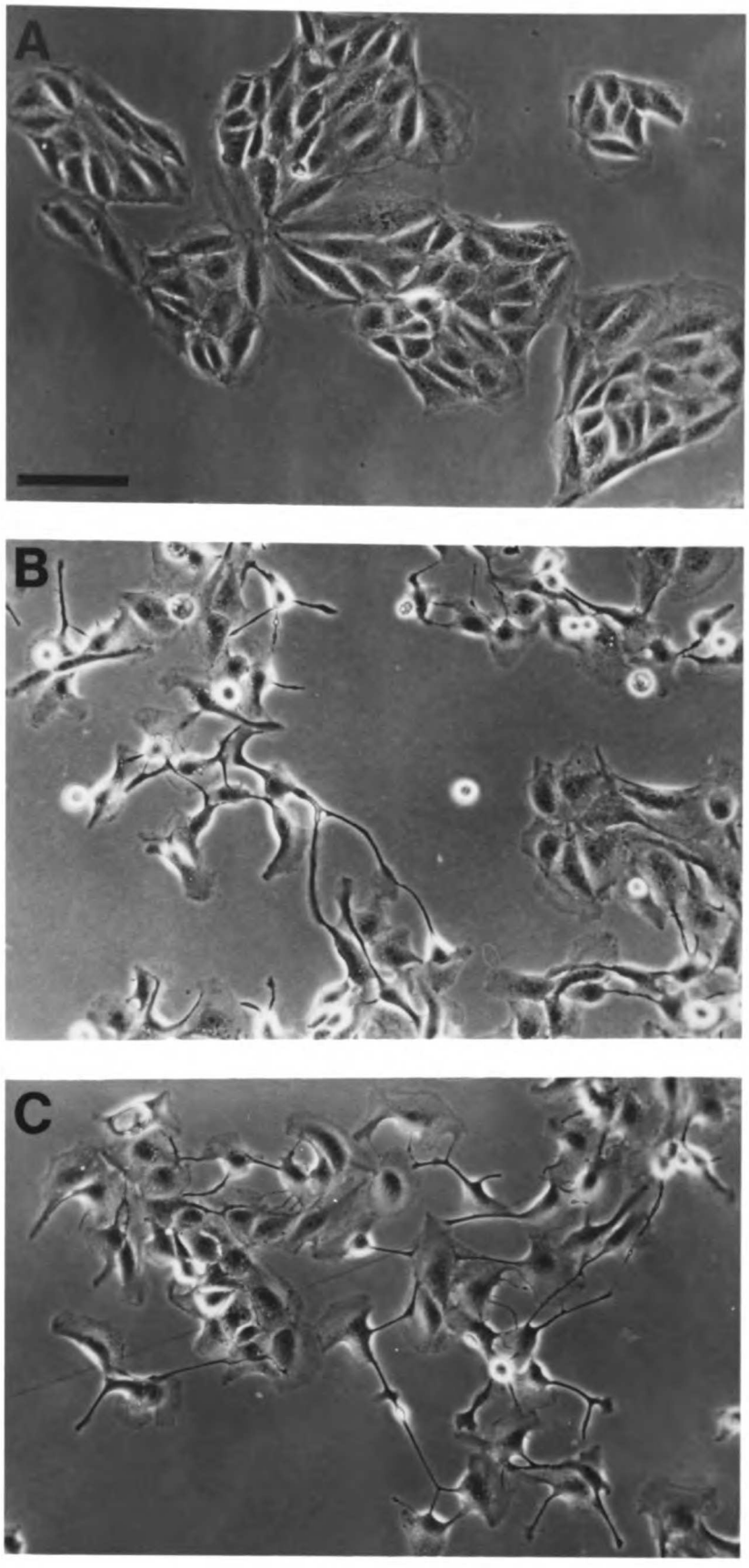

250
200
150
100
50
0
Transepithelial Resistance (ohm-cm^2)
0
1
10
100
200
SF/HGF (ng/ml)

Figure 3.5. Direct activation of c-met with mAb DO24 stimulates scattering but has minimal effect on TER. (*A-C*) MDCK cells transfected with polymeric immunoglobulin receptor were plated at low density and cultured for 9 hours to form small islands. Cells were cultured for an additional 26-28 hours in the absence (*A*) or presence of 2 nM (*B*) or 10 nM (*C*) mAb DO24 prior to photographing. Bar equals 100 μm. (*D*) MDCK cells expressing the polymeric immunoglobulin receptor were cultured on Transwell filters to form confluent monolayers. Culturing was continued for 20 hours in the absence or presence of mAb DO24 prior to measurement of TER. Each point represents the mean +/- SEM for pooled results of 3-9 samples for each concentration tested.


A
B
C

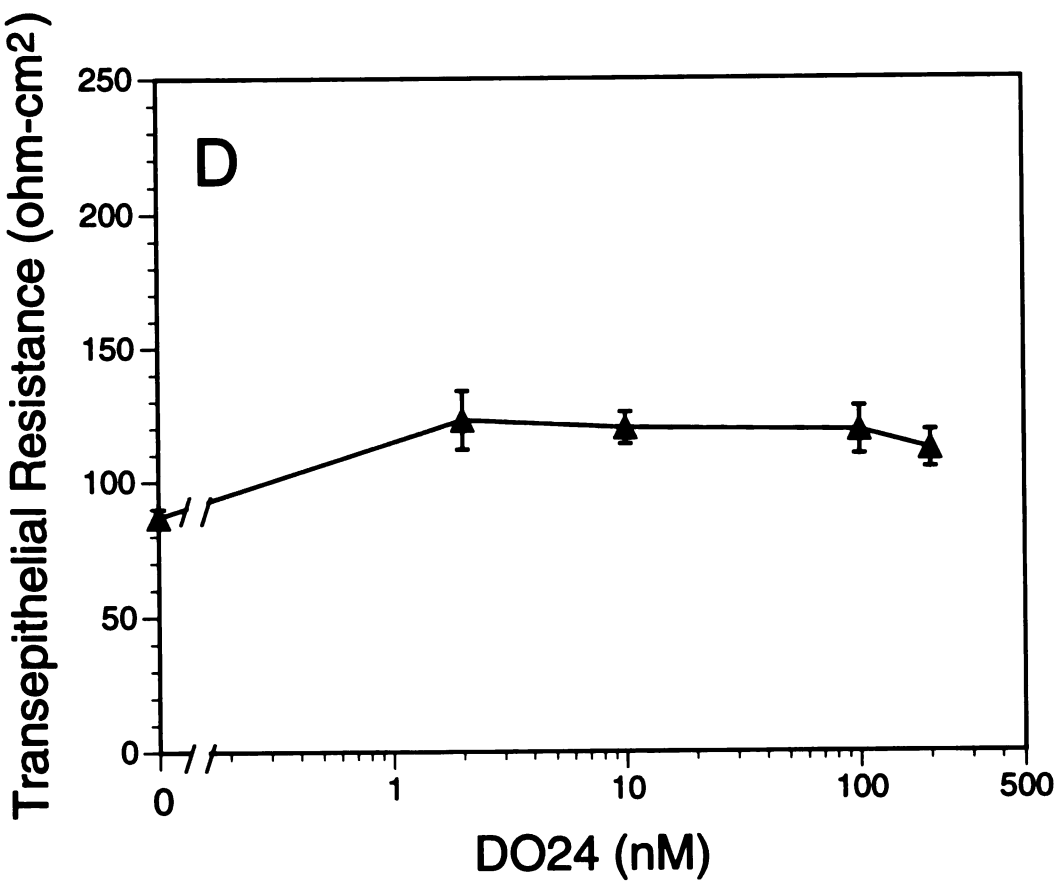

D
Transepithelial Resistance (ohm-cm2)
250
200
150
100
50
0
0
1
10
100
500
DO24 (nM)

Figure 3.6. SF/HGF and NGF have equivalent effects on scattering of MDCK cells transfected with a trk/met chimeric receptor. (*A-H*) MDCK cells expressing either the neo resistance gene (*A, C, E, G*) or the trk/met chimeric receptor (*B, D, F, H*) were plated at low density and cultured for 9 hours to form small islands. Cells were cultured for an additional 26-28 hours in the absence (*A, B*) or presence of 2.5 ng/ml SF/HGF (*C, D*), 2.5 ng/ml NGF (*E, F*), or 250 ng/ml NGF (*G, H*) prior to photographing. Bar equals 100 μm.

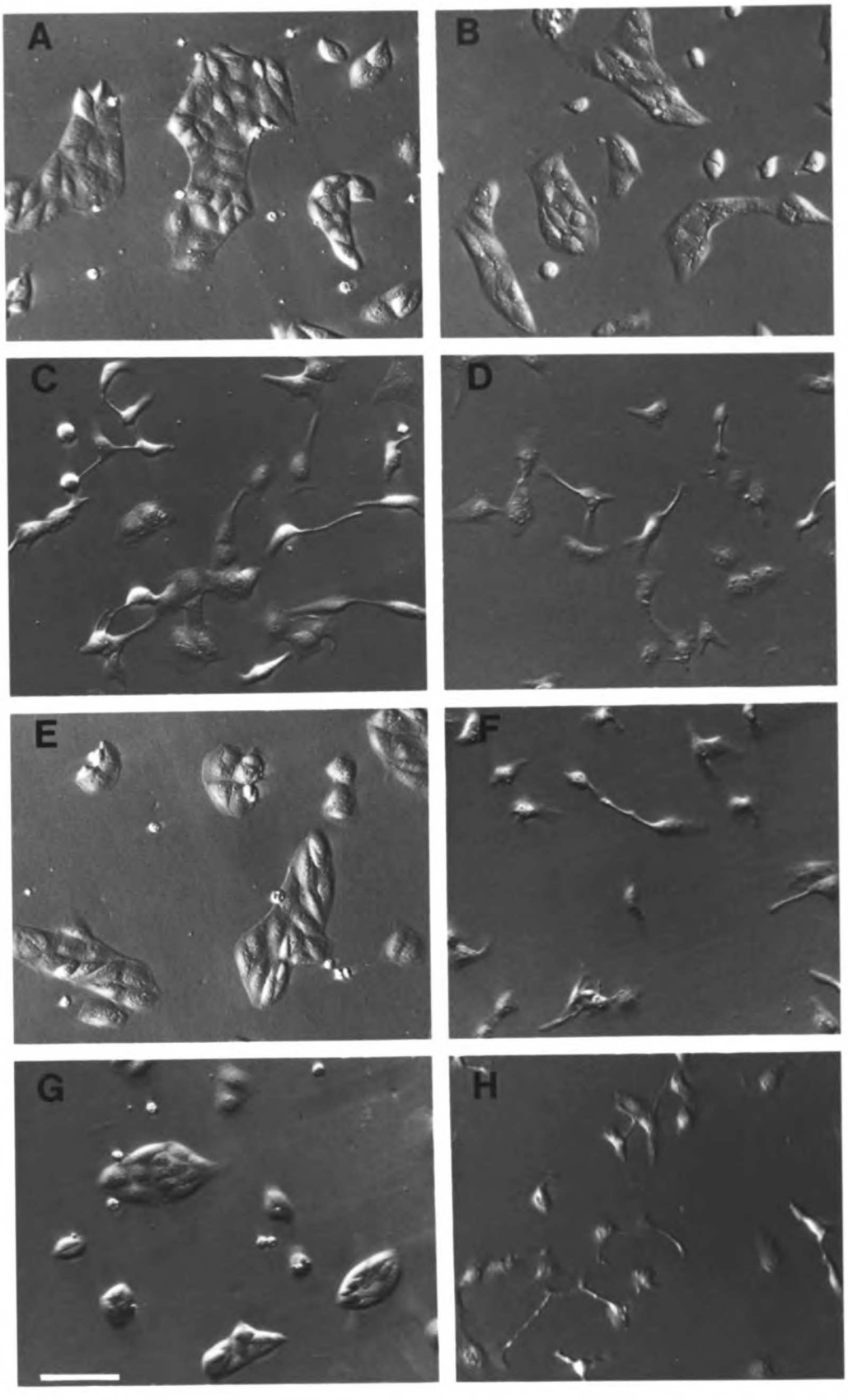
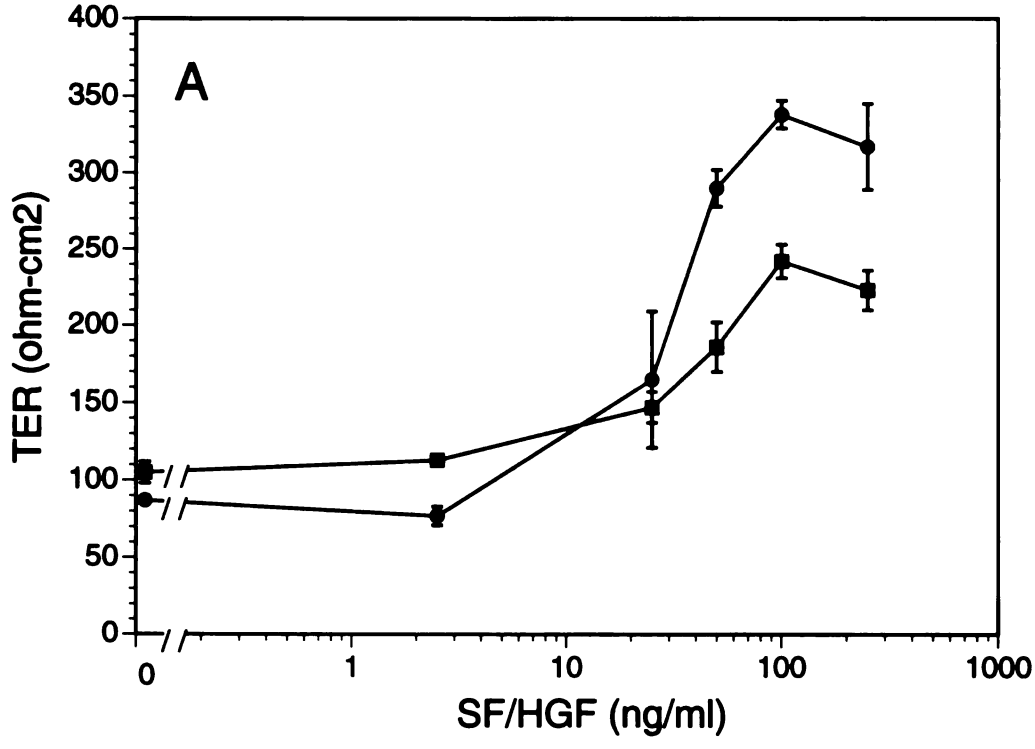
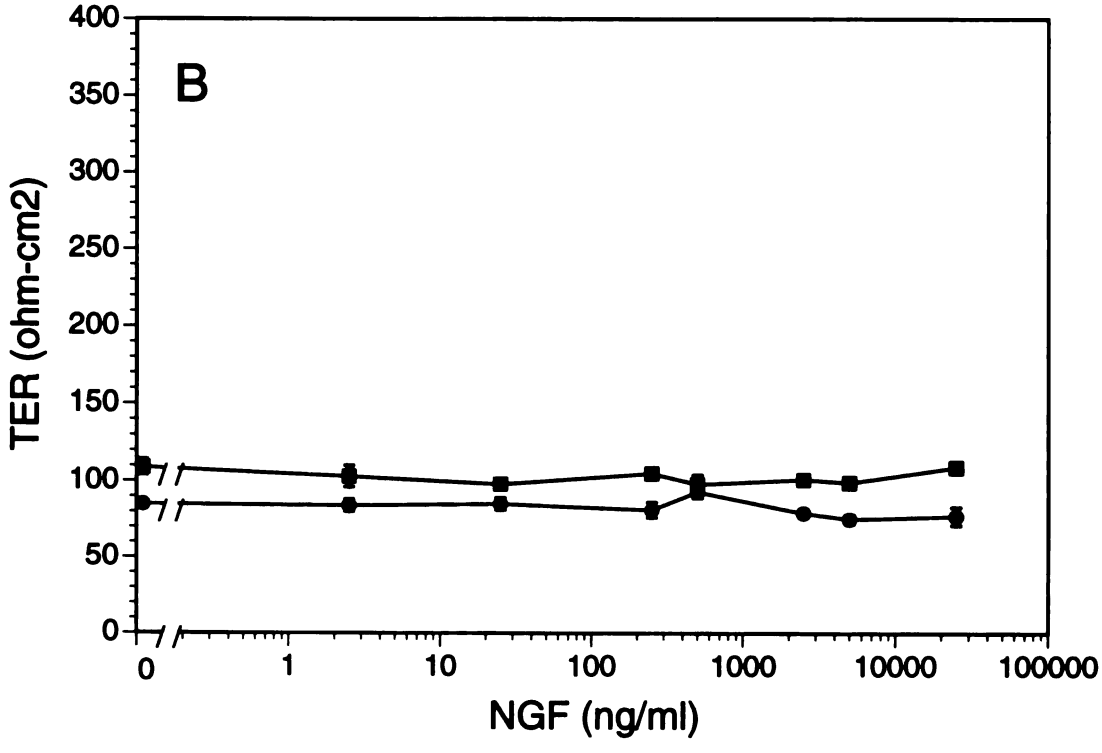

A
B
C
D
E
F
G
H

Figure 3.7. Stimulation of the trk/met chimeric receptor with NGF has no effect on TER. MDCK cells expressing either the neo resistance gene (■) or the trk/met chimeric receptor (●),were cultured on Transwell filters to form confluent monolayers. Culturing was continued for 20 hours with either (*A*) SF/HGF (0-250 ng/ml) or (*B*) NGF (0-25,000 ng/ml) prior to measurement of TER. Each point represents the mean +/- SEM for pooled results of 4-10 samples for each concentration tested.


A
TER (ohm-cm2)
400
350
300
250
200
150
100
50
0
0
1
10
100
1000
SF/HGF (ng/ml)


B
TER (ohm-cm2)
400
350
300
250
200
150
100
50
0
0
1
10
100
1000
10000
100000
NGF (ng/ml)

SUMMARY

SUMMARY

The results of this thesis address the interplay between epithelial cell polarity, cell-cell adhesion and growth factor regulation of epithelial morphogenesis. SF/HGF is a growth factor that has been previously shown to stimulate epithelial tubulogenesis in vitro and is proposed to be an endogenous mediator of tubulogenesis in vivo. My approach has been to study SF/HGF-induced epithelial morphogenesis in two in vitro MDCK cell culture systems. In one model system, polarized MDCK cell cysts are established and then stimulated by SF/HGF to form tubules. In a second system, the response of polarized MDCK cell monolayers to SF/HGF is examined. In chapter one I describe the localization of the SF/HGF receptor, c-met, in polarized epithelial cells. Chapter two describes an electron and confocal microscopic analysis of epithelial cell polarity and cell-cell adhesion during morphogenesis induced by SF/HGF. I demonstrate that, in addition to stimulating tubulogenesis in vitro, SF induces monolayers to form pseudostratified layers. I present new information on the rearrangements of plasma membrane subdomains and the functional integrity of tight junctions during these morphogenetic processes. In chapter three a novel effect of SF/HGF on transepithelial resistance is presented, for which a new signalling mechanism is proposed and discussed in terms of the relationship of this effect to epithelial morphogenesis.

The initial studies presented in this thesis demonstrate for the first time that, both in vivo and in vitro, the c-met protooncogene is maintained almost exclusively at the basolateral membrane of polarized epithelial cells. I also demonstrate that the polarized distribution of c-met is established by direct targeting, after synthesis, from the trans golgi network to the basolateral cell surface. The localization of c-met at the basolateral cell membrane positions this receptor at the cell surface at which it is available for interaction with its ligand, SF/HGF.

Tubulogenesis is a multistep process that involves extensive reorganization of cell morphology and position within a multicellular structure. Models have been proposed which suggest that tubules form by dissociation and migration of individual cells followed by reassociation into highly organized tubular structures. However, in vivo and organ explant studies of kidney organogenesis have indicated that compaction of cell aggregates and cell polarization are early steps in tubulogenesis. After careful analysis, I have defined four sequential stages of tubulogenesis in vitro and provide new information about epithelial cell membrane polarity at individual stages of tubulogenesis. My results show that during tubulogenesis apical/basolateral polarity is transiently lost. Initially, the membrane delineated by apical membrane components diminishes and becomes undetectable while basolateral membrane components are randomly distributed on the cell surface. At later stages, basolateral membrane components remain randomized while apical membrane components colocalize with basolateral constituents at cell-cell borders. As tubules mature, lumen formation occurs in discontinuous regions along the length of tubules and membrane polarity is reestablished in regions of lumen development. I demonstrate that while apical and basolateral membrane subdomains undergo extensive rearrangements, cell-cell contacts are maintained throughout tubulogenesis. My results indicate that tight junction functional integrity is critical for maintaining cell-cell adhesion, while modulation of desmosomal and adherens junctional contacts is important for cell rearrangements during tubule morphogenesis. My studies of pseudostratified layer morphogenesis confirm that tight junction functional integrity is maintained during the rearrangement of epithelial cells into new multicellular structures. The important conclusion that can be drawn from these results is that SF/HGF induces a differential regulation of cell-cell adhesive mechanisms that results in localized modulation of adhesion and stimulation of cell migration without complete loss of cell-cell contact.

Based on the ability of SF/HGF to stimulate cell motility and scattering, it was expected that SF/HGF's effects on polarized epithelial cells would be to stimulate cell

dissociation. However, tests of inulin and ruthenium red leakage, shown in chapter two, provided functional evidence that pseudostratification is stimulated while tight junctions remained intact. In chapter three I present an analysis of the effect of SF/HGF on transepithelial resistance during pseudostratification. Transepithelial resistance increased transiently in response to SF/HGF during the formation of pseudostratified layers, providing convincing confirmation that tight junctions remain functionally intact during SF/HGF-stimulated morphogenesis.

All previously identified effects of SF/HGF have been shown to be induced by direct activation of c-met. The effect of SF/HGF on TER that I present in chapter three is the first evidence for an effect of SF/HGF that is not inducible by direct activation of c-met. The activation of multiple sites on the cytoplasmic domain of c-met has been demonstrated to activate multiple intracellular signalling pathways and regulate which cellular response is transduced. This has been suggested as the mechanism to explain how multiple effects can be stimulated by the interaction of the single ligand, SF/HGF, by the single receptor, c-met. However, how distinct cytoplasmic domains of c-met are activated, or how a general activation would induce only certain responses has not been elucidated. My results suggest that some effects of SF/HGF may require low affinity interactions with coreceptors or cofactors. Evidence is available which suggests that low affinity sites for SF/HGF interaction are cell surface sulfoglycolipids or HSPGs. Whether these molecules can modulate the effect of SF/HGF on TER is yet to be explored.

In conclusion, the detailed analysis of cell polarity and adhesion during SF/HGF-induced epithelial morphogenesis that is presented in this thesis provides a foundation upon which to further analyze the specific role of each of these components in tubulogenesis. How SF/HGF induces transient alterations in cell polarity and cell adhesion is still an open question that may be important for a multitude of morphogenetic systems. There is clearly reciprocal regulation between SF/HGF activity, cell adhesion and cell polarity that needs to be further explored. Future experiments testing the effect of disrupting E-cadherin-based

adhesion on the ability of SF/HGF to stimulate tubulogenesis may provide insight into regulation of the delicate balance of cell growth, migration adhesion and polarization that is necessary for outgrowth and organization of cells into tubules. One approach to address this question is to test dominant negative effects of an overexpressed mutant β-catenin on tubule development. These experiments are currently in progress.

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