Membrane-associated HB-EGF modulates HGF-induced cellular responses in MDCK cells

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Summary

In MDCK cells, hepatocyte growth factor/scatter factor (HGF/SF) induces epithelial cell dissociation, scattering, migration, growth and formation of branched tubular structures. By contrast, these cells neither scatter nor form tubular structures in response to the epidermal growth factor (EGF) family of growth factors. Heparin-binding EGF-like growth factor (HB-EGF) is a member of the EGF family of growth factors and is synthesized as a membrane-associated precursor molecule (proHB-EGF). ProHB-EGF is proteolytically cleaved to release a soluble ligand (sHB-EGF) that activates the EGF receptor. Although recent studies suggest possible physiological functions, the role of proHB-EGF remains largely undefined. Using MDCK cells stably expressing proHB-EGF, a noncleavable deletion mutant of proHB-EGF or soluble HB-EGF, we show that epithelial cell functions differ depending on the form of HB-EGF being expressed. Expression of noncleavable membrane-anchored HB-EGF promoted cell-matrix and cell-cell interactions and decreased cell migration, HGF/SF-induced cell scattering and formation of tubular structures. By contrast, expression of soluble HB-EGF induced increased cell migration, decreased cell-matrix and cell-cell interactions and promoted the development of long unbranched tubular structures in response to HGF/SF. These findings suggest that HB-EGF can not only modulate HGF/SF-induced cellular responses in MDCK cells but also that membrane-bound HB-EGF and soluble HB-EGF give rise to distinctly different effects on cell-cell and cell-extracellular matrix interactions.

Key words: Membrane-anchored HB-EGF, MDCK II, Scattering, Tubulogenesis, Transepithelial resistance

Introduction

The maintenance of the structural and functional integrity of epithelia requires highly dynamic cell-cell and cell-matrix interactions. Under steady-state conditions, the assembly and maintenance of intercellular junctions appear to be tightly regulated (Braga, 2002; Mitic and Anderson, 1998). However, during development, tissue repair and tissue metamorphosis, concerted cell-cell dissociation and cell migration occur and appear to be intimately linked, such as is observed with epithelial 'scattering' (Tsukamoto and Nigam, 1999). Also, during cell division or inflammation, as well as invasion and metastasis of tumor cells, cell-cell junctions are disassembled and sometimes internalized as cells diminish their contacts and become motile (Bauer et al., 1998; Gumbiner, 1996). Alterations in cell polarity and cell-cell and cell-matrix interactions are also well described in renal epithelial cells in response to acute ischemic injury (Molitoris and Wagner, 1996; Racusen, 1997).

The Madin-Darby canine kidney II (MDCK II) cell line has been utilized as an in vitro model to help define the mechanisms underlying complex epithelial cell movements such as scattering, tubulogenesis and dedifferentiation (Balkovetz, 1998; Stuart et al., 1995), especially in response to hepatocyte growth factor/scatter factor (HGF/SF). HGF/SF is a polypeptide cytokine produced by mesenchymal cells that induces a range of responses in epithelial cells (Matsumoto and Nakamura, 2001; Stella and Comoglio, 1999). In addition to causing proliferation, HGF/SF also induces normal epithelial cells grown on plastic to 'scatter' because of increased motility and reduced cell-cell interactions and results in cells growing as a dispersed culture in carcinoma cells, where invasiveness is increased by HGF/SF (Bardelli et al., 1997; Maulik et al., 2002). HGF/SF also induces epithelial cells grown in three-dimensional collagen gels to form branching tubules reminiscent of epithelial ducts in the breast and kidney (Barros et al., 1995; Pollard, 2001; Zhang and Vande Woude, 2003). Interestingly, although exogenous epidermal growth factor (EGF) induces DNA synthesis and cell proliferation in MDCK cells (Hauguel-DeMouzon et al., 1992), it does not induce scattering or branching tubulogenesis (Tanimura et al., 1998), nor does it modulate the response of HGF/SF (Liang and Chen, 2001; Tanimura et al., 1998).

Heparin-binding EGF-like growth factor (HB-EGF) was first identified in the conditioned medium of a macrophage-like cell line, U937 (Higashiyama et al., 1991) but is now known to be widely expressed in cells of epithelial, endothelial and mesenchymal origin. As with other members of the EGF family (Iwamoto and Mekada, 2000; Raab and Klagsbrun, 1997), HB-EGF is synthesized as a type-1 transmembrane protein that can be shed enzymatically to release a soluble 14-20 kDa growth factor (Goishi et al., 1995; Higashiyama et al., 1992). However, a considerable amount of proHB-EGF remains on the cell surface under normal physiological conditions (Goishi et al., 1995). The cleavage of proHB-EGF has been shown to occur through

metalloproteinase activation, attributed in various cells to ADAM (a disintegrin and metalloproteinase)-9, -10, -12 and/or -17 (Asakura et al., 2002; Izumi et al., 1998; Sunnarborg et al., 2002; Yan et al., 2002), as well as MMP-3 and -7 (Suzuki et al., 1997; Yu et al., 2002), and it can be blocked by metalloproteinase inhibitors such as Batimastat (BB-94) (Chen et al., 2002; Prenzel et al., 1999). Both the transmembrane (proHB-EGF) form of the molecule, as well as the soluble form, are suggested to be active signaling molecules (Iwamoto and Mekada, 2000; Raab and Klagsbrun, 1997). HB-EGF serves as a ligand for EGFR1 (erbB1) as well as erbB4 (Elenius et al., 1997; Higashiyama et al., 1992; Riese et al., 1996). The soluble form of HB-EGF can interact with its receptors in an autocrine and/or paracrine manner, is a potent mitogen and induces proliferation and migration in a variety of cells (Faull et al., 2001; Piepkorn et al., 1998; Tokumaru et al., 2000b). The transmembrane form of HB-EGF interacts with other integral membrane proteins, especially integrins, via the tetraspanin, CD9/DRAP27 (Lagaudriere-Gesbert et al., 1997; Nakamura et al., 1995). It is noteworthy that the HB-EGF-CD9-integrin complex and EGFR are both targeted to cell-cell junctions in some epithelial cells (Fukuyama and Shimizu, 1991; Nakamura et al., 1995). HB-EGF also complexes with heparin sulfate proteoglycans (Paria et al., 1999).

Targeting of the HB-EGF complex and EGFR to immediately juxtaposed cellular regions has been proposed to facilitate juxtacrine signaling (Higashiyama et al., 1995). During the process of organ development, juxtacrine signaling may be an efficient method to deliver selective signals to adjacent cells. Ectodomain shedding of membrane proteins changes their fate, localization and mode of action, thus affecting the biological activities of membrane proteins (Davis et al., 1994; Massague and Pandiella, 1993; Werb and Yan, 1998).

ProHB-EGF may serve a variety of functions depending on its cellular context and has been reported to be mitogenic and cytoprotective in some cells while growth inhibitory and pro-apoptotic in others (Iwamoto et al., 1999; Nishida et al., 2000; Takemura et al., 1997). ProHB-EGF has also been suggested to promote cell-cell adhesion (Kimber, 2000; Takemura et al., 1997). The fact that a considerable amount of proHB-EGF is uncleaved and remains membrane-bound under normal physiological conditions further complicates the analysis of juxtacrine signaling versus tightly coupled autocrine/paracrine signaling due to soluble HB-EGF. The present study was undertaken to investigate the respective roles of soluble and membrane-associated HB-EGF in epithelial cell morphogenesis. We utilized MDCK II cells stably expressing proHB-EGF, a noncleavable membrane-anchored mutant form of proHB-EGF or soluble HB-EGF. We show that the expression of soluble or membrane-anchored HB-EGF molecules results in distinct cell phenotypes, with major differences in both cell-extracellular matrix and cell-cell interactions. Furthermore, we show that expression of membrane-bound HB-EGF inhibits HGF/SF-induced cell scattering and formation of branched tubular structures. This is the first report to describe distinct roles of soluble and membrane-anchored HB-EGF and show that an EGFR ligand can modulate HGF/SF-induced scattering and tubulogenesis in MDCK II cells.

Materials and Methods

Cell culture and transfections

MDCK type II cells were grown in modified Dulbecco Eagle''s medium (DMEM) containing Earle's balanced salt solution supplemented with 5% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 mg/ml amphotericin B in 5% CO_2/95% air at 37°C. To develop MDCK cells stably expressing HB-EGF mutant constructs, cells (1×10^5 cells/35 mm dish) were transfected with 1 μg of pcDNA3-neo containing the HB-EGF mutant cDNAs, using LipofectAMINE Reagent (Gibco BRL, Life Technologies, Grand Island, NY). The cells were cultured for five passages in DMEM containing G418 (1 mg/ml; Sigma Chemical Co., St Louis, MO), and colonies derived from single cells were picked to generate clones stably expressing the molecule of interest. Cell lines expressing the HB-EGF mutants were identified by immunoblotting with the anti-FLAG (M2) and anti-HB-EGF antibodies. At least two independent stably transfected clones with comparable protein expression of the transfected molecule for each mutant were maintained and used for experiments. The data presented throughout the manuscript are from clone# 1 of the cells transfected with HB-EGF mutants and are representative of the findings from two separate clones unless stated otherwise.

Antibodies and reagents

The rabbit anti-rat HB-EGF polyclonal antibody was a generous gift from Li Feng (Department of Immunology, The Scripps Research Institute, La Jolla, CA). The mouse anti-FLAG (M2) antibody, heparinase I and the HGF/SF were purchased from Sigma Chemical Co. The anti-EGFR and anti-P-EGFR antibodies were purchased from Upstate Biotechnology (Lake Placid, NY), the anti-phospho-c-Met antibody was purchased from Biosource International (Camarillo, CA), and the anti-ZO-1 and anti-E-cadherin antibodies were purchased from Zymed Laboratories (South San Francisco, CA) and BD Biosciences Transduction Laboratories, respectively. The anti-CD9 antibody was purchased from abcam.com. The P-ERK 1/2, ERK (extracellular signal-regulated kinase), P-JNK and JNK (Jun N-terminal kinase) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and the P-Akt and Akt antibodies were obtained from Cell Signaling (Beverly, MA).

Transepithelial resistance

MDCK II cells were plated on transwell filters (12 mm, 0.4 μm pore size; Corning Costar, Cambridge, MA) at confluence (~2×10^5 cells) and allowed to attach overnight to form a confluent monolayer in normal Ca^{2+}-containing medium (NC). Transepithelial resistance (TER) was measured after 12 hours of plating (day 1) and every 24 hours thereafter using a Millipore (Bedford, MA) electrical resistance measurement system, and the results are expressed in Ω/cm^2.

Heparin-sepharose chromatography

Confluent vector and HB-EGF transfected cells were washed twice with PBS and rendered quiescent by exposure to serum-free culture medium for 48 hours. Conditioned media (CM) were centrifuged and soluble HB-EGF was purified as described by Chen et al. (Chen et al., 2002). Equal amount of eluants were subjected to SDS-PAGE and immunoblotted with anti-HB-EGF antibody.

Cell adhesion assay

Rat-tail collagen I (Collaborative Research, Bedford, MA) was diluted in PBS [(–) without Ca^{++} and Mg^{++}] according to the manufacturer's directions, and plates were coated as described by Kanai et al. (Kanai

et al., 1999). In each assay, six wells were used for each experimental condition. Three wells received a suspension of 1×10^5 viable cells, while the other three were used for background measurements. The plated cells were then incubated covered at 37°C/5% CO_2 for 60 minutes, the time necessary for MDCK to adhere maximally to collagen I (data not shown). Plates were then gently washed twice with 200 μl/well of PBS (+), and fixed with 100 μl/well of 3.7% formaldehyde for 30 minutes at room temperature. The plates were then stained for 20 minutes with 100 μl/well of 0.1% crystal violet filtered through a 0.22 μm filter unit (Millipore), washed four times with 200 μl/well of PBS (+), and air-dried before the dye was dissolved in 20% acetic acid (100 μl/well) for 10-20 minutes. The amount of staining, which is proportional to the number of attached cells, was measured with a 96-well plate reader (Molecular Devices Corporation, Sunnyvale, CA) at 570 nm. The average absorbance of wells without cells was measured and subtracted from the average of wells with cells to yield the absorbance due to the adherent cells only. Each graph represents the data from four independent experiments.

Cell spreading assay

Rat collagen I was prepared as above and 35 mm tissue culture dishes were coated with 2.5, 5 and 10 μg/ml of collagen I overnight at 4°C and blocked with 1% BSA+PBS as above. Dishes were washed with 1×PBS, and 5×10^4 cells were plated for 4 hours at 37°C/5% CO_2. Thereafter, cells were washed with PBS, fixed with 3.7% formaldehyde and photographed under phase contrast using a Nikon Diaphot TMD inverted microscope.

Cell scattering

3×10^3cells were plated in 12-well tissue culture dishes and cells were allowed to grow as discrete colonies. After 48 hours cells were transferred into medium containing 0.2% fetal calf serum and treated with 20 ng/ml HGF/SF. 12-16 hours later, cells were fixed in 3.7% formaldehyde and photographed under phase contrast using a Nikon Diaphot TMD inverted microscope.

Collagen gel culture

For the collagen gel culture, cells were suspended at 5×10^3 cells/ml in a neutralized collagen solution (Collaborative Biomedicals), dispensed into 96-well plates and incubated at 37°C. After the collagen solution had gelled, the complete culture medium with HGF/SF (20 ng/ ml) was added and renewed every day. One group of cells did not receive HGF/SF and served as control. The cultures were photographed after 7-9 days of culture under phase contrast using a Nikon Diaphot TMD inverted microscope.

Cell migration assay

For migration assays, cells were prepared as for the cell-adhesion assay and resuspended in serum-free media. The migration assay was performed using transwell chambers (12 mm, 0.8 μm pore size; Corning Costar, Cambridge, MA) with the lower chambers filled with serum-free media containing rat-tail collagen (10 μg/ml). Each well was plated with 1×10^4 cells in 500 μl serum-free media. The transwell chamber was placed at 37°C in a humidified 5% CO_2 atmosphere. After 4 hours, cells on the upper surface of the membrane were removed using a cotton swab. The membrane was then fixed with 3.7% formaldehyde for 30 minutes at room temperature and stained with crystal violet (0.1%, Sigma Chemical Co.) for 1 hour. Cells were washed extensively after staining and the cells on the lower surface were counted under a light microscope at high-power magnification (40×). Multiple fields (four to eight) were counted for each well and the mean values were obtained.

Immunoprecipitation and immunoblotting

Cells were washed with 2 M NaCl before washing with PBS to remove any soluble HB-EGF bound to the membrane. Preparation of total cell lysate and immunoprecipitation and immunoblotting was performed as described by Dhawan and Richmond (Dhawan and Richmond, 2002). Immunoprecipitation samples for HB-EGF were resolved on 10% Tricine gels (Invitrogen, Carlsbad, CA) while other western blot analyses were performed using regular SDS-PAGE. Signals were detected using an ECL detection kit (Amersham-Pharmacia Biotech, Piscataway, NJ).

Immunofluorescence microscopy

Cells plated on transwell filters or grown on glass coverslips were rinsed in PBS (with Ca^{++}, Mg^{++} and 0.2% BSA) and fixed with 3.7% formaldehyde in PBS for 30 minutes on ice. The fixed cells were treated with 0.2%Triton X-100 in PBS for 10 minutes on ice and washed three times with PBS. After soaking in PBS containing 5% normal goat serum and 1% BSA in PBS, the samples were incubated with primary antibodies for 1 hour in a moist chamber at 37°C. Samples were then washed three times with PBS, followed by incubation for 30 minutes with the respective conjugated second antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). The samples were washed with PBS three times, embedded in glycerol-PBS-based mounting medium and examined using a Zeiss-410 confocal microscope at the Vanderbilt Imaging Core facility. The image analysis was performed using Metamorph cell-imaging program (Universal Imaging Corporation, Downingtown, PA).

Statistics

Graphic data are presented as mean±s.e.m. Statistical analysis was performed, where appropriate, using the Student *t*-test, or one-way analysis of variance (ANOVA), followed by the Tukey's Multiple Comparison Test. Differences with $P<0.05$ were considered statistically significant.

Results

Construction of HB-EGF mutant plasmid constructs

The cDNA encoding the open reading frame for the native proHB-EGF molecule was amplified from rat kidney total RNA using PCR primers based on the rat HB-EGF sequence (Acc. # L05489.1). The resultant PCR product was subcloned into PCR II and was used as a template for generation of HB-EGF mutant cDNAs. To generate a cDNA corresponding to soluble HB-EGF, PCR primers were designed to amplify amino acids 1-148 of the rat proHB-EGF, while amino acids PVENP (aa 149-153), the putative cleavage site or the complete juxtamembrane domain (aa 149-160) (Hirata et al., 2001; Suzuki et al., 1997) were deleted to generate the 5 aa and 12 aa deletion mutant constructs (Fig. 1). To differentiate between effects mediated by the membrane-anchored and soluble forms of HB- EGF, we developed stably transfected renal epithelial cells (MDCK II cells) expressing soluble HB-EGF (aa 1-148; $MDCK^{sol}$), proHB-EGF (aa 1-208; $MDCK^{pro}$) and deletion mutant constructs of proHB-EGF lacking the amino terminal 5 ($MDCK^{5aa}$) and 12 amino acids ($MDCK^{12aa}$) of the juxtamembrane extracellular domain (Fig. 1). One group of cells was transfected with empty vector ($MDCK^{con}$) and served as control. To the HB-EGF constructs we added a COOH terminus FLAG epitope, which allowed immunoprecipitation of membrane-anchored HB-EGF with anti-FLAG antibody M2 and immunolocalization with anti-

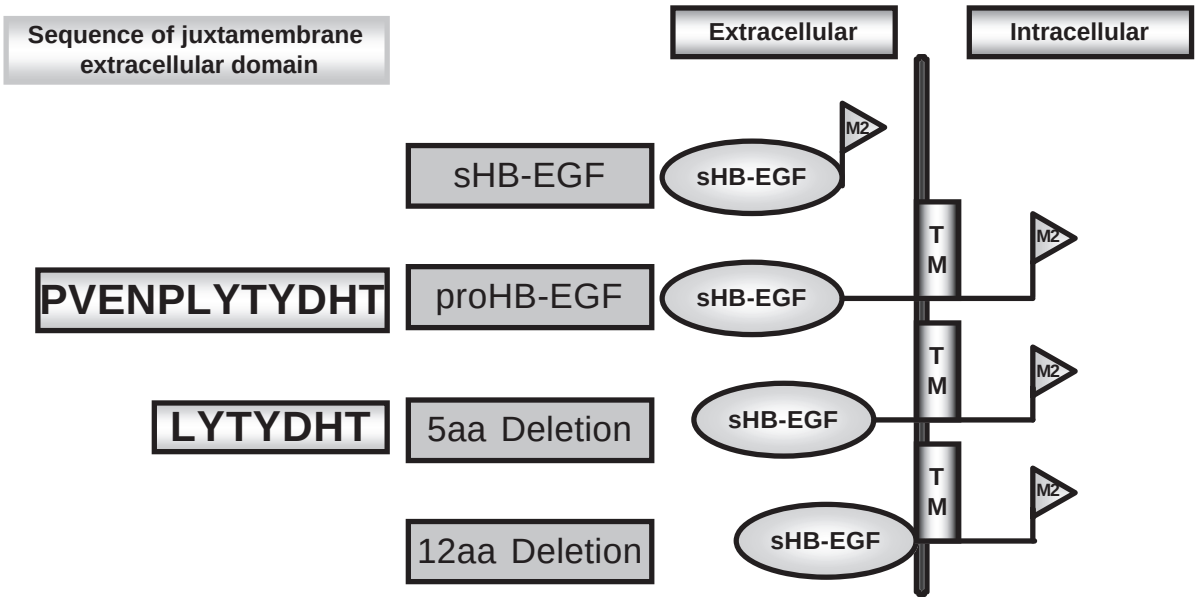

Fig. 1. Graphical presentation of cDNA constructs for membrane-bound HB-EGF (proHB-EGF), soluble HB-EGF (sHB-EGF)and the deletion mutant constructs. The sequence of the juxtamembrane extracellular domain and the amino acids deleted in the mutant constructs are shown. A C-terminal FLAG epitope (M2) was added to each construct.

HB-EGF antibodies. Immunoblotting using a polyclonal anti-rat HB-EGF antibody showed similar expression levels of transfected molecules, whereas sHB-EGF was not detected in the total cell lysate (Fig. 2A) but instead was secreted in the medium (Fig. 2B).

Deletion of putative cleavage site(s) inhibited cleavage of proHB-EGF

Membrane-associated HB-EGF (proHB-EGF) is partially cleaved in response to metalloproteinase activation under normal physiological conditions (Suzuki et al., 1997). To determine whether deletion of the putative cleavage site inhibited the normal metalloproteinase-dependent cleavage of the membrane-anchored HB-EGF mutants, conditioned media from transfected and control cells were purified using heparin-sepharose columns. Equal volumes of eluants were subjected to SDS-PAGE and immunoblotted with an anti-HB-EGF antibody. sHB-EGF expression was detected in the conditioned media from MDCK^{sol} and MDCK^{pro} cells, but no sHB-EGF was detected in media from MDCK^{5aa} or MDCK^{12aa} cells, suggesting lack of cleavage (Fig. 2B).

To determine whether the secreted HB-EGF in the conditioned medium was biologically active, we examined EGF receptor activation in response to the addition of equal amounts of eluants from the conditioned media to quiescent A431 cells, a cell line known to overexpress EGF receptor (Yoshimoto and Imoto, 2002). After 5 minutes, cells were washed and lysed, and equal amounts of protein were subjected to immunoblotting using a phosphospecific anti-EGFR antibody. As indicated in Fig. 2C, conditioned media from MDCK^{sol} cells induced significant EGFR tyrosine phosphorylation, and detectable EGFR activation was also seen with the conditioned media from MDCK^{pro} cells, whereas media from MDCK^{5aa}, MDCK^{12aa} and control cells did not induce detectable tyrosine phosphorylation of the EGF receptor, although equal concentrations of total EGFR were detected (Fig. 2C).

Protein kinase C activation with phorbol myristate acetate ester (PMA) induces cleavage of proHB-EGF, and 1 μM of PMA has been shown to cleave the majority of proHB-EGF in 30 minutes (Goishi et al., 1995; Izumi et al., 1998). To ascertain whether deletion of the putative cleavage site would block cleavage of proHB-EGF, we exposed quiescent control and transfected cells to PMA (1 μM) for 30 minutes. Thereafter, cell lysates were subjected to immunoprecipitation using anti-FLAG M2 antibody and immunoblotted with the same antibody. As shown in Fig. 2D, in MDCK^{pro} cells, transfected proHB-EGF was recognized as 24-28 kDa bands; in addition, there was a less prominent smaller (approximately 12-15 kDa) FLAG-labeled band, representing residual transmembrane (TM) and cytoplasmic tail regions of HB-EGF after cleavage of the proHB-EGF molecule. Phorbol ester (PMA) treatment induced cleavage of HB-EGF protein in the MDCK^{pro} cells, as evidenced by the marked decrease in the ~28 kDa band, representing the full-length HB-EGF protein and a corresponding increase in the 12-15 kDa FLAG-labeled fragment. By contrast, phorbol ester treatment of the cells did not alter the expression of the ~28 kDa band in the MDCK^{5aa} or MDCK^{12aa} cells, and essentially none of the 12-15 kDa FLAG-labeled band could be detected, suggesting lack of cleavage. As expected, in cells expressing soluble HB-EGF, no FLAG-labeled cell-associated product could be detected, as the protein is secreted into the medium (Fig. 2D). We could not detect any endogenous expression of HB-EGF in nontransfected MDCK II cells with our antibodies.

With autocrine/paracrine activation of EGF receptors, ligand-receptor binding initiates receptor internalization and degradation, which results in the downregulation of receptor expression (Carpenter, 2000). In MDCK^{sol} cells, EGF receptor

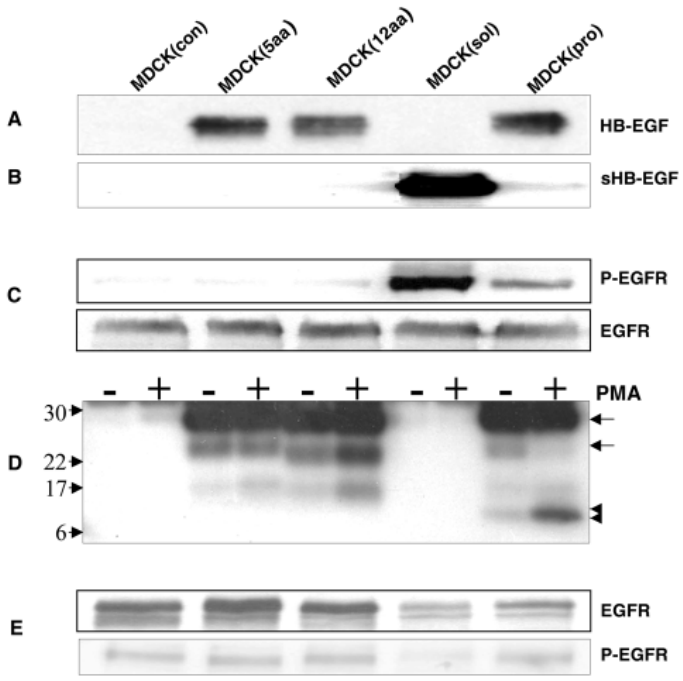

Fig. 2. (A) Western blot analysis using a polyclonal rabbit anti-rat HB-EGF antibody and total cellular lysate from the cells used in the studies. (B) Release of soluble HB-EGF in the culture medium. Serum free conditioned medium from the control and HB-EGF-transfected cells was loaded onto pre-equilibrated heparin-sepharose columns, washed with 10 mM Tris-cl, pH 7.4 and 100 mM NaCl and eluted with 2 M NaCl. Equal amount of eluants was resolved on 12% SDS-PAGE and probed with anti-HB-EGF antibody. The observations are representative of data from two independent clones for each transfected molecule and three independent experiments. (C) A Paracrine assay to establish the lack of cleavage of proHB-EGF in cells expressing deletion mutant constructs of HB-EGF. Equal amounts of the eluants from the purified conditioned media of control and HB-EGF-transfected cells were added to the quiescent A431 cells for 5 minutes. Cells were washed with PBS and lysed in RIPA buffer. Equal amounts of protein were resolved on 6% SDS-PAGE and probed with anti-phospho-EGFR antibody. The observations are representative of data from two independent clones for each transfected molecule and at least three independent experiments. The same blot was stripped and probed with the anti-EGFR antibody to verify equal protein loading. (D) Effect of phorbol myristate acetate ester (PMA; 1 μM) on the cleavage of proHB-EGF and deletion mutant constructs of HB-EGF. Quiescent cells were treated with 1 μM of PMA for 30 minutes. Cells were washed with 2 M NaCl to remove any soluble HB-EGF bound to the cell surface. Equal amount of protein was immunoprecipitated with anti-FLAG (M2) antibody. Samples were resolved on 10% Tricine gel and probed with anti-FLAG antibody. The (+) indicates cells treated with PMA and (–) indicates nontreated cells. The full-length HB-EGF and residual transmembrane/cytoplasmic domain remaining after cleavage of proHB-EGF are indicated by arrows and arrowheads, respectively. The observations are representative of data from two independent clones for each transfected molecule and at least three independent experiments. (E) EGF receptor expression in confluent MDCK control and HB-EGF-transfected cells. The basal EGF receptor tyrosine phosphorylation in quiescent MDCK control and HB-EGF-transfected cells. The observations are representative of data from two independent clones for each transfected molecule and five independent experiments.

expression was found to be markedly downregulated, and in $MDCK^{pro}$ cells EGFR expression was detectably lower than in control cells, whereas in $MDCK^{5aa}$ and $MDCK^{12aa}$ cells, EGFR expression appeared to be similar to vector transfected cells (Fig. 2E). Basal EGF receptor tyrosine phosphorylation in $MDCK^{pro}$, $MDCK^{5aa}$ and $MDCK^{12aa}$ cells was similar to that seen in control cells, whereas lower basal EGFR tyrosine phosphorylation was observed in $MDCK^{sol}$ cells (Fig. 2E).

Deletion of the putative cleavage site(s) did not affect juxtacrine signaling or membrane localization

Membrane-anchored HB-EGF has been suggested to signal in a juxtacrine manner (Iwamoto and Mekada, 2000; Raab and Klagsbrun, 1997). To verify that the deletion of the amino acids from the juxtamembrane extracellular domain did not affect the ability of deletion mutants to initiate juxtacrine signalling, we performed a juxtacrine assay (Higashiyama et al., 1995). Subconfluent A431 cells were overlain with formalin-fixed MDCK cells transiently transfected with different HB-EGF mutant constructs. After 10 minutes, the MDCK cells were washed away, membranes were prepared from A431 cells, EGF receptor was immunoprecipitated with an anti-EGFR antibody and an in vitro kinase assay was performed. All mutant constructs induced EGFR phosphorylation, indicating their ability to signal in a juxtacrine manner (Fig. 3A). The cells expressing the 5 amino acid deletion mutant were chosen for further studies.

To determine whether the deletion of amino acids from the juxtamembrane extracellular domain of pro-HB-EGF altered membrane localization, $MDCK^{con}$, $MDCK^{5aa}$ and $MDCK^{pro}$ were grown on transwell filters and immunofluorescent staining was performed using an anti-rabbit polyclonal anti-FLAG (M2) antibody followed by confocal microscopy. Results from this study confirmed a predominantly lateral membrane localization of both transfected proHB-EGF and 5 amino acid deletion mutant molecules (Fig. 3B,C).

Membrane-associated HB-EGF promoted collagen I-dependent cell adhesion and spreading and inhibited cell migration

In vitro expression of proHB-EGF promoted cell adhesion in renal tubular epithelial cells (Takemura et al., 1999; Takemura et al., 1997); however, it has not been determined whether the effect is due to membrane-bound HB-EGF or to sHB-EGF released following cleavage of proHB-EGF. Because our stably expressed juxtamembrane deletion mutants were not cleaved, we tested the potential role of juxtacrine signaling in cell-matrix interactions. We determined integrin-mediated collagen I-dependent cell adhesion in control and HB-EGF transfected cells using a standard cell-adhesion assay (described in Materials and Methods). The cells showed differences in their attachment at all concentrations of collagen tested; at the concentration of 10 μg/ml of collagen, $MDCK^{5aa}$ cells showed 25% greater cell adhesion compared with control cells and 37% increased adhesion compared with $MDCK^{sol}$, which had lower cell adhesion compared with all other cell types tested. $MDCK^{pro}$ cells had similar collagen I-dependent cell adhesion to $MDCK^{con}$ cells (Fig. 4A). Pretreating cells with β_1-integrin blocking antibody completely abolished cell-adhesion in all

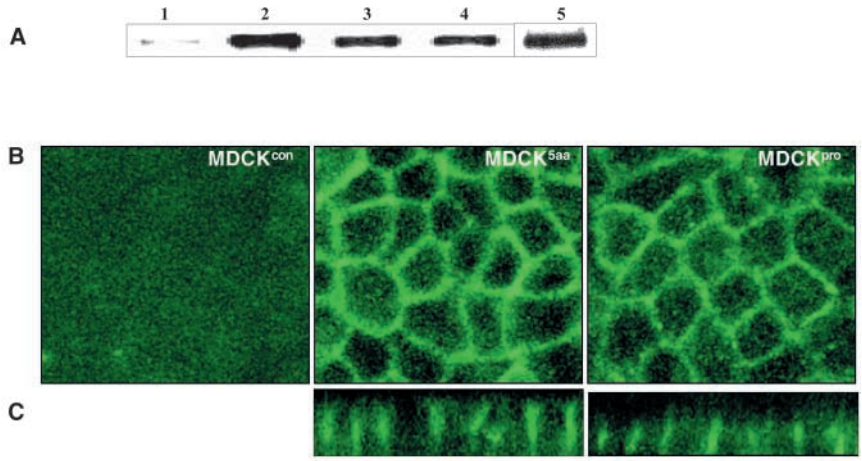

Fig. 3. (A) Juxtacrine assay with transiently transfected MDCK II cells. Lane 1: vector transfected cells; 2: proHB-EGF; 3: 5 aa deletion; 4: 12 aa deletion; 5: soluble HB-EGF administration. Subconfluent A431 cells were overlain with formalin fixed MDCK cells transiently transfected with different HB-EGF constructs. After 10 minutes, the MDCK cells were washed away, membranes were prepared from A431 cells, EGF receptor was immunoprecipitated with an anti-EGFR antibody and an in vitro kinase assay was performed. Soluble HB-EGF-induced EGF receptor tyrosine phosphorylation was used as a positive control. (B) Immunofluorescent staining of the control, MDCK^{5aa} and MDCK^{pro} cells with the anti-FLAG (M2) antibody. Cells were plated on transwells and stained with a polyclonal anti-FLAG (M2) antibody followed by an anti-rabbit conjugated with Rhodamine-X. Photographs were taken using a Zeiss-410 confocal microscope. The photographs shown are three-dimensional reconstructions of the Z-stack captured by confocal microscope and are representative of observations from two independent clones for each transfected molecule. Original magnification ×400. **(**C) The YZ orthogonal view of the sectioning for the FLAG-immunofluorescent staining for the MDCK^{5aa} and MDCK^{pro} cells through the stacks obtained using confocal microscopy.

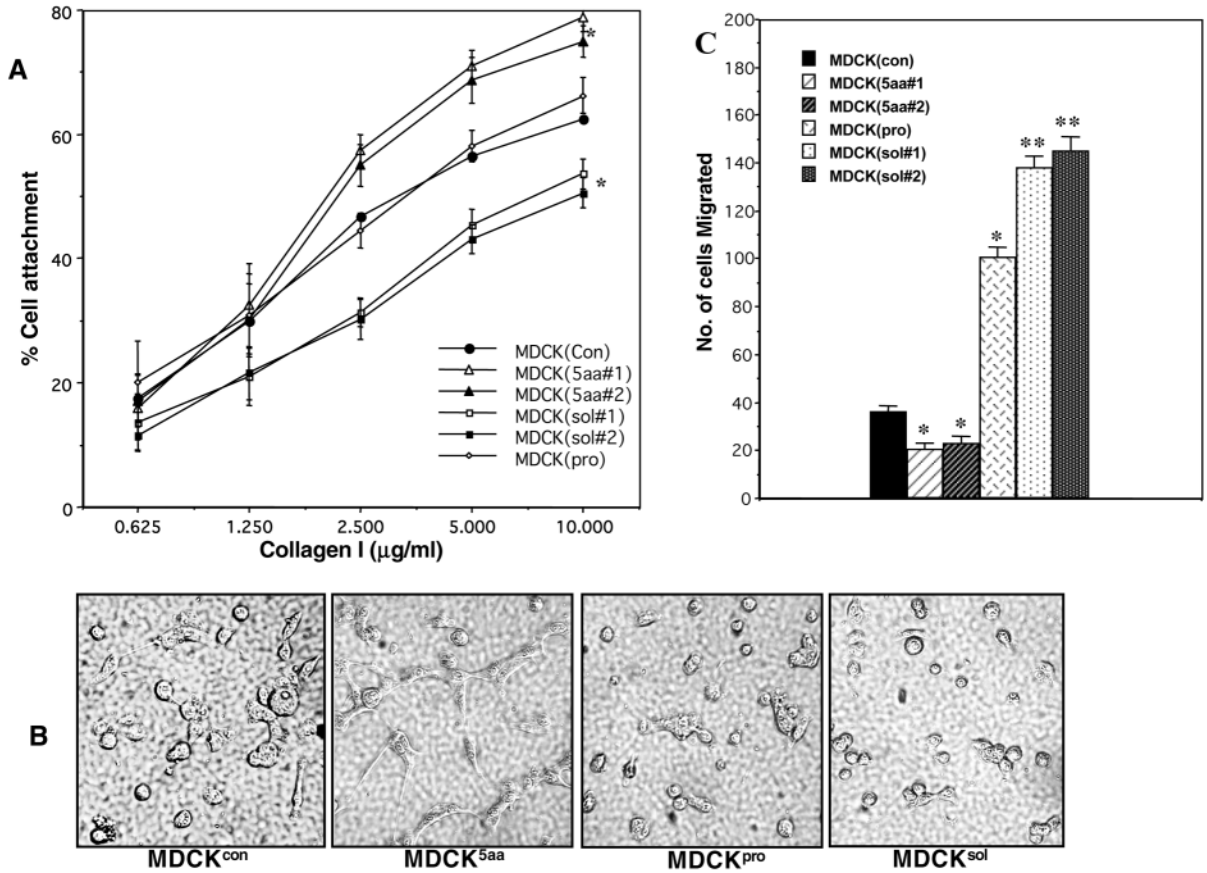

Fig. 4. (A) Collagen I-dependent cell adhesion. Equal numbers of cells were plated on collagen I-coated culture wells (U-bottom 96-well plates) for one hour at 37°C/5%CO_2. Cells were washed with PBS, fixed with 3.7% formaldehyde and stained with crystal violet (0.1% solution). Thereafter, cells were lysed in 20% acetic acid and values were determined at 570 nm in a plate reader. Values shown represent the mean of the values obtained from at least four independent experiments ± s.e.m. *P<0.001. (B) Cell spreading. Assay was performed as described in Materials and Methods. These photographs are representative of data from two independent clones for each transfected mutant molecule and at least four independent experiments. MDCK^{5aa} cells showed extended cellular extensions, whereas MDCK^{con} cells were all rounded. Original magnification ×100. (C) Cell migration. Experiments were performed as described in Materials and Methods. Values shown represent the mean of the values obtained from at least four independent experiments ± s.e.m. *P<0.05; **P<0.001.

cells, showing that the cell-adhesion was integrin dependent. There was no difference in β_1-integrin expression among the cell types, as determined by FACS and western blot analysis using anti-β_1-integrin antibody (data not shown), confirming that the differences in adhesion were not due to changes in β_1-integrin expression.

We next determined whether these cells also exhibited differences in their ability to spread on a matrix after attachment. MDCK^{5aa} cells expressing membrane-anchored HB-EGF showed elaborate cell spreading with extensive cellular extensions at all collagen concentrations; however, the differences were most pronounced at 10 μg/ ml of collagen I. By contrast, MDCK^{sol} cells remained rounded and did not spread within the time period of the assay, whereas MDCK^{pro} cells exhibited an intermediate phenotype, with a minority of the cells spreading with rudimentary cellular extensions (Fig. 4B).

Because expression of membrane-anchored and soluble HB-EGF affected cell adhesion in distinctly opposite ways and soluble HB-EGF has been implicated as an important mediator of wound healing and cell migration in keratinocytes (Tokumaru et al., 2000a), we performed cell migration assays (described in Materials and Methods). MDCK^{sol} cells showed significantly higher cell migration compared with control cells, and expression of the 5 amino acid deletion mutant inhibited cell migration compared with control cells (Fig. 4C).

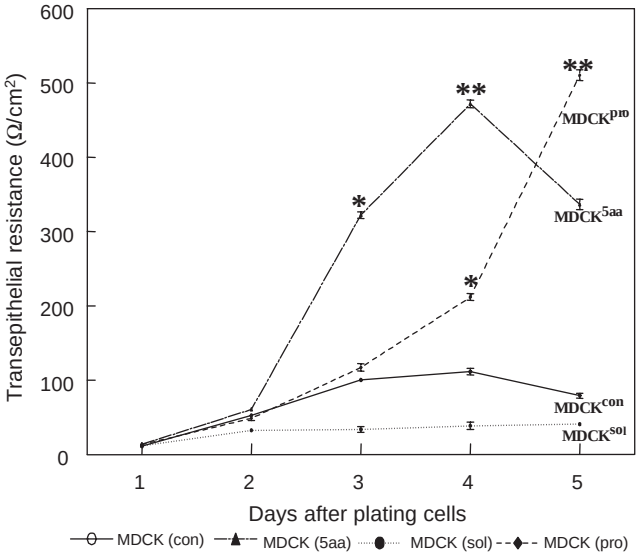

Fig. 5. Transepithelial resistance in polarized cells (cells grown on transwells). Final values were obtained by subtracting the value from blank wells. Values shown represent the mean of the values obtained from at least three independent experiments and are representative of the data obtained from two independent clones for each transfected molecule ± s.e.m. *P<0.01; **P<0.001.

Expression of membrane-anchored HB-EGF resulted in increased transepithelial resistance

Epithelial cells polarize when grown on permeable filter supports, and TER across the confluent epithelial cell layer is an established measure of the degree of cell-cell contact (Hopkins et al., 2000). Cells were plated on clear-transwells at superconfluent density and TER was measured every 24 hours. MDCK^{5aa} cells developed significantly higher TER than control cells. By contrast, the MDCK^{sol} cells developed minimal resistance, whereas MDCK^{pro} cells showed low resistance initially but achieved significantly higher TER than MDCK^{con} and MDCK^{sol} cells by day 4-5 after plating (Fig. 5).

To determine whether release of sHB-EGF due to cleavage of proHB-EGF was responsible for the initial low TER observed in MDCK^{pro} cells, we used a specific metalloproteinase inhibitor Batimastat (BB-94). BB-94 (5 μM) has been shown to inhibit the cleavage of proHB-EGF completely (Chen et al., 2002; Prenzel et al., 1999). The cells were plated on transwells as above and after 10 hours of plating (the time necessary to obtain a confluent cell layer), BB-94 (5 μM) was added. Addition of the metalloproteinase inhibitor eliminated the initial delay in the increase in TER in MDCK^{pro} cells, and the TER values and timing were similar to those of MDCK^{5aa} cells. Addition of BB-94 had no significant effect on TER in any other cell type under study, including MDCK^{con} cells (Table 1).

Cells expressing membrane-anchored HB-EGF did not scatter in response to HGF/SF

MDCK II cells normally scatter in response to HGF/SF. For scattering to occur, cells must dissociate from each other and from the substratum (Balkovetz, 1998; Stella and Comoglio, 1999). Thereafter, cells develop a fibroblast-like appearance and migrate away from each other. Although the exogenous administration of soluble EGF induces cell scattering in certain cells, it neither induces nor inhibits cell scattering in MDCK cells (Liang and Chen, 2001; Tanimura et al., 1998). At 16 hours post-treatment with HGF/SF, the scattering pattern of MDCK^{sol} and MDCK^{pro} cells was not different from

Table 1. Transepithelial resistance (TER) in polarized control and HB-EGF mutant transfected cells with and without Batimastat (BB-94)

	Batimastat (BB-94)	Day 1	Day 2	Day 3	Day 4	Day 5
MDCK^{con}	–	13.33±1.56	49.50±2.813	107.50±3.04	130.16±2.93	85.83±3.53
	+	13.66±1.41	60.66±2.60	107.16±3.31	134.83±3.72	97.83±4.93
MDCK^{5aa}	–	13.16±1.58	60.16±3.00	310.50±3.99**	436.66±4.93**	306.66±4.90**
	+	15.00±1.77	63.50±2.83	332.16±7.91**	447.16±3.895**	318.33±4.19**
MDCK^{pro}	–	13.50±1.73	48.33±2.49	122.83±3.50	215.16±4.88*	512.33±6.70**
	+	11.66±1.12	76.83±3.44	357.00±4.56**	538.66±4.66**	401.16±6.24**
MDCK^{sol}	–	12.00±1.064	30.83±2.59	39.66±2.06	35.16±2.65	48.16±2.44
	+	12.16±1.05	39.00±2.017	47.66±2.42	48.50±3.11	54.33±1.96

Values represent TER±s.e.m.; *P<0.01; **P<0.001. The data are representative of the data obtained from two independent clones for each transfected mutant molecule from three independent experiments.

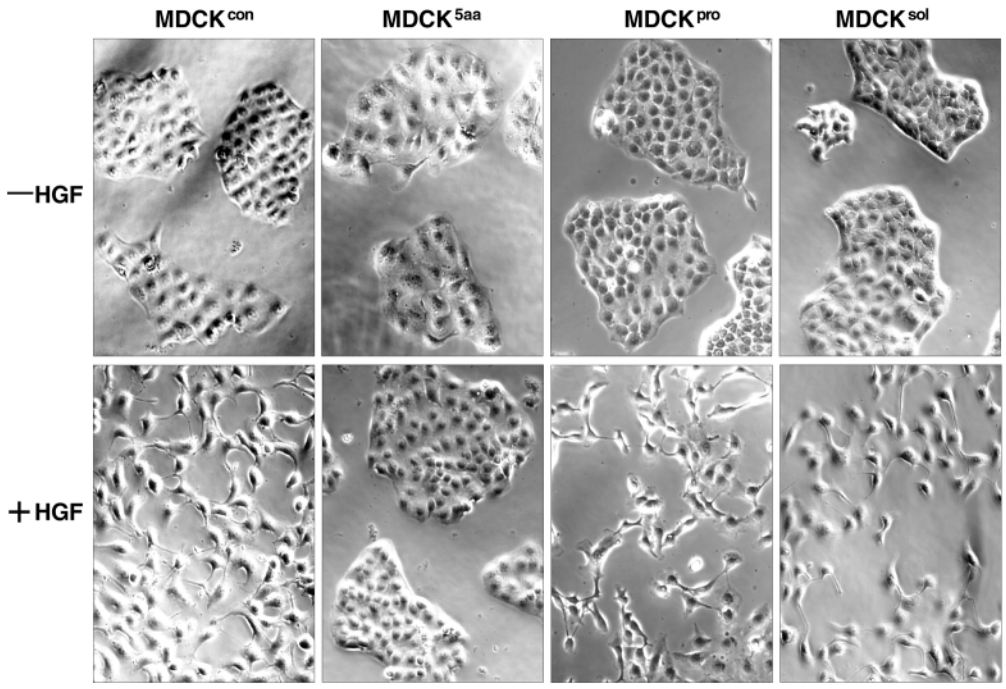

Fig. 6. Cell scattering. Experiments were performed as described in Materials and Methods. Upper panel (– HGF) shows cells without HGF/SF treatment; lower panel (+ HGF) shows cells 16 hours after HGF/SF (20 ng/ml) treatment. These findings are representative of at least five independent experiments from two independent clones for each transfected molecule. Original magnification ×100.

MDCK^{con}. By contrast, MDCK^{5aa} did not dissociate or migrate apart from each other (Fig. 6). Exogenous administration of either recombinant EGF (20 ng/ml) or soluble HB-EGF (20 ng/ml) 30 minutes before HGF/SF administration did not alter HGF/SF-induced scattering in the control cells, nor did it alter the inhibition of scattering observed in MDCK^{5aa} (data not shown). Furthermore, PD 153035, a specific and stable inhibitor of EGF receptor tyrosine phosphorylation (Bancroft et al., 2002; Pai et al., 2002) (Fig. 7A) failed to inhibit or induce cell scattering in the HGF-treated MDCK^{con} or MDCK^{5aa} cells, respectively (Fig. 7B).

Western blot analysis using a specific antibody for c-Met, the HGF/SF receptor, showed identical receptor expression among HB-EGF-transfected and control cells (data not shown). Furthermore, analysis with phosphospecific antibodies for c-Met or EGFR, although confirming the possibility of cross-talk between the c-Met and the EGFR activation (Jo et al., 2000; Scheving et al., 2002), indicated no differences in HGF-induced tyrosine phosphorylation of either receptor in the MDCK^{5aa} cells compared with control cells (data not shown). We could not detect any ErbB4 protein expression in our cells using multiple antigen-specific antibodies (data not shown).

Inhibition of proHB-EGF cleavage inhibited HGF-induced cell scattering in MDCK^{pro} cells, while blocking CD9 and heparin interactions induced scattering in MDCK^{5aa} cells

Because inhibition of metalloproteinase activity in MDCK^{pro} cells eliminated the initial delay in the increase in TER in MDCK^{pro} cells such that the TER values and timing were similar to those of MDCK^{5aa} cells, we determined whether the HGF-induced cell scattering could also be modulated by the inhibition of metalloproteinase activity. Experiments were performed as for cell scattering except that cells were exposed to Batimastat (5, 10 and 20 μM) for 3-5 hours before adding HGF/SF (20 ng/ml). MDCK^{con} cells scattered in the presence of all the doses of Batimastat (data not shown), but the HGF-induced scattering was markedly inhibited in MDCK^{pro} cells+Batimastat (20 μM) compared with MDCK^{pro} cells that received only HGF (Fig. 8A).

Because proHB-EGF is known to complex with CD9 and heparin-sulfate proteoglycans (Lagaudriere-Gesbert et al., 1997; Nakamura et al., 1995), we tested whether abrogating this complex formation could induce cell scattering in MDCK^{5aa} cells in response to HGF. We used a monoclonal CD9 blocking antibody and heparinase I for this purpose. Cells were pretreated with CD9 blocking antibody (1 μg/ml) or heparinase I (1 U/ml) for 3-5 hours before exposing cells to HGF. As a control for the CD9 antibody, a group of cells was exposed to mouse IgG (1 μg/ml). Although neither CD9 blocking antibody nor heparinase I affected the HGF-induced scattering in MDCK^{con} cells, they both induced scattering in MDCK^{5aa} cells in response to HGF compared with the MDCK^{5aa} cells receiving only HGF (Fig. 8B)

Expression of membrane-anchored HB-EGF inhibits tubulogenesis

MDCK cells form cysts when cultured in three-dimensional collagen gels, and addition of exogenous HGF/SF induces these cysts to develop into branched tubules (Balkovetz, 1998; Stuart et al., 1995). However, as in the case of cell scattering, EGF administration does not have any effect on tubulogenesis in MDCK II cells (Barros et al., 1995). Because expression of membrane-anchored HB-EGF inhibited HGF/SF-induced cell scattering, we investigated whether it could also modulate HGF/SF-induced tubulogenesis. Experiments were performed as described in Materials and Methods, and cells were cultured for 7-9 days before photographs were taken. Interestingly, in nonHGF/SF-treated cells, all HB-EGF-transfected cells showed significant increases in the size of cysts compared with

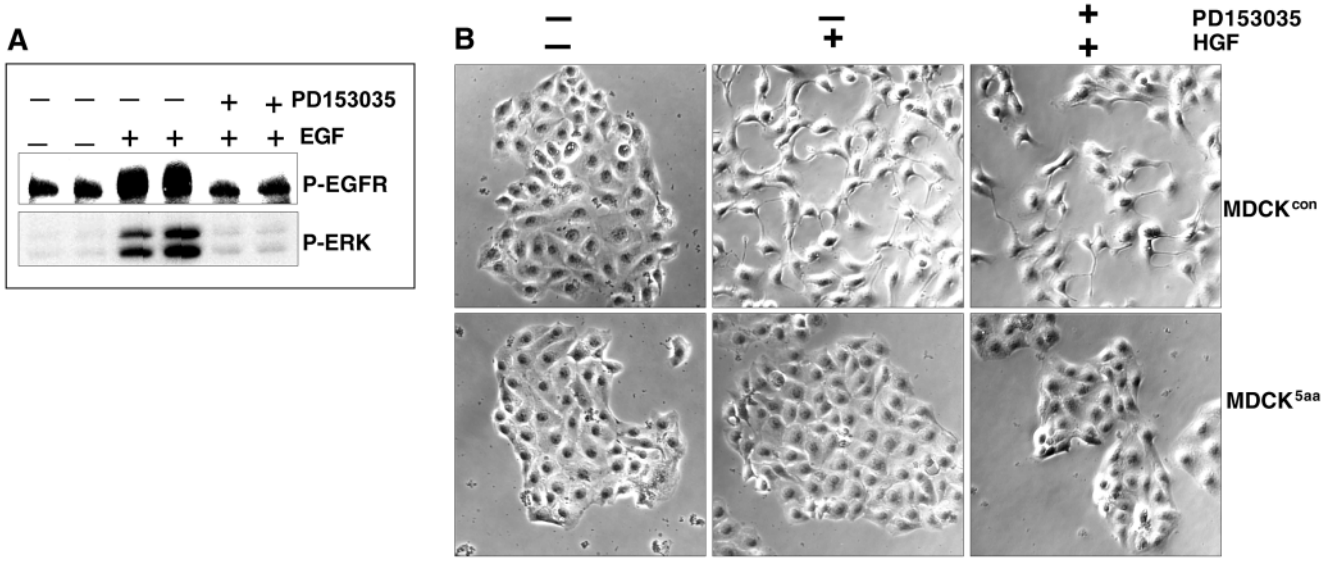

Fig. 7. (A) Effect of PD153035, a specific and stable inhibitor of EGF receptor tyrosine phosphorylation, on EGF-induced EGFR and ERK 1/2 phosphorylation. Quiescent MDCK II cells were treated with 20 ng/ml of EGF with or without PD153035 (2.5 μM) for 30 minutes before EGF treatment. (B) Effect of PD153035 on HGF/SF-induced cell scattering in MDCK^{con} and MDCK^{5aa} cells. Experiments were performed as in Fig. 6 except that one group of cells received PD 153035 (2.5 μM) 30 minutes before the addition of HGF (20 ng/ml). The top panel shows MDCK^{con} cells and the lower panel shows MDCK^{5aa} cells. Original magnification ×100.

MDCK^{con} cells, indicating a possible proliferative role for membrane-anchored as well as soluble HB-EGF in collagen. However, tubular structures induced by HGF/SF treatment were very different among all transfected cells as well as compared with control cells. MDCK^{5aa} cells formed small irregular masses with many small-spike or needle-shaped outgrowths without any tubular extensions, whereas MDCK^{sol} cells developed into long tubules with reduced branching compared with MDCK^{con}. MDCK^{pro} cells once again showed an intermediate phenotype, with tubules exhibiting wider lumens compared with MDCK^{con} (Fig. 9).

HGF/SF-induced disruption of cell-cell junction was inhibited in MDCK^{5aa} cells

During cell scattering, the adherent and tight junctions are disrupted/dissociated, and the adherent junction protein E-cadherin and the tight junction protein ZO-1 are both translocated to the cytoplasm (Grisendi et al., 1998; Kamei et al., 1999). During HGF/SF-induced tubulogenesis, epithelial cell polarity is transiently lost and components of cell-cell junctional complexes, including E-cadherin and ZO-1, undergo profound rearrangements (Pollack et al., 1998). Given that MDCK^{5aa} cells neither scattered nor formed well-defined tubular structures in response to HGF/SF, we determined the changes in E-cadherin and ZO-1 localization in control and MDCK^{5aa} cells in response to HGF/SF at various time-points. Cells were plated on glass coverslips and 48 hours post-seeding, cells were subjected to HGF/SF treatment as in the scattering studies. Cells were fixed at 4, 8 and 12 hours after the addition of HGF/SF. One group of cells did not receive any HGF/SF and served as a control. Immunofluorescent staining using anti-E-cadherin and anti-ZO-1 antibody showed that HGF/SF treatment induced disruption of adherent junctions by 4 hours post-treatment; at 12 hours after HGF/SF treatment almost all the adherent junctions were lost and E-cadherin was translocated to the cytoplasm in the MDCK^{con} cells. Disruption of tight junctions, as evidenced by ZO-1 localization, was delayed compared with adherent junctions disruption, but some degree of cytoplasmic translocation of ZO-1 was evident by 4 hours of HGF/SF treatment. At 12 hours post-treatment most of the tight junctions were disrupted, but the departing cells were still connected by individual strands of tight junction. By contrast, although there was a minor amount of cytoplasmic translocation of E-cadherin and ZO-1 in the MDCK^{5aa} cells, disruption of both adherent and tight junctions was largely prevented, even after 12 hours of HGF/SF treatment (Fig. 10a,b).

HGF/SF-induced activation of ERK 1/2 and phosphoinositide 3-kinase was largely unaffected in MDCK^{5aa} cells

HGF/SF treatment of MDCK cells has been shown to result in activation of ERK 1/2 and phosphoinositide 3-kinase/Akt pathways, and specific pharmacological inhibitors of these pathways inhibit cell scattering induced by HGF/SF (Khwaja et al., 1998; Sipeki et al., 1999; Tanimura et al., 1998). Also, in a recent report, decreased JNK/SAPK phosphorylation was implicated in HGF/SF-induced cell scattering in MDCK cells (Paumelle et al., 2000). Therefore, we determined whether differences in the activation of one or more of these signaling pathways could be responsible for the lack of scattering observed in MDCK^{5aa} cells. The change in the phosphorylation status of Akt has been used as an index of HGF/SF-induced phosphoinositide 3-kinase activation, and inhibition of cell scattering with a specific pharmacological inhibitor (LY294002) of phosphoinositide 3-kinase also inhibits HGF/SF-induced Akt phosphorylation (Khwaja et al., 1998). HGF/SF treatment resulted in the slow but sustained activation

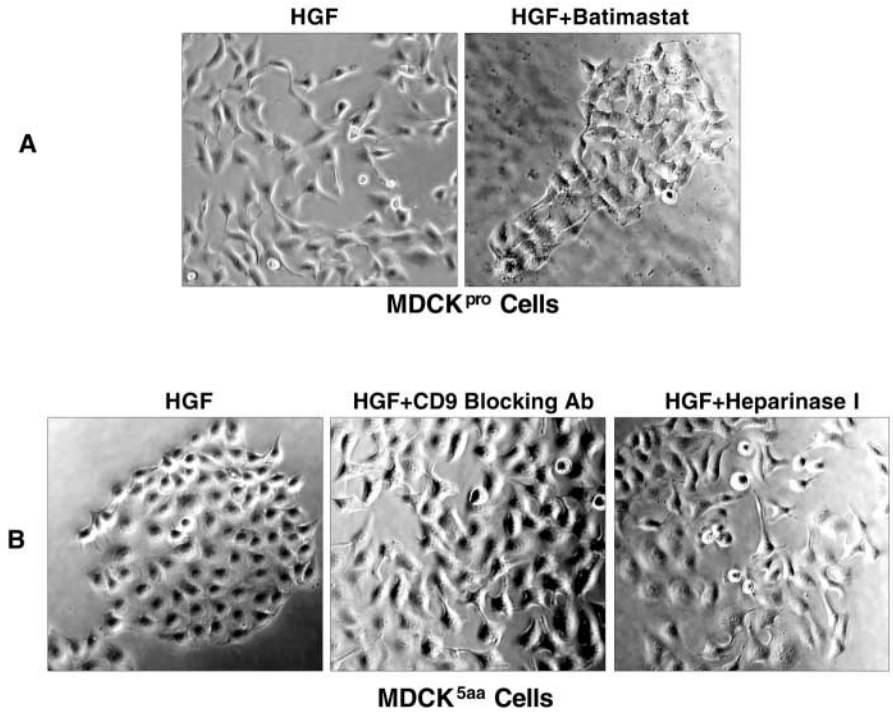

Fig. 8. (A) Effect of Batimastat on HGF/SF-induced cell scattering in MDCK^{pro} cells. Experiments were performed as in Fig. 6 except that one group of cell received Batimastat (20 μM) for 3-5 hours before the addition of HGF (20 ng/ml). (B) Effects of anti-CD9 antibody and heparinase I on HGF/SF-induced cell scattering in MDCK^{5aa} cells. Experiments were performed as above except that cells received anti-CD9 antibody (1 μg/ml) or Heparinase I (1 U/ml) for 3-5 hours before the addition of HGF (20 ng/ml). Original magnification ×100.

of ERK 1/2 and Akt in the quiescent control cells and no significant differences were observed in the transfected cells compared with control cells. The basal activity as well the inducible activity of ERK and Akt were relatively lower in MDCK^{sol} cells, although the relative degree of overall activation of these proteins in response to HGF in these cells was not different than other cells under the study (Fig. 10). However, notably, the antibody against P-Akt recognized an additional band of relatively higher molecular weight in addition to the ~65 kDa band for P-Akt in the MDCK^{5aa} cells, and the phosphorylation patterns of these two bands were different. While the higher molecular band showed transient phosphorylation with the peak at 15 minutes followed by a decrease in the phosphorylation, the ~65 kDa band showed a slow but sustained phosphorylation similar to control cells. A similar higher molecular weight band but of lesser intensity was also recognized in the MDCK^{pro} cells (Fig. 11). Pretreatment of the cells with LY294002, a specific inhibitor of the phosphoinositide 3-kinase, resulted in complete inhibition of both HGF-induced bands recognized by the anti-phospho-Akt antibody in the MDCK^{5aa} cells (data not shown). The identity of this higher molecular weight band remains to be determined. No differences in the phosphorylation of JNK/SAPK were observed at any time-point after the treatment in the control cells or among HB-EGF transfected cells (data not shown).

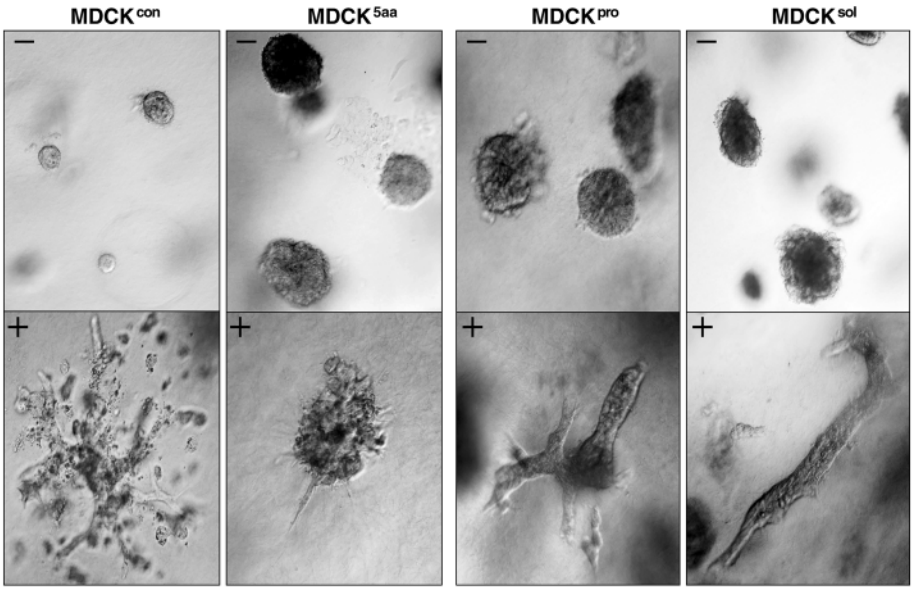

Fig. 9. Cells grown in collagen. Experiment was performed as described in Materials and Methods. After 9 days of culture, cells were fixed with 3.7% formaldehyde and photographed. Upper panel (–) shows cells without treatment and lower panel (+) shows cells grown in the presence of HGF/SF (20 ng /ml). These findings are representative of at least six independent experiments and are representative of the data obtained from two independent clones for each transfected molecule. Original magnification ×200.

Discussion

Among membrane-anchored growth factors, HB-EGF is of special interest because it forms complexes with different proteins, functions as a receptor for Diphtheria toxin, serves as a ligand for two different receptors (erbB1 and erbB4) and is a precursor for a soluble growth factor (Iwamoto and Mekada, 2000; Raab and Klagsbrun, 1997). HB-EGF has been shown to be involved in various physiological, as well as pathological, processes; however, to date no study of the biological effects attributed to HB-EGF has unequivocally differentiated between mediation by the membrane-associated versus the soluble form of the growth factor, in which the processing of the transmembrane form may alter juxtacrine to autocrine/paracrine signaling. In the present study, we have defined distinct roles for membrane-associated HB-EGF and soluble HB-EGF in epithelial cell morphogenesis and behavior, using cultured polarized epithelial cells stably expressing either soluble secreted HB-EGF or a noncleavable membrane-anchored form of HB-EGF.

We used these stably transfected cell lines to show that in MDCK cells: first, deletion of the putative cleavage site of the proHB-EGF inhibited its metalloproteinase-dependent cleavage and did not affect the membrane localization or the ability of the molecule to act in a juxtacrine manner; second, the expression of membrane-anchored HB-EGF enhanced collagen-dependent cell-adhesion and induced tighter cell-cell

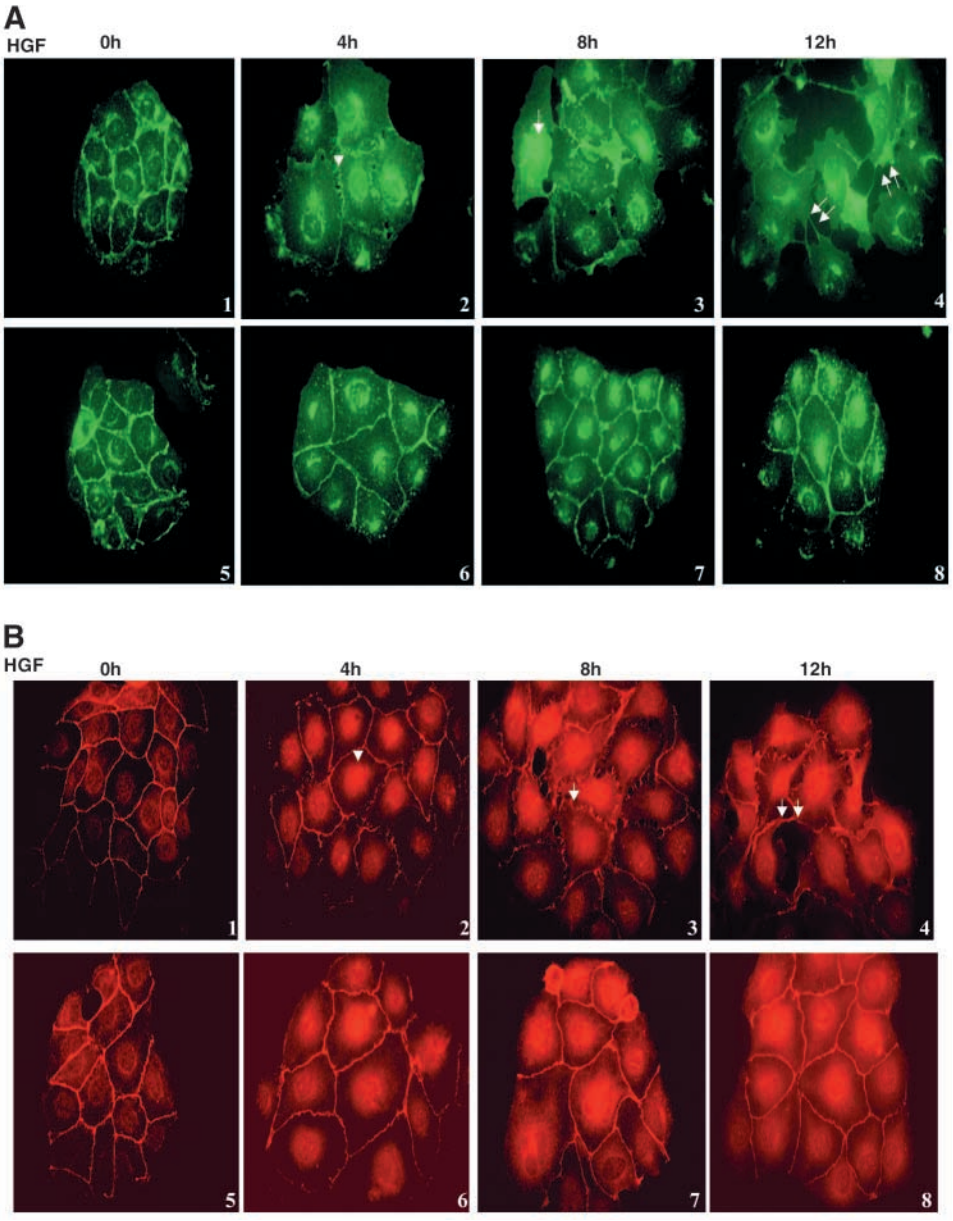

Fig. 10. Disruption of adherent and tight junctions by HGF/SF was prevented by expression of membrane-anchored HB-EGF mutant. Experiments were performed as for the scattering experiment except that the cells were plated on glass coverslips. Forty-eight hours after the plating, cells were exposed to HGF/SF 20 ng/ml and cells were washed and fixed at 4, 8 and 12 hours after the HGF/SF treatment and subjected to immunofluorescent staining. (a) Adherens junction's integrity was assessed using anti-E-cadherin antibody followed by an anti-mouse FITC-conjugated antibody. 1-4: $MDCK^{con}$ cells at 0, 4, 8 and 12 hours after HGF/SF treatment. The adherent junctions began dissociating 4 hours after HGF/SF treatment (see arrowheads) and had mostly disappeared after 8 hours, with the majority of E-cadherin translocated into the cytoplasm (see arrows), and after 12 hours post-treatment cells had begun to move apart. However, they were still connected with individual strands of adherent junctions (see double arrows). At this time-point most of the E-cadherin staining was lost possibly because of endocytosis. 5-8: $MDCK^{5aa}$ cells at 0, 4, 8 and 12 hours after HGF/SF treatment. Although there was a small amount of cytoplasmic translocation of E-cadherin in response to HGF/SF treatment, the junctions were intact even after 12 hours after the treatment. Original magnification ×400. (b) Tight-junction integrity was assessed using anti-ZO-1 antibody followed by anti-rabbit-rhodamine-X conjugated antibody. 1-4: $MDCK^{con}$ cells at 0, 4, 8 and 12 hours after HGF/SF treatment The cytoplasmic translocation of ZO-1 was observed by 4 hours after HGF/SF treatment (see arrowhead) but the tight junctions were still largely intact. However, by 8 hours of HGF/SF treatment, tight junctions were markedly disrupted (see arrows) and by 12 hours post-treatment, almost all of junctional ZO-1 had translocated into the cytoplasm and cells had begun to move apart. However, they were still connected with strands of tight junctions (see double arrows). 5-8: $MDCK^{5aa}$ cells at 0, 4, 8 and 12 hours after HGF/SF treatment. Although there was little cytoplasmic translocation of ZO-1 in response to HGF/SF treatment, the tight junctions were intact like adherent junctions in the $MDCK^{5aa}$ cells even at 12 hours after the treatment. Original magnification ×400.

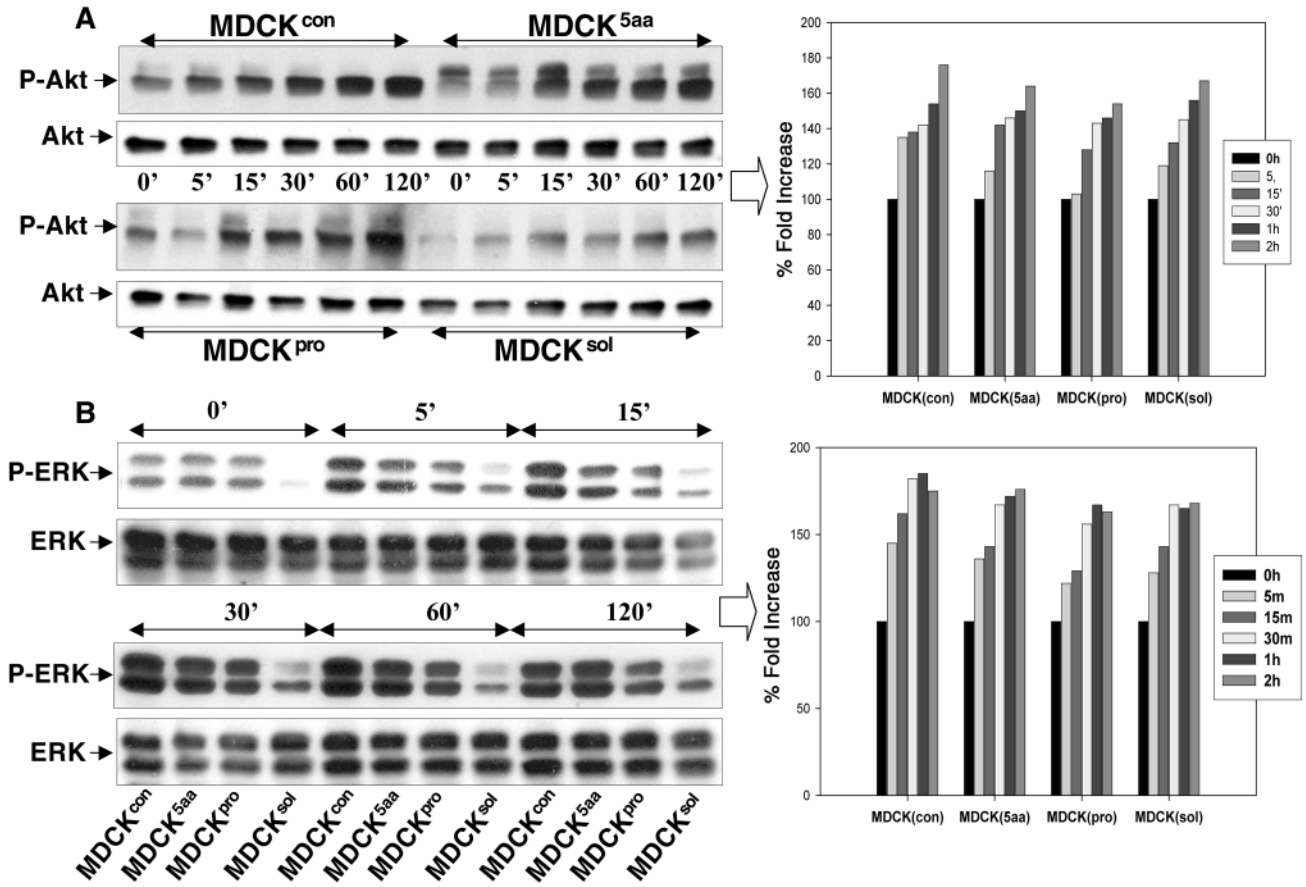

Fig. 11. Effects of HGF/SF on phosphorylation of ERK 1/2 and Akt proteins. Quiescent cells were treated for the indicated times with HGF/SF (20 ng/ml). One group of cells did not receive any HGF and were considered 0 hours. Immunoblot analysis was performed with the use of the (A) anti-phospho-Akt kinase antibody or (B) anti- phospho-ERK 1/2 kinase antibody. The filters used for examination of phospho-ERK 1/2 and Akt were stripped and reprobed with their respective anti-ERK 1/2 and Akt antibodies to determine equal protein loading. The bar diagrams on the right panel represent the densitometric values for the respective western blot analysis.

contact while inhibiting cell migration; third, soluble HB-EGF decreased collagen-dependent cell-adhesion and cell-cell contact and significantly enhanced cell migration; and fourth, the expression of membrane-anchored HB-EGF inhibited HGF/SF-induced cell-cell dissociation and the formation of branched tubular structures.

The observation that expression of a noncleavable form of membrane-associated HB-EGF resulted in a significant increase in collagen I-dependent cell-adhesion supports our earlier observation that overexpression of proHB-EGF enhanced cell attachment in tubular epithelial cells (NRK52-E cells) (Takemura et al., 1997), and indicates that this effect is not cell specific. Differences in cell adhesion were not due to differential expression of β_1-integrins. Our observation that membrane-anchored HB-EGF enhanced cell adhesion is also consistent with the biological effects attributed to membrane-anchored HB-EGF that is induced in the mouse luminal epithelium solely at the site of blastocyst apposition before the attachment reaction (Paria et al., 1999).

Expression of soluble HB-EGF resulted in significantly decreased cell adhesion and increased migration compared with MDCK^{5aa} cells. Ectodomain shedding of EGF receptor ligands, particularly HB-EGF, is required for keratinocyte migration in cutaneous wound healing (Piepkorn et al., 1998; Tokumaru et al., 2000b). In addition, N-arginine dibasic convertase (NRDc), a metalloendopeptidase of the M16 family, which has been recently identified as a receptor for HB-EGF, induces HB-EGF-dependent cell migration (Nishi et al., 2001). Thus, our data are in agreement with the previous reports suggesting a role for sHB-EGF in wound healing and enhancing cell migration.

HGF/SF-induced cell scattering/motility in MDCK cells involves changes in cell-matrix interactions and disassembly of cell-cell junctions, with adherent junctions disassembled before the disruption of tight junctions (Grisendi et al., 1998; Kamei et al., 1999). Immunofluorescent localization of E-cadherin and ZO-1 indicated persistent E-cadherin at adherent junctions and ZO-1 at the tight junctions in MDCK^{5aa} cells compared with control cells after exposure to HGF/SF. Compared with MDCK^{con} or MDCK^{sol} cells, MDCK^{5aa} cells also developed significantly higher resistance, suggesting tighter cell-cell contact. Cell-cell junctions and polarity undergo profound although transient changes during the process of tubulogenesis (Pollack et al., 1998), and persistence of tight and adherens junction integrity may underlie the failure of MDCK^{5aa} cells to develop normal branched structures.

By contrast, MDCK^{sol} cells developed only minimal TER when grown on permeable membrane supports. In this regard, decreased cell-adhesion, increased motility and lack of resistance due to compromised cell junctions are characteristics of transformed cells (Schoenenberger et al., 1991), and the members of EGF family of growth factors are known to induce epithelial-mesenchymal transformation (Cattan et al., 2001).

Increases in TER were delayed in MDCK^{pro} cells compared with MDCK^{5aa} cells, and HGF/SF-induced scattering was not prevented in MDCK^{pro} cells. We postulate that the initial low resistance in MDCK^{pro} cells was due to sHB-EGF released by cleavage of proHB-EGF at the time of plating and cell attachment, and the high TER at later points represented a shift to the predominant expression of membrane-associated HB-EGF. This hypothesis was supported by the fact that pretreatment of the MDCK^{pro} cells with a metalloproteinase inhibitor Batimastat (BB-94) not only selectively eliminated the delayed increase in TER in MDCK^{pro} cells, such that the timing of increase in TER was similar to MDCK^{5aa} cells, but also markedly inhibited the HGF-induced scattering in these cells.

In the present study, HGF/SF treatment induced comparable levels of both c-Met and EGFR tyrosine phosphorylation in MDCK^{con} and MDCK^{5aa} cells. Furthermore, identical patterns and degrees of activation of both ERK 1/2 and Akt were detected. HGF/SF treatment of MDCK cells is known to induce activation of ERK 1/2 and phosphoinositide 3-kinase, as well as phosphoinositide 3-kinase-dependent Akt activation (Khwaja et al., 1998; Tanimura et al., 1998). Interestingly, lower levels of baseline phosphorylation of Akt and ERK 1/2 were observed in MDCK^{sol} cells compared with control cells; however, the trend of HGF/SF-induced activation of Akt and ERK 1/2 in MDCK^{sol} cells was not different to control cells. We postulate that the lower basal and inducible activation of the ERK 1/2 and Akt proteins in these cells could be a result of constitutive activation of EGF receptors.

Interestingly, the antibody against P-Akt recognized an additional band of relatively higher molecular weight in addition to the ~65 kDa band for P-Akt, and the signal intensity and timing of phosphorylation for the higher molecular weight band (~67-70 kDa) were different between MDCK^{con} and MDCK^{5aa} cells. Pretreatment of the cells with LY294002 inhibited not only the activation of the ~65 kDa (Akt) band but also the activation of the higher molecular weight band (data not shown). Recently, three Akt isoforms, Akt1/PKBα, Akt2/PKBβ and Akt3/PKBγ have been reported (Datta et al., 1999). Although the literature regarding the role of different Akt-isoforms is still in its nascent phase, these isoforms have been shown to be differentially regulated by EGF stimulation (Okano et al., 2000). It is tempting to speculate that the observed unidentified band could be an Akt isoform and its activation is induced by the expression of membrane-anchored HB-EGF. However, the identity and significance of this band remains to be determined because of the current lack of specific reagents for canine cells.

One of the most striking findings of the present study was the persistence of the cell-junction integrity and significantly higher TER in MDCK^{5aa} cells. Furthermore, administration of exogenous EGF/soluble HB-EGF or inhibition of EGFR tyrosine phosphorylation did not alter HGF/SF-induced cell scattering in the control cells. Also, no major differences were observed in EGF receptor tyrosine phosphorylation under basal conditions or in response to HGF administration among the cells under study, whereas a blocking antibody against the extracellular domain of CD9, as well as pretreatment of cells with heparinase I, both induced cell scattering in MDCK^{5aa} cells in response to HGF. These findings suggest that juxtacrine EGFR activation per se may not be necessary and/or sufficient for the observed phenotypic changes in MDCK^{5aa} cells and that membrane-anchored HB-EGF may be functioning as a scaffolding protein, as proHB-EGF is known to complex with CD9, a member of the tetraspanin superfamily (Lagaudriere-Gesbert et al., 1997). CD9 associates only with membrane-associated proHB-EGF but not soluble HB-EGF by interacting with the extracellular heparin-binding domain (Nakamura et al., 2000). CD9 interacts with selected integrins, especially β1 integrins, which are important in cell-cell and cell-extracellular matrix adhesive properties (Nakamura et al., 1995). In Vero cells expressing HB-EGF and CD9, HB-EGF-CD9 complexes localize to the cell-cell contact interface in association with α-catenin and vinculin, and CD9 interacts specifically with α3β1 integrins at the adherens junctions (Nakamura et al., 1995). Membrane-associated HB-EGF also interacts with cell-surface heparin-sulfate proteoglycans (Paria et al., 1999). Therefore, our results suggest that the phenotypic changes observed in MDCK^{5aa} cells may result from membrane-bound HB-EGF interacting with proteins such as the tetraspanin, integrins or heparin-sulfate proteoglycans, rather than by juxtacrine receptor activation.

Disruption of cell-cell and cell-matrix interaction is of central importance in epithelial cell injury, including renal injuries such as ischemia-reperfusion (Racusen, 1997; Sutton and Molitoris, 1998). As discussed above, owing to their basolateral localization in the epithelial cells, EGF receptor, proHB-EGF, CD9 and α3β1 integrins are capable of making a large protein complex involving heparin-sulfate proteoglycans at the cell-cell contact site. We postulate that proHB-EGF helps to maintain the integrity of epithelial sheet through these interactions, providing stronger cell-cell and cell-matrix interaction. This hypothesis is supported by significantly higher cell-matrix and cell-cell adhesion in our cells expressing the noncleavable mutant of proHB-EGF. When the integrity of a tissue is disrupted, surrounding cells such as platelets, monocytes/macrophages and other cells in the damaged tissue release several factors including lysophosphatidic acid (LPA). Activation of G protein-coupled receptors induces ectodomain shedding of proHB-EGF (Prenzel et al., 1999). LPA is one of the ligands of GPCR that stimulates ectodomain shedding of proHB-EGF (Hirata et al., 2001). LPA and other factors possibly induce ectodomain shedding of proHB-EGF to yield sHB-EGF. sHB-EGF may then help in the regeneration of the damaged tissue by stimulating proliferation as well as migration of cells. This proliferation and migration of the cells would reinstate the cell-cell contact, and interactions of proHB-EGF with EGFR and other proteins would be reconstituted and cell-cell integrity re-established.

In summary, we have developed novel engineered MDCK cell lines that allow the study of membrane-associated and soluble HB-EGF. Using these cell lines we have shown that expression of membrane-anchored HB-EGF inhibited HGF/SF-induced epithelial cell scattering and tubulogenesis.

We have also shown that the expression of membrane-anchored HB-EGF resulted in enhanced cell adhesion as well as tighter cell-cell contact and inhibited cell migration and HGF/SF-induced cell scattering and tubulogenesis. By contrast, expression of soluble HB-EGF resulted in reduced cell-extracellular matrix and cell-cell interactions and enhanced cell migration. These studies describe the distinctive roles of membrane-associated and soluble HB-EGF in epithelial cell morphogenesis and indicate that an EGFR ligand can modulate HGF/SF-induced responses in MDCK cells.

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