Epilysin (MMP-28) induces TGF-β mediated epithelial to mesenchymal transition in lung carcinoma cells

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Accepted 4 July 2006
Journal of Cell Science 119, 3856-3865 Published by The Company of Biologists 2006
doi:10.1242/jcs.03157

Summary

Epilysin (MMP-28) is the newest member of the matrix metalloproteinase (MMP) family. Although it is expressed in a number of tissues, no biological substrates or functions for this enzyme have been identified yet. We have expressed recombinant epilysin in A549 lung adenocarcinoma cells and found that this resulted in stable and irreversible epithelial to mesenchymal transition (EMT) accompanied by loss of cell surface E-cadherin, proteolytic processing of latent TGF-β-complexes and increased levels of active TGF-β. The cascade of events leading to the onset of EMT is prevented by the MMP inhibitor GM6001 or antibodies neutralizing the activity of TGF-β. Once EMT had occurred the cell phenotype could, however, not be reversed by the MMP-inhibitor. Importantly, the expression of epilysin also resulted in upregulation of MT1-MMP and gelatinase-B (MMP-9) and in the collagen invasive activity of A549 cells. Further, we found that epilysin and the recombinant hemopexin domain were targeted to the surface of epithelial cells. This cell surface interaction was sensitive to the proteolytic activity of MT1-MMP, and was lost after EMT. Current results indicate that epilysin can induce EMT and cell invasion through a TGF-β-dependent mechanism suggesting novel biological roles for this enzyme in the regulation of epithelial cell function and in the induction of carcinogenesis.

Key words: Matrix metalloproteinase, Epithelial cell, EMT, TGF-β, Collagen invasion

Introduction

Epithelial to mesenchymal transition (EMT) is a fundamental biological process where epithelial cells lose their polarity and adopt a morphology appropriate for migration. EMT occurs both during embryonic development and in the progression of carcinomas. Hallmarks of EMT are loss of cell-cell adhesion receptors, loss of cell-matrix contacts and changes in the composition of the intermediate filament cytoskeleton (Thiery, 2002; Kalluri and Neilson, 2003). The multifunctional transforming growth factor-β (TGF-β), in addition to a number of other growth factors and cytokines, is known to initiate and maintain EMT in a number of biological systems (Miettinen et al., 1994; Zavadil and Böttinger, 2005). In the extracellular matrix (ECM) the mature TGF-β is associated non-covalently with its propeptide (latency-associated peptide, LAP) and to a latent TGF-β-binding protein (LTPB) (reviewed in Hyytiäinen et al., 2004). A pool of inactive TGF-β is hence maintained in the ECM from where the mature molecule can be activated when needed (reviewed in Annes et al., 2003).

Matrix metalloproteinases (MMPs) comprise a family of 24 endopeptidases capable of degrading most ECM components as well as several cell surface and pericellular proteins, providing them with the means not only to degrade and remodel the ECM, but also to release bioactive molecules and fragments (reviewed in Mott and Werb, 2004). MMPs can thus modulate the behavior of cells according to the information from the surrounding ECM and neighboring cells. EMT is an example of a biological process where MMPs play an important role in the regulation of cell behavior (Bissell and Radisky, 2001). Overexpression of stromelysin-1 (MMP-3) causes EMT by mechanisms that lead to the degradation of the cell-cell adhesion receptor E-cadherin (Lochter et al., 1997; Noe et al., 2001; Radisky et al., 2005). Matrilysin (MMP-7) can also cleave E-cadherin allowing tracheal epithelial cells to detach from each other and migrate in response to injury (McGuire et al., 2003). During EMT, several other MMPs, such as gelatinases A and B, collagenase-3 and MT1-MMP (MMPs -2, -9, -13 and -14, respectively) are frequently upregulated to drive the invasion of the phenotypically altered cells through basement membranes and into surrounding tissues (reviewed in Murphy and Gavrilovic, 1999; Seiki and Yana, 2003), further emphasizing the roles of MMPs in carcinogenesis. Gelatinases A and B, collagenase-3 and MT1-MMP have also been implicated in the regulation of osteoblast, chondrocyte and epithelial cell functions through TGF-β activation (Yu and Stamenkovic, 2000; Dangelo et al., 2001; Karsdal et al., 2002; Mu et al., 2002).

Epilysin (MMP-28) is the newest member of the MMP-family, which we originally cloned from human keratinocyte and testis cDNA libraries (Lohi et al., 2001; Illman et al., 2001). *Epilysin* mRNA is expressed in various human tissues, particularly testis, lung, heart and the gastrointestinal tract, but specific epilysin producing cells within these tissues have not been identified yet. The only specific human cell type recognized to express epilysin is the basal keratinocyte in the skin, where epilysin expression is upregulated during wound repair (Lohi et al., 2001; Saarialho-Kere et al., 2002). In the rhesus monkey *epilysin* mRNA is expressed by a number of

epithelial cell types in the placenta during early pregnancy (Li et al., 2003). *Epilysin* mRNA has also been detected by RT-PCR in a number of adenocarcinoma cell lines (Marchenko and Strongin, 2001). Epilysin consists of the classical MMP-domains: a signal sequence that targets it for secretion from the cell, and a pro domain that keeps the enzyme inactive, followed by catalytic and hemopexin domains (Lohi et al., 2001). A specific feature for epilysin is the functional furin activation sequence following the pro domain (Illman et al., 2003). All membrane-bound MMPs contain this activation sequence, but among the soluble MMPs it exists only in epilysin and stromelysin-3 (MMP-11). Epilysin is very well conserved as 85% of the amino acids are identical between human and mouse with the greatest similarity in the catalytic domain [97% identical residues; Illman et al. (Illman et al., 2003)].

In the present study we have expressed recombinant epilysin both transiently and stably in human lung adenocarcinoma A549 cells. We found that recombinant epilysin is attached to the surface of epithelial cells and that this interaction is sensitive to cleavage by MT1-MMP. Stable expression of epilysin in A549 cells resulted in EMT of these cells accompanied by the loss of cell surface E-cadherin, increased cell invasion and migration and upregulation of MT1-MMP, MMP-9 and TGF-β. The onset of EMT could be prevented by a synthetic MMP inhibitor as well as by TGF-β neutralizing antibodies. Our results reveal for the first time that epilysin is attached to the cell surface, and that its proteolytic activity is sufficient to induce EMT and an invasive phenotype in A549 cells through a TGF-β-dependent mechanism.

Results

Epilysin induces a stable epithelial to mesenchymal transition in A549 Cells

Epilysin expression has so far been detected only in cells of epithelial origin (Lohi et al., 2001; Marchenko and Strongin, 2001; Saarialho-Kere et al., 2002). To gain more information about the biological functions of epilysin we generated A549 human lung adenocarcinoma cell pools stably overexpressing wild-type epilysin (Epi) and a catalytically inactive mutant of epilysin (Epi-E/A). We took advantage of the pEF-IRES-P vector (see Materials and Methods), which expresses the inserted cDNA and a puromycin resistance gene as a single mRNA molecule with an internal ribosomal entry site in-between to force all selected puromycin resistant cells to maintain the expression of epilysin. Comparable levels of the mature 48 kDa form of wild type and E/A epilysin were detected in the conditioned media harvested from the corresponding cell pools (Fig. 1A). This is consistent with constitutive intracellular activation of epilysin by pro-protein convertases of the furin-family (Illman et al., 2003). Unexpectedly, during the selection process, when the epilysin content of the cell pools was gradually increased, the morphology of the cells was dramatically altered. The epilysin-expressing epithelial A549 cells started to detach from each other, elongate and adopt a fibroblast-like appearance consistent with EMT (Fig. 1B, middle left panel). This phenomenon was repeatedly observed in four independent experiments, and was also observed in MDCK kidney epithelial cells (data not shown). Neither the vector-transfected control cell pools (Fig. 1B, upper left panel) nor the cell pools expressing the catalytically inactive mutant of epilysin showed signs of EMT (Fig. 1B, lower left panel).

A hallmark of EMT is the loss of the cell surface protein E-cadherin, which forms adherens junctions between epithelial cells (reviewed in Kalluri and Neilson, 2003; Zavadil and Böttinger, 2005). No E-cadherin could be detected on the surface of cells expressing wild type epilysin that had undergone EMT (Fig. 1B, middle right panel) but, as expected, the E-cadherin staining on the cell surface of control and Epi-E/A pools was abundant (Fig. 1B, upper and lower right panel). Accordingly, higher levels of the 80 kDa ectodomain of E-cadherin (Wheelock et al., 1987; Noe et al., 2001) that had been

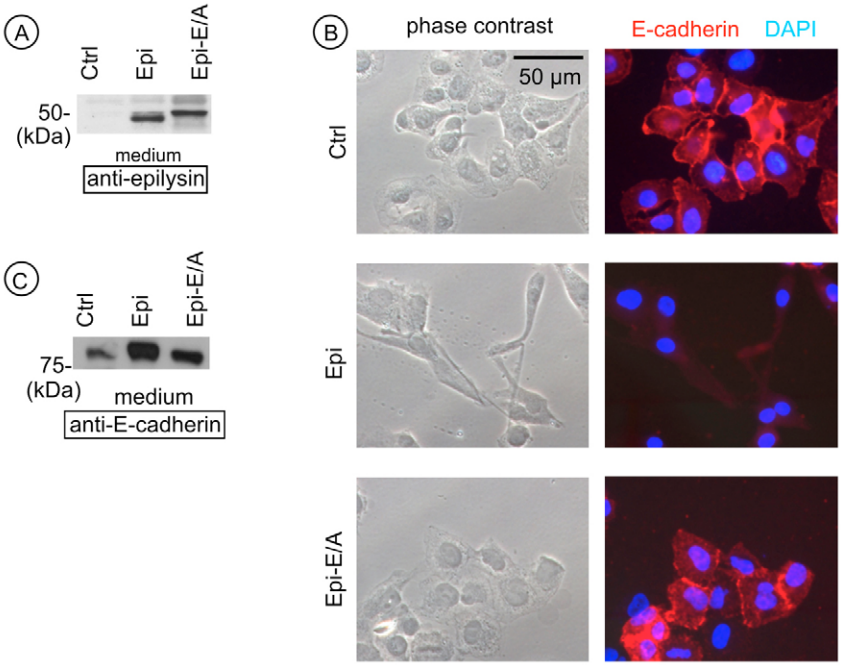

Fig. 1. Epilysin induces EMT in A549 cells. (A) Cell pools stably expressing epilysin were prepared by puromycin selection of A549 cells transfected with expression constructs for either wild-type (Epi) or a catalytically inactive mutant (Epi-E/A) of epilysin. Ctrl denotes empty vector-transfected controls that have undergone the same selection procedure. Conditioned serum-free media from the cell pools were harvested and polypeptides were detected with epilysin specific antibodies (active epilysin: 48 kDa). (B) Immunofluorescence studies were performed with antibodies specific for the extracellular part of E-cadherin. To avoid internalization of the antibodies during the procedure, living cells were incubated on ice with primary antibodies, fixed with PFA and incubated with secondary Alexa-conjugated antibodies. Coverslips were mounted using a mounting medium containing DAPI. E-cadherin: red; DAPI: blue. (C) Immunoblotting of E-cadherin was performed with antibodies recognizing its ectodomain (shed degradation product 80 kDa).

shed from the cell surface were detected in the conditioned medium of epilysin-expressing cells than in the media of control and Epi-E/A cell pools (Fig. 1C). These results indicate that enhanced E-cadherin shedding and the loss of E-cadherin mediated cell-cell adhesion coincide with the transition of epilysin-expressing A549 cells from epithelial to mesenchymal cell morphology.

Epilysin increases TGF-β expression and activity in A549 cells

Because of the well-known capacity of TGF-β to initiate and maintain EMT (reviewed in Zavadil and Böttinger, 2005), we next determined the levels of active and total TGF-β in the A549 cell pools using the mink lung epithelial cell (Mv1Lu) indicator assay. Because the A549 cells deposit very sparse amounts of ECM in culture (Alitalo et al., 1981), only medium samples were subjected to this assay. The levels of active (1.8-fold) and total (2.8-fold) TGF-β were both markedly increased in the medium of the epilysin-expressing cells (Fig. 2A).

Mature TGF-β remains latent through association with its latency-associated peptide (LAP), which can be sequestered to the ECM through association with the latent TGF-β-binding proteins (LTBPs) (Koli et al., 2001). Latent TGF-β can be activated by proteolytic processing of the latent complex. To determine whether the processing of large latent TGF-β complexes takes place in the epilysin-expressing pools, antibodies specific for latent TGF-β1 complexes (TGF-β1-LAP) and for LTBPs were used to identify polypeptides from their conditioned medium. Interestingly, the results revealed only degraded TGF-β1 complexes in the medium from the wild-type epilysin-expressing cell pool (encircled in Fig. 2B), whereas only intact large latent complexes were detected in the medium from the control and Epi-E/A pools (indicated with an arrow in Fig. 2B). Similarly, LTBP-1 was truncated and digested in the epilysin pools (indicated with a bracket in Fig. 2C) whereas TGF-β-containing large LTBP-1 complexes were found in the conditioned medium from both the control and Epi-E/A pools (indicated with an arrow in Fig. 2C). LTBP-4, which is another LTBP with TGF-β1-binding activity (Saharinen and Keski-Oja, 2000), was not detected by immunoblotting (data not shown). Taken together, these results reveal proteolytic degradation of latent TGF-β1/LTBP-1 complexes in cultures of the epilysin-expressing pool, evidently resulting in the enhanced secretion and activation of TGF-β.

TGF-β mediates the epilysin-induced EMT

To determine whether the observed increase in TGF-β activity in the conditioned medium from the epilysin-expressing cells was a cause or a consequence of EMT, we selected A549 cell pools stably expressing epilysin in the constant presence of the synthetic MMP inhibitor GM6001. The phenotypic conversion of the epilysin-expressing cells could not be reversed by GM6001, but addition of the inhibitor already at the initiation of the selection procedure significantly delayed the epilysin-induced EMT (data not shown, see Fig. 3B and Fig. 6). Before the cells showed any signs of EMT, they were changed to medium supplemented with neutralizing antibodies against either TGF-β or the hepatocyte growth factor (HGF) or left with GM6001 or without any supplements. As expected, the TGF-β levels in pools kept with GM6001 were comparable to those of the control and Epi-E/A pools after 72 hours, (Fig. 3A) and the cells showed no phenotypic alterations at this point (Fig. 3B, upper panel). Interestingly, the cells incubated with TGF-β neutralizing antibodies similarly retained their epithelial morphology (Fig. 3B) and low total TGF-β levels (Fig. 3A). On the contrary, analyses of the conditioned media from the pools incubated either without any supplements or with anti-HGF antibodies revealed an increase in the levels of both active and total TGF-β (Fig. 3A). These cells also showed alterations in their morphology as they started to detach from each other and elongate within 72 hours (Fig. 3B, upper panel).

To characterize the epilysin-induced EMT, the cells were stained with antibodies against E-cadherin and the phosphorylated form of the TGF-β signaling molecule SMAD2 (P-SMAD2, Fig. 3B). SMAD2 is phosphorylated in response to TGF-β stimulation through TGF-β receptors on the cell surface and is an essential mediator of TGF-β-induced

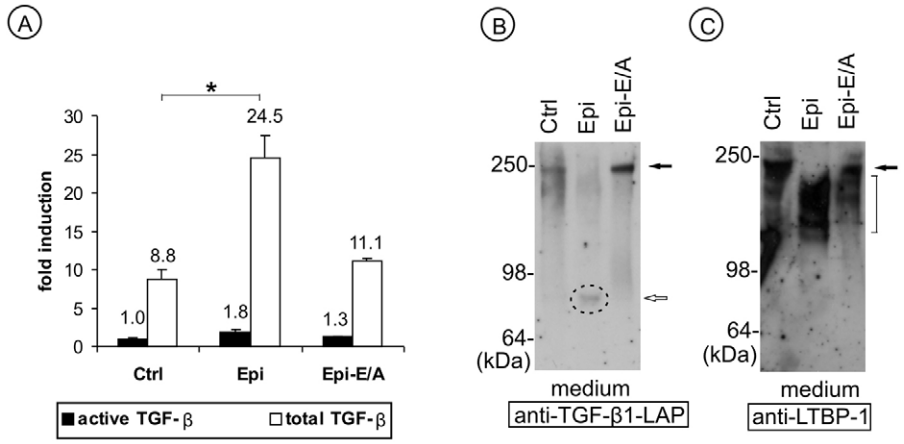

Fig. 2. TGF-β is upregulated and latent TGF-β complexes are degraded in the morpholgically altered epilysin-expressing A549 cells. (A) Conditioned serum-free media were harvested from A549 cell pools, and the concentrations of TGF-β in the untreated (active TGF-β) or heat-treated (total TGF-β) media were determined using indicator cells. The results are shown as relative TGF-β activity where the activity in the medium from the control pool has been set to 1. The asterisk denotes statistically significant difference in the total TGF-β levels over the control (determined using the Student's *t*-test, $P<0.05$). (B,C) Polypeptides in the conditioned media from the cell pools were immunodetected with antibodies specific for latent complexes containing TGF-β1 (anti-TGF-β1-LAP) and LTBP-1 as indicated. In panel B the intact large latent TGF-β1 complexes in the Ctrl and Epi-E/A samples (filled arrow) and proteolytically processed complexes in the Epi sample (unfilled arrow, band encircled) have been indicated. In panel C the intact TGF-β/LTBP-1 complexes have been indicated with a filled arrow, and the proteolytically processed, truncated forms of LTBP-1 with a bracket.

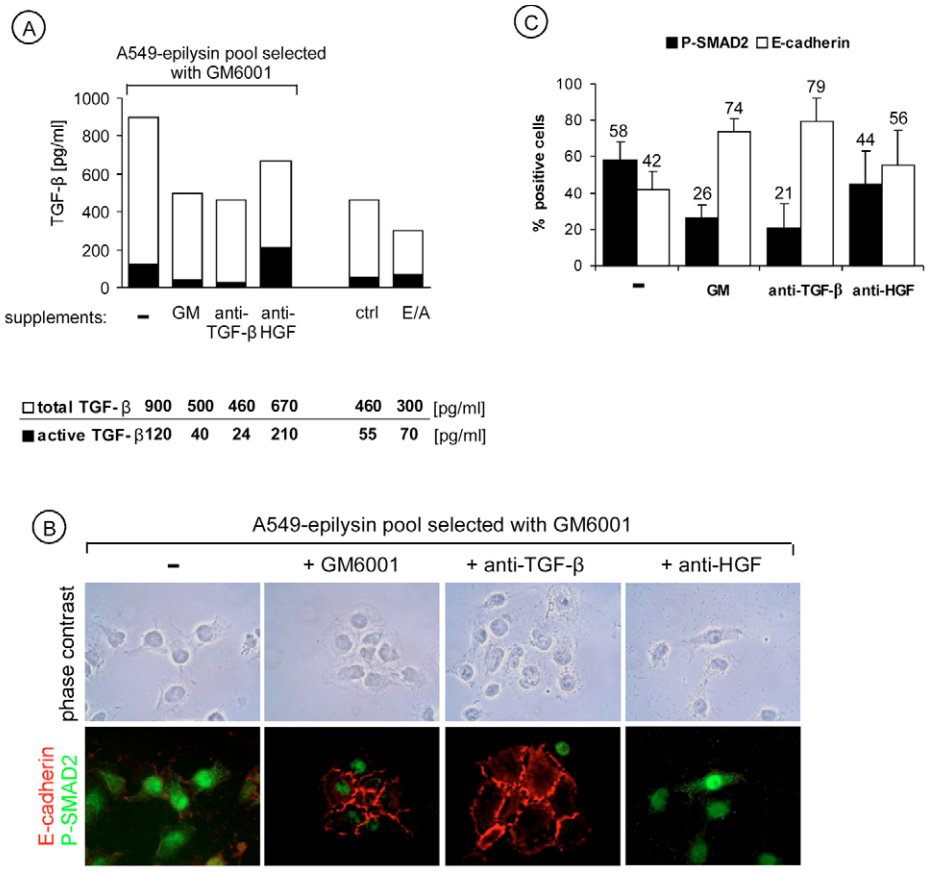

Fig. 3. Epilysin-induced EMT is prevented by the MMP-inhibitor GM6001 and by anti-TGF-β antibodies. (A) Epilysin-expressing A549 pools were selected in the presence of GM6001 (1 μM), which prevented the epilysin-induced EMT. The cells were seeded on glass coverslips in 10% serum containing medium with the indicated supplements. After 48 hours the cells were changed to serum free medium containing the same supplements. After 24 hours the amounts of total and active secreted TGF-β in the media were determined. The TGF-β levels in the control and E/A pools are shown on the right. (B) Living cells were incubated with anti-E-cadherin antibodies, fixed with PFA, permeabilized and then stained with antibodies against the phosphorylated form of SMAD2. (C) The numbers of E-cadherin and P-SMAD2 positive cells in each culture were counted. The proportions of positive cells in each culture are given.

EMT in tumor progression (Valcourt et al., 2005). Interestingly, cells with abundant surface E-cadherin staining simultaneously showed weak staining for nuclear P-SMAD2, in contrast to the E-cadherin negative cells that showed more prominent nuclear P-SMAD2 staining (Fig. 3B, lower panel). Of the GM6001 and anti-TGF-β antibody-treated cells 75% were E-cadherin positive while only 40% of the cells incubated without any supplements and 55% of the cells incubated with the anti-HGF antibodies were E-cadherin positive (Fig. 3C). On the contrary, the numbers of P-SMAD2-positive cells were about twofold higher among the cells kept without supplements or supplemented with anti-HGF over the cells incubated with either GM6001 or TGF-β antibodies, indicating enhanced TGF-β signaling upon withdrawal of GM6001. These results indicate that neutralizing anti-TGF-β antibodies, like the MMP inhibitor GM6001, can antagonize epilysin-induced EMT in A549 cells revealing TGF-β as a critical mediator of this process.

Upregulation of gelatinase B and MT1-MMP coincide with epilysin-induced EMT in A549 cells

Given the ability of several MMPs to act in cascades to activate other soluble MMPs (reviewed in Sternlicht and Werb, 2001) we next determined the levels in the epilysin-expressing cell pools of stromelysin-1 (MMP-3) and matrilysin (MMP-7), whose functions have been linked to the shedding of E-cadherin (Noe et al., 2001; McGuire et al., 2003) and EMT (Lochter et al., 1997). No stromelysin-1 was immunodetected in the medium from any of the cell pools (data not shown). A faint band likely representing matrilysin was detected in the medium from all the cell pools, but no major difference could be detected between the different pools (Fig. 4A, pro-form: 29

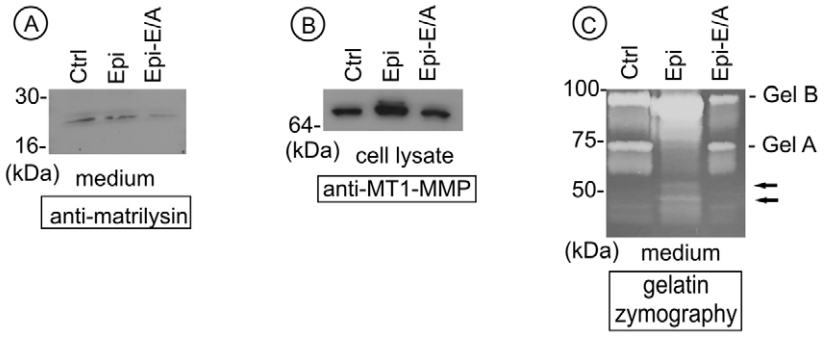

Fig. 4. MT1-MMP and MMP-9 are upregulated in the epilysin-expressing pools. (A,B) Polypeptides in the conditioned media from the A549 cell pools were immunodetected with antibodies against matrilysin (A) and MT1- MMP (B). (C) The content of gelatinases in the conditioned media from the A549 cell pools was analyzed by gelatin zymography. Bands corresponding to gelatinase B (MMP-9) and gelatinase A (MMP-2) are indicated. Two additional unidentified gelatinolytic bands in the epilysin-expressing pool have been indicated with arrows.

kDa, active: 19 kDa). Thus the epilysin-induced E-cadherin degradation is not likely to involve stromelysin-1 or matrilysin activation or activity. Further, the alternatively spliced form of the small GTPase Rac1 (Rac1b) that underlies stromelysin-1 induced EMT in transfected mammary epithelial cells (Radisky et al., 2005) could not be detected by RT-PCR in any of the pools (data not shown) suggesting that the TGF-β-dependent EMT mechanism in epilysin-expressing cells is distinct from the one adopted by stromelysin-1.

Among MMPs, gelatinases A (MMP-2) and B (MMP-9) and MT1-MMP (MMP-14), all widely induced in transformed cells, have been suggested to mediate TGF-β activation and invasion of transformed cells (Yu and Stamenkovic, 2000; Mu et al., 2002). Interestingly, both MT1-MMP and gelatinase B were significantly increased in the epilysin-expressing cell pool as determined by immunoblotting (Fig. 4B) and gelatin zymography (Fig. 4C), respectively, whereas gelatinase A was basically undetectable in this cell pool (Fig. 4C). Two minor gelatinolytic bands corresponding to the sizes of the pro and active forms of epilysin were also detected in the medium from the epilysin-expressing pools (indicated by arrows in Fig. 4C). These bands were, however, not characterized further and could also correspond to a number of other MMPs with minor gelatinolytic activity.

Epilysin is associated with the surface of epithelial cells through its hemopexin domain

To determine how the structure of epilysin is related to its function, we carried out transient transfection experiments of A549 cells. For this purpose we generated expression constructs coding for wild-type epilysin (Epi*), a catalytically inactive mutant of epilysin (Epi-E/A*) and deletion constructs containing either the pro and catalytic domains (Cat*), or the hemopexin domain (Pex*) of epilysin, all with C-terminal V5(*)-tags (Fig. 5A). All these recombinant proteins were detectable by immunoblotting in both the corresponding

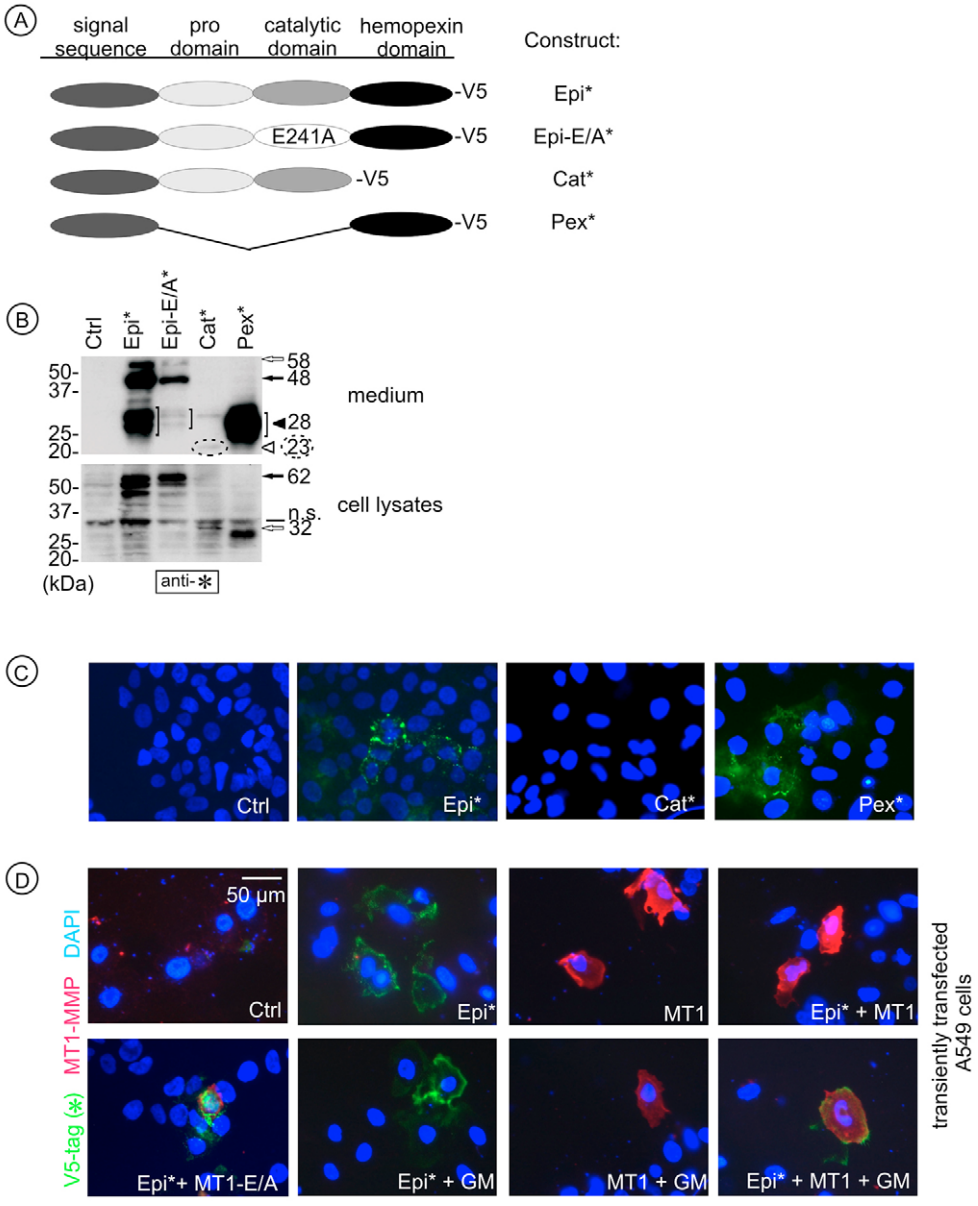

Fig. 5. Recombinant epilysin associates with the surface of epithelial cells through the hemopexin domain. (A) Schematic presentation of cDNA-constructs coding for wild type epilysin (Epi*), a catalytically inactive mutant (Epi-E/A*), the pro and catalytic domains (Cat*) or the hemopexin domain (Pex*) in the pEF1/V5-His vector generating recombinant proteins with C-terminal V5-tags (denoted with *). (B) Transiently transfected A549 cells were changed to serum free medium 24 hours post transfection. The conditioned media (upper panel) were harvested and total cell lysates (lower panel) prepared after 24 hours. Comparable amounts of polypeptides from the media and cell lysates were immunodetected with antibodies specific for the V5-tag. Upper panel: 58 kDa: pro-form of Epi* and Epi-E/A* (unfilled arrow); 48 kDa: mature form of Epi* and Epi-E/A* (filled arrow); 28 kDa: Pex* (filled arrowhead) and 23 kDa: mature form of Cat* (unfilled arrowhead, encircled). Brackets indicate degradation products of Epi*, Epi-E/A* and Pex*. Lower panel: 62 kDa: prepro-form of Epi* and Epi-E/A* (filled arrow); 32 kDa: pro-form of Cat* (unfilled arrow); n.s.: nonspecific band. Ctrl denotes empty vector-transfected control. (C,D) A549 cells were transiently transfected as indicated and living, non-permeabilized cells were stained with antibodies specific for the V5-tag(*) and MT1-MMP. MT1 denotes wild type MT1-MMP and MT1-E/A an inactive mutant of MT1-MMP.

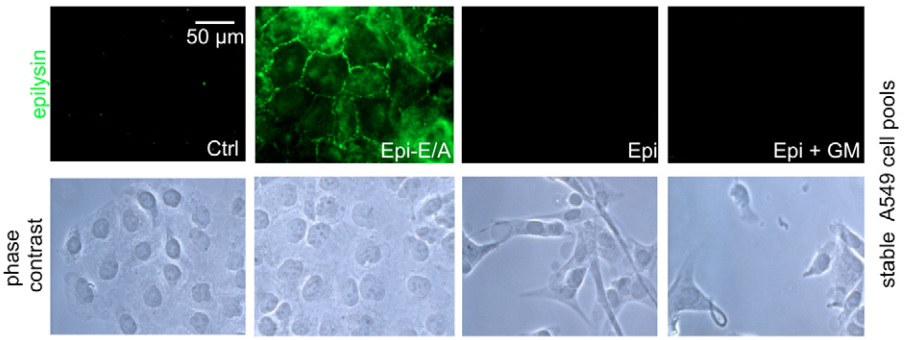

Fig. 6. Cell surface association of epilysin is abolished after EMT. Non-permeabilized, stably transfected A549 cell pools were stained with antibodies specific for epilysin. Phase contrast images show the corresponding cell morphologies. The MMP-inhibitor GM6001 (GM, 1 μM) was included in the incubation medium as indicated.

conditioned medium and cell lysates of the transfected A549 cells. The wild type and E/A forms of epilysin were mostly found in the active form (48 kDa) but also in the pro form (58 kDa, Fig. 5B, uppermost panel). As expected, the Cat* protein was also detected in both pro and active forms (32 kDa and 23 kDa, respectively), and the Pex* protein migrated as a broad 28 kDa band (Fig. 5B). The Cat* protein was repeatedly detected at much lower levels than the other proteins, possibly due to instability or autodegradation.

The transient transfection of epilysin (5-10% transfection efficiency; Fig. 5) did not result in either significant induction/secretion of TGF-β into the conditioned medium or clearly detectable morphological changes of the cells (data not shown). Unexpectedly, however, although epilysin itself does not contain a transmembrane domain or membrane anchoring sequence, epilysin, as well as the Pex* protein and the inactive Epi-E/A* mutant (data not shown) were detected by immunofluorescence on the surface of the transfected, non-permeabilized A549 cells (Fig. 5C). By contrast, the protein containing only the catalytic domain was not detectable on the cell surface. Interestingly, immunofluorescence analyses of A549 cells transiently co-transfected with different epilysin and MT1-MMP-constructs showed that all cells expressing MT1-MMP on the cell surface were negative for epilysin (Fig. 5D). Co-expression of epilysin and MT1-MMP could, however, be detected on the cell surface in the presence of GM6001 or when the catalytically inactive mutant of MT1-MMP (MT1-E/A) was expressed (Fig. 5D). Further, both the Pex* and Epi-E/A* proteins were removed from the cell surface by MT1-MMP (data not shown). Recombinant epilysin was localized on the cell surface also in the other examined cell lines of epithelial origin (A431 epidermoid carcinoma cells and MDCK canine kidney epithelial cells), whereas no recombinant epilysin could be detected on the surface of HT-1080 fibrosarcoma cells (data not shown). Taken together these results indicate that the hemopexin domain of epilysin is sufficient for its binding onto the surface epithelial cells.

Cell surface association of epilysin is lost upon EMT

To determine whether epilysin binds to the cell surface after EMT, stable epilysin-expressing cell pools were subjected to immunofluorescence analysis. In these cells abundant surface expression of the catalytically inactive E/A mutant epilysin was immunodetected by specific antibodies consistent with the binding of epilysin onto an epithelial cell surface structure (Fig. 6). By contrast, no cell surface expression of wild type epilysin was observed in the stable epilysin-expressing cell pools that had undergone EMT (Fig. 6). Both forms of epilysin were secreted into the medium at comparable levels (Fig. 1A). Addition of GM6001 to the wild type epilysin-expressing pool, which did not reverse the phenotype, did not restore epilysin on the cell surface either (Fig. 6). These results indicated that either the receptor attaching epilysin to the cell surface was downregulated during EMT or the turnover of epilysin on the surface of the converted cells was more rapid than that of the Epi-E/A mutant in the cells displaying the epithelial phenotype.

Epilysin increases the motility of A549 cells

A characteristic feature of EMT is the facilitated motility of the transformed epithelial cells (reviewed in Kalluri and Neilson, 2003; Zavadil and Böttinger, 2005). To determine whether the expression of epilysin affects cell motility, stable A549 cell pools were seeded on type I collagen-coated cell culture inserts (8 μm pore membranes). After 4 hours, the cells that had migrated to the lower side of the membrane were fixed and counted. The results showed a marked increase in the migration of epilysin-expressing cells over control cells (4.2±1.5-fold) (Fig. 7). By contrast, the expression of the catalytically inactive Epi-E/A mutant did not increase the migration of the A549 cells. Interestingly, the MMP inhibitor GM6001 did not display any significant effect on the migration of the epilysin-expressing A549 cell pool (Fig. 7; 3.5±1.1-fold). On the contrary, transfection of epilysin into HT-1080 cells, which normally display mesenchymal cell morphology,

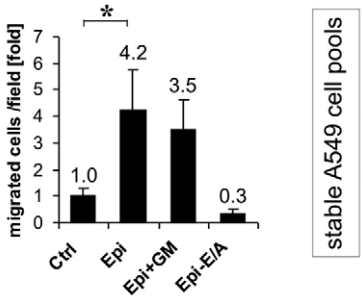

Fig. 7. Epilysin increases the migration of A549 cells. Cell culture plate inserts with 8 μm pore membranes were coated with type I collagen (0.1 mg/ml). Stable A549 cell pools were added to the upper chamber. The MMP-inhibitor GM6001 (10 μM) was then added to both chambers as indicated. After 4 hours cells that had migrated through the membrane were stained and counted. The asterisk denotes statistically significant variation between indicated samples (Student's *t*-test, $P<0.05$).

did not affect their migratory properties (data not shown). These results suggest that the epilysin-induced change from a quiescent to a motile phenotype rather than the cell migration itself requires MMP activity.

Epilysin-induced EMT results in a collagen invasive phenotype

A critical feature of transformed and mesenchymal cells is their ability to invade collagen-rich tissues. To define whether the epilysin-induced EMT provides the cells with the means to invade collagen matrices, stable A549 cell pools were seeded on top of type I collagen gels in cell culture inserts. After 8 days in culture, prominent invasion of epilysin-expressing cells into the underlying collagen was observed by microscopy of HE-stained paraffin sections (Fig. 8A). By contrast, no invading cells were found in the control or Epi-E/A cell pools. The MMP inhibitor GM6001 significantly inhibited the invasion of the epilysin-expressing cells (Fig. 8A). Consistent with the less evident morphological changes and the absence of TGF-β induction in cells transiently transfected with epilysin, only a few of these invaded into the collagen gel (Fig. 8B). The few invaded cells, however, all showed an altered phenotype (Fig. 8B). In accordance with the previously identified role of MT1-MMP in collagen invasion (Hotary et al., 2000; Sabeh et al., 2004), the A549 cells transiently transfected with MT1-MMP invaded readily into the collagen gel (Fig. 8B). Taken together these results suggest that stable epilysin-induced EMT provides the cells with collagen invasive properties.

Discussion

Since the cloning of epilysin (MMP-28) (Lohi et al., 2001) its function has remained largely unknown. Considering the epithelial cell-specific expression pattern of epilysin (Lohi et al., 2001; Marchenko and Strongin, 2001; Saarialho-Kere et al., 2002), our unexpected observation that the expression of epilysin causes stable EMT in A549 adenocarcinoma cells points out a novel role for epilysin in the regulation of the phenotype of epithelial cells. Consistent with this epithelial cell-specific function of epilysin, a similar phenomenon was observed also in MDCK kidney epithelial cells, whereas epilysin expression caused no observed effects on the

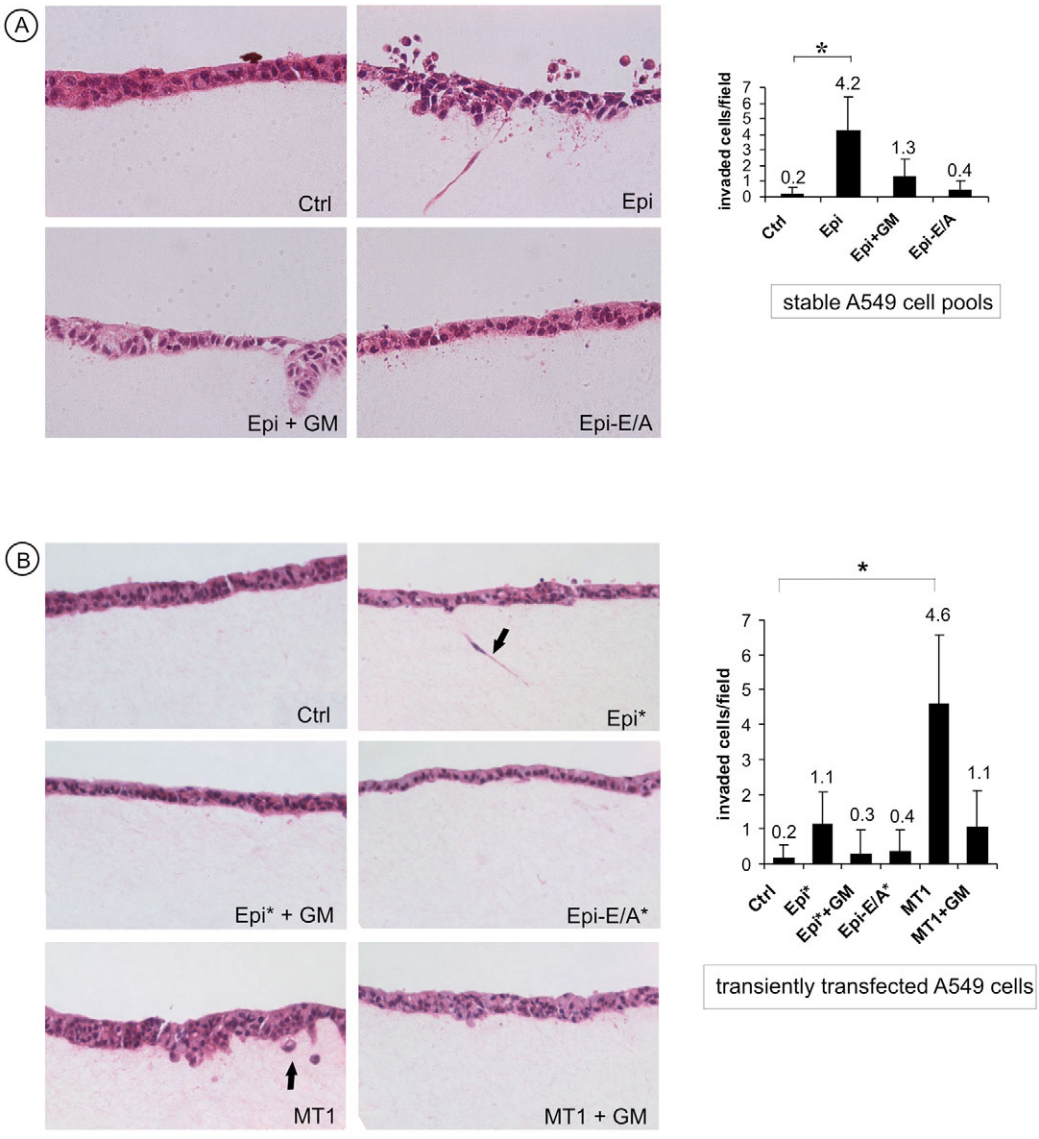

Fig. 8. Morphologically altered epilysin-expressing A549 cells and MT1-MMP transfected A549 cells have an increased ability to invade type I collagen. Type I collagen gels were made into cell culture plate inserts. (A) Stable A549 cell pools and (B) A549 cells transiently transfected with epilysin and MT1-MMP were seeded to the upper chamber as indicated and medium containing HGF (10 ng/ml) as a chemoattractant was added to the lower chamber. The epilysin-induced morphological changes were not affected by the neutralizing anti-HGF antibodies in the EMT assay (Fig. 3B). The MMP-inhibitor GM6001 (10 μM) was added to both chambers as indicated. The cells were incubated on the collagen gels for 8 days changing the medium every third day. The gels were finally fixed with 3% PFA, dehydrated and embedded in paraffin. Sections were then cut and stained with hematoxylin and eosin. Cells that had invaded into the collagen gel were counted under a light microscope. The asterisks denote statistically significant variation as compared with the control (Student's *t*-test, $P<0.05$).

morphology and migration of HT-1080 fibrosarcoma cells (data not shown). Interestingly, an inactivating point mutation of epilysin (E241A), the MMP inhibitor GM6001, and neutralizing antibodies against TGF-β all prevented the onset of EMT, indicating that this is a TGF-β mediated process triggered by the catalytic activity of epilysin. The epilysin-induced EMT was irreversible as the altered phenotype was maintained despite the later addition of the MMP inhibitor. In accordance with the current results, Lin et al. (Lin et al., 2006) have recently proposed a role for epilysin in oral squamous cell carcinoma. In physiological processes, however, epilysin activity may also trigger less permanent changes in TGF-β signaling and cell phenotype, for example when it is upregulated in basal skin keratinocytes during wound healing (Lohi et al., 2001; Saarialho-Kere et al., 2002).

Members of the TGF-β family can induce and maintain EMT in a variety of biological and pathological systems like wound healing (Grose and Werner, 2004), cancer and fibrosis (Zavadil and Böttinger, 2005). Interestingly, we found proteolytic degradation of latent TGF-β complexes accompanied by increased levels of both active and latent TGF-β and increased phosphorylation of the TGF-β signaling molecule SMAD2 in the morphologically altered epilysin-expressing cell pools. Neutralizing anti-TGF-β antibodies as well as the MMP inhibitor GM6001 prevented the epilysin-induced release of active TGF-β and soluble TGF-β complexes and, hence prevented EMT in the cell pools. In accordance with increased TGF-β signaling, the mRNA levels of the transcription factor Snail, which is a downstream target of TGF-β signaling (Peinado et al., 2003) and a critical regulator of EMT (reviewed in Barrallo-Gimeno and Nieto, 2005), were upregulated about 1.5-fold in the epilysin-expressing cell pools (data not shown). Snail induces EMT through numerous target molecules (De Craene et al., 2005). Among these we found upregulation of gelatinase B (MMP-9) (Jorda et al., 2005) and MT1-MMP (MMP-14) (Miyoshi et al., 2004) in the morphologically altered cells. By contrast, the expression of MMP-2 was downregulated in these cells further highlighting the extensive change in the MMP expression profile upon this transcriptional EMT program. Interestingly, both MMP-9 and MT1-MMP have the ability to activate TGF-β by direct cleavage of the LAP complex (Yu and Stamenkovic, 2000; Mu et al., 2002). Further investigations aiming at the identification of direct epilysin substrates will illuminate the more exact cascade of events leading to TGF-β activation and EMT. The current results, nevertheless, indicate that transient activity of epilysin is sufficient to induce a coordinated TGF-β-dependent program leading to the loss of the epithelial phenotype and to the gain of characteristics of invasive cancer cells. With some analogy to these results, another MMP, stromelysin-1 (MMP-3), can also initiate EMT (Lochter et al., 1997; Radisky et al., 2005), and both stromelysin-1 and matrilysin (MMP-7) can cleave E-cadherin (Noe et al., 2001), which is a critical event in the onset of EMT (reviewed in Thiery, 2002). In contrast to our results, however, stromelysin-1 induced EMT is mediated by Rac1b and reactive oxygen species, but does not involve TGF-β. Therefore, even though we find here that the epilysin-induced EMT also involves E-cadherin shedding, the mechanism by which epilysin induces EMT differs from that of stromelysin-1. Further, we observed that the epilysin-induced EMT does not involve the activation of either stromelysin-1 or matrilysin.

MMPs exhibit a considerable overlap in their in vitro substrates. In vivo the substrate specificity is achieved through the regulation of their affinity towards different substrates and by strict temporal and spatial regulation of the active proteases, so called compartmentalization. Even though most MMPs are secreted as soluble molecules, many of them can interact with specific receptor molecules on the cell surface to direct their proteolytic activity to the pericellular environment (Brooks et al., 1996; Yu and Stamenkovic, 2000; Yu and Woessner, Jr, 2000; Dumin et al., 2001; Yu et al., 2002). Current data indicate that epilysin, though lacking a transmembrane domain, associates with the surface of epithelial cells through mechanisms involving the hemopexin domain. Accordingly, high molecular weight complexes containing epilysin can be detected on the surface of A549 cells that have not yet undergone EMT (S.A.I. and J.L., unpublished observation). These results identify spatial targeting of epilysin as a novel means of regulating its activity and bringing it into close proximity of potential cell surface targets in epithelial cells.

Directed pericellular proteolysis is crucial in cell migration and invasion through the ECM (Murphy and Gavrilovic, 1999). We found enhanced migration through type I collagen coated membranes of the morphologically altered A549 cell pools stably overexpressing epilysin. This enhanced migration was not, however, inhibited by GM6001 indicating that epilysin is required only transiently for the epithelial cells to acquire a migratory phenotype, whereas the transwell migration itself of these cells is independent of MMP activity. Conversely, invasion of both fibroblasts and tumor cells into cross-linked collagen gels in vitro as well as into the chicken chorioallantoic membrane in vivo are both processes dependent on the catalytic activity of MT1-MMP (Hotary et al., 2000; Sabeh et al., 2004). We found that A549 cells transiently expressing MT1-MMP invaded readily into collagen gels, as did the stable epilysin-expressing cells that had undergone EMT. The collagen invasion of all cells was inhibited by GM6001 emphasizing the role of MMPs in the process. It has not been determined yet whether epilysin itself possesses any collagenolytic activity. However, as we observed upregulation of MT1-MMP in the transformed cells, the invasive properties can, in agreement with previous data (Hotary et al., 2000; Sabeh et al., 2004), most likely be ascribed to this protease. Further, collagenases MMP-1 and -13, which are induced by TGF-β in squamous cell carcinoma cells (Leivonen et al., 2006), might also contribute to the invasion of the transformed A549 cells. Given the well-established importance of the cell surface targeting of proteinases for cell invasion (Murphy and Gavrilovic, 1999; Hotary et al., 2000; Sabeh et al., 2004), the loss of pericellular localization of epilysin in the morphologically altered and invasive epilysin-expressing A549 cells also supports a less significant role of epilysin activity in collagen degradation. Epilysin would thus regulate cell invasion in an indirect fashion through the induction of other MMPs.

Current observations reveal a unique role for epilysin among the MMPs in the regulation of epithelial cell behavior through the activation of TGF-β, induction of EMT and acquirement of a type I collagen invasive phenotype. The current results point out novel potential in vivo functions for epilysin and offer new directions for research concerning links between epilysin

and TGF-β in TGF-β regulated processes such as wound healing, fibrosis and inflammation. The current results further highlight the potential involvement of epilysin in carcinogenesis and in the induction of MMP mediated cell invasion.

Materials and Methods

Cell culture

Human A549 lung adenocarcinoma (CCL-185; American Type Culture Collection, ATCC, Manassas, VA), human A431 epidermoid carcinoma (CRL-1555; ATCC), MDCK canine kidney epithelial (CCL-34; ATCC) and human HT-1080 fibrosarcoma cells (CCL-121; ATCC) were grown in Eagle's minimal essential medium (MEM), containing 10% heat-inactivated fetal calf serum (FCS), 100 IU/ml penicillin, and 50 μg/ml streptomycin. Stable, transfected pools of A549 cells were maintained in medium supplemented with 20 μg/ml puromycin (Sigma, St Louis, MO) and the synthetic MMP-inhibitor GM6001 (1 μM; Calbiochem, San Diego, CA) where indicated.

Antibodies

Pretested dilutions of antibodies against the hinge domain of MT1-MMP (RP1MMP14; Triple Point Biologics, Forest Grove, OR; used in immunofluorescence experiments), the extracellular domain of E-cadherin (HECD-1; Zymed, San Fransisco, CA), the V5-tag (R960-25; Invitrogen, Carlsbad, CA) and MMP-3 (AB19150; Chemicon, Temecula, CA) were used where indicated. Rabbit polyclonal antibodies against the catalytic domain of epilysin (Lohi et al., 2001), the catalytic domain of MT1-MMP (Lehti et al., 2000) (used in immunoblotting experiments), the TGF-β1/LAP (Taipale et al., 1994) and LTBP-4 (Koli et al., 2005) were as described. Rabbit polyclonal antibodies specific for the phosphorylated form of SMAD-2 (P-SMAD2) were a kind gift from Peter ten Dijke (The Nederlands Cancer Institute, Amsterdam), polyclonal rabbit antibodies against human LTBP-1 (Ab39) from Carl-Henrik Heldin (Ludwig Institute for Cancer Research, Uppsala, Sweden) and polyclonal rabbit antibodies against human MMP-7 from Carole L. Wilson (University of Washington, Seattle, WA).

cDNA constructs

Constructs encoding epilysin and a catalytically inactive form of epilysin (Epi-E241A) (Illman et al., 2003) were generated using PCR with primers including restriction sites at both ends of the full length cDNA and inserted into the pEF-IRES-P vector (Hobbs et al., 1998). Pools of A549 cells stably expressing epilysin were then selected as described (Illman et al., 2003). For transient expression the cDNAs were ligated into the pEF1/V5-His vector (Invitrogen), generating constructs for epilysin and the epilysin-E/A mutant with C-terminal V5-tags (Epi* and Epi-E/A*, where the asterisk denotes the tag). Deletion constructs containing either the signal sequence, pro and catalytic domains (aa: 1-284, Cat*) or the signal sequence and the hemopexin domain (aa: 1-22 + 285-520, Pex*), both with C-terminal V5-tags, were generated by PCR and ligated into the same vector (Fig. 5A). Expression constructs for MT1-MMP (Lohi et al., 1996) and the catalytically inactive mutant of MT1-MMP (MT1-E/A) (Valtanen et al., 2000), were generated in our laboratory. All constructs were verified by sequencing. Transfections were carried out using FuGENE 6 (Roche, Mannheim, Germany).

Immunofluorescence

Forty eight hours after transfection the living cells on glass coverslips were washed with cold phosphate buffered saline (PBS) and incubated with primary antibodies on ice for 30 minutes. Cells were fixed using 3% paraformaldehyde (PFA), and nonspecific binding sites were saturated with bovine serum albumin (BSA, 5% solution in PBS). Where indicated, the cells were fixed with PFA and permeabilized with 0.1% Triton X-100 in PBS prior to incubation with the primary antibodies. For indirect immunofluorescence the cells were incubated with Alexa-conjugated secondary antibodies (Molecular Probes, Eugene, OR), and mounted using VectaShield (Vector Laboratories, Burlingame, CA) containing DAPI to stain the nuclei. Images were obtained using fluorescence microscopy and a digital camera.

Immunoblotting and gelatin zymography

Confluent cell cultures were washed and incubated for 24 hours in serum free MEM. Transiently transfected cells were incubated for 24 h after transfection before transferring to serum free medium. The conditioned media were then harvested and cell lysates prepared as described (Lehti et al., 1998). The medium was supplemented with the MMP inhibitor GM6001 (5 μM, final concentration) where indicated. The conditioned media were clarified by centrifugation and concentrated 10-fold using Microcon concentrators (Millipore, Bedford, MA). Aliquots of conditioned medium from the same number of cells were analyzed by SDS-PAGE and immunoblotting as described (Lohi et al., 2001), using 7.5% standard or 4-15% gradient polyacrylamide gels (Bio Rad, Hercules, CA).

For gelatin zymography the medium polypeptides were dissolved in non-reducing Laemmli sample buffer and separated on 10% polyacrylamide gels containing gelatin (Invitrogen). The polypeptides were permitted to refold and the gels were stained as described (Lohi et al., 1996).

Transwell migration assays and type I collagen invasion

Transwell migration assays were performed using type I collagen (0.1 mg/ml; Upstate Biotechnology, Waltham, CA) coated Falcon cell culture inserts with 8 μm pore membranes in 24-well cell culture plates. A549 cells (10^5) in serum free MEM were plated in the upper chambers, and MEM supplemented with 0.5% fetal calf serum was added to the lower chambers. The cells were allowed to migrate for 4 hours after which they were fixed and stained (40% methanol, 10% acetic acid and 0.1% Coomassie Blue in H_2O). Cells were removed from the upper side of the insert, and the cells migrated to the lower side of the membrane were counted. Transiently transfected cells were co-transfected with a construct coding for the green fluorescent protein (pCM5-EGFP; Invitrogen), and transfected cells only were included in the results.

Type I collagen invasion assays were performed essentially as described (Hotary et al., 2000). The collagen solution was neutralized with NaOH and diluted to a final concentration of 2.2 mg/ml in MEM. Collagen gels were then cast into the upper chamber of Falcon cell culture inserts with 8 μm pore membranes in 24-well cell culture plates and allowed to set at +37°C for 1 hour. A549 cells (10^6 per insert) in MEM containing 10% serum were added on top of the gel, and MEM containing 10% FCS supplemented with 10 ng/ml recombinant HGF to act as a chemoattractant was added to the lower chamber. Where indicated GM6001 (10 μM, final concentration) was added to both chambers. The cells were grown for 8 days changing the media every third day, after which the gels were removed from the inserts, fixed in 3% PFA, dehydrated and embedded into paraffin. Sections were stained with hematoxylin and eosin (HE-staining), and the invading cells were counted and photographed under a light microscope. Both experiments were repeated three times with similar results. The diagrams show the mean values from one representative experiment.

TGF-β activity assay

Conditioned medium from the same number of cells was analyzed for TGF-β activity either directly (active TGF-β) or after heat treatment (total TGF-β). Heat treatment activates latent forms of TGF-β (Brown et al., 1990). Mink lung epithelial cells stably transfected with a fragment of the PAI-1 promoter fused to the luciferase gene (TMLC) were a kind gift from Daniel B. Rifkin (New York University School of Medicine, New York, NY). These cells produce luciferase activity in response to TGF-β. TGF-β standards and medium samples were analyzed as described (Abe et al., 1994). The luciferase values were compared with the values of a dilution series of standards containing 7.8-500.0 pg/ml of recombinant TGF-β1. All assays were repeated three times. The table shows the result of one representative experiment. Neutralizing polyclonal antibodies to TGF-β (AB-100-NA; R&D Systems, Minneapolis, MN) and of HGF (AF-294-A, R&D Systems) were used according to the manufacturer's instructions where indicated.

Statistical analyses

Statistical analyses were performed comparing series of results to the values of the control sample using the Student's *t*-test. Samples showing statistically significant differences have been denoted with asterisks ($P<0.05$).

We thank Katri Koli for discussions and comments on the manuscript. We further thank Sami Starast and Anne Remes for excellent technical assistance. This work was supported by the Academy of Finland, Finnish Cancer Foundation, Sigrid Juselius Foundation, Biocentrum Helsinki, National Technology Agency (TEKES), Novo Nordisk Foundation, Helsinki University Hospital Fund, Helsinki Biomedical Graduate School and the University of Helsinki.

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