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Research Article

Matrix metalloproteinase 19 processes the laminin 5 gamma 2 chain and induces epithelial cell migration

T. Sadowski[a,‡], S. Dietrich[a], F. Koschinsky[a], A. Ludwig[a], E. Proksch[b], B. Titz[a] and R. Sedlacek[a,*]

[a] Institute of Biochemistry, University of Kiel, Olshausenstrasse 40, 24098 Kiel (Germany),
Fax: +49 431 880 5007, e-mail: radek.sedlacek@biochem.uni-kiel.de
[b] Department of Dermatology, University of Kiel, Kiel (Germany)

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Abstract. In this study we analyzed the proteolytic activity of MMP-19 and its impact on keratinocyte migration. In the HaCaT keratinocyte cell line overexpressing wild-type MMP-19 (HaCaT-WT), transmigration through fibrin and type IV collagen matrices was significantly increased compared to cells harboring a catalytically inactive mutant (HaCaT-EA). Studying the expression of MMP-19 in early stages of squamous cell cancer (SCC), we found co-localization of MMP-19 and laminin 5 at the invading tumor front but not in suprabasal epidermis of the tumor. Examination of laminin 5 processing revealed increased processing of the γ2 chain in the medium and matrix of HaCaT-WT cells and degradation by recombinant human MMP-19 to 105-kDa and 80-kDa fragments. Parental HaCaT grown on the matrix of HaCaT-WT and HaCaT-EA cells displayed differential tyrosine phosphorylation. Using integrin blocking and stimulating antibodies we could attribute these differences to a shift from β4-integrin-dependent signaling on the HaCaT-EA matrix toward α3-integrin-dependent signaling on the HaCaT-WT matrix. As a consequence, parental HaCaT showed increased migration on the matrix of HaCaT-WT cells. These data suggest that the MMP-19-dependent processing of the γ2 chains leads to the integrin switch favoring epithelial migration and that MMP-19 actively participates in the early stages of SCC invasion.

Key words. Matrix metalloproteinase; extracellular matrix; laminin; tumor invasion; integrin signaling; keratinocyte.

Tumorigenesis in humans is a multistep process that requires essential alterations in cell physiology [1]. Among these, capability for invasion and metastasis enables tumor cells to escape the primary tumor mass and spread to distant sites of the body. These processes necessitate the activity of extracellular proteases that facilitate tumor cell invasion into various tissues by degradation and remodeling of the extracellular matrix (ECM). In particular, matrix metalloproteinases (MMPs) have been implicated in the progression of malignant tumor of various origin [2]. Beside their classical role in degradation of ECM, MMPs are responsible for the release and activation of some growth factors, and shedding of diverse cell surface proteins, all of which influences the tumor microenvironment as well as the fate of tumor cells [3–5].

Although most MMPs are generally absent from healthy skin, their expression has been observed in the development and progression of skin cancers [6, 7]. Thus, MMP-1 mRNA was detected in tumor and stromal cells in squamous cell carcinoma (SCC) [8], while MMP-11 was typically expressed in stromal cells [9]. MMP-13 mRNA was expressed by tumor cells at the invading front [10] whereas its potential activator, MMP-3, was expressed by stromal cells [11]. MMP-7 as well as MMP-9 was expressed in SCC cells at the stromal interface surrounding tumor nests [12, 13]. Various murine models also

* Corresponding author.
‡ Present address: Aventis Pharma Deutschland GmbH, DI&A DG Cardiovascular, Frankfurt (Germany)

showed that MMPs contribute to the tumor growth. For example, MMP-2-deficient mice exhibited reduced tumor progression in a murine model of melanoma [14]. Thus, the activity of MMPs has been proposed to contribute to the aggressive and metastatic growth of malignant melanoma [15].

Laminin 5 is a major adhesive component of many epithelial basement membranes that promotes assembly of hemidesmosomes. The secreted precursor form of laminin 5 is a heterotrimer containing a 200-kDa α3 chain, a 140-kDa β3 chain, and a 155-kDa γ2 chain. These chains can be proteolytically processed by plasminogen activators [16], MMPs [17, 18], or bone morphogenetic proteins (BMPs) [19, 20]. In particular, processing of the γ2 chain has been demonstrated to trigger migration of epithelial cells [17, 18]. Furthermore, overexpression of the γ2 subunit has been previously described as a marker of invasion in epithelial cancers [21].

Unlike most other MMPs, MMP-19 is expressed in healthy epidermis and appears to be confined to undifferentiated keratinocytes of the basal layer [22, 23]. However, its expression is altered in cutaneous diseases exhibiting increased epidermal proliferation. Thus MMP-19 was also found in suprabasal and spinous epidermal layers in psoriasis, eczema, and tinea [23, 24]. In addition, MMP-19 was detected in hyperproliferative keratinocytes in basal cell carcinoma (BCC) and SCC [24]. We have recently reported that overexpression of MMP-19 in HaCaT keratinocytes increases cellular proliferation as well as migration and adhesion to type I collagen, and that these effects of MMP-19 were mediated by providing bioavailable insulin-like growth factor (IGF) through proteolysis of IGFBP-3 [22]. In the present study, we present evidence that MMP-19 facilitates migration of keratinocytes through type IV collagen and fibrin matrices and that processing of the γ2 chain of laminin 5 by MMP-19 leads to an integrin switch, which promotes keratinocyte migration. Based on the co-expression of laminin 5 and MMP-19 in the invading front of SCC, we propose that MMP-19 actively participates in tumor invasion.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM) was purchased from PAA (Linz, Austria), the Vectastain ABC Elite kit and 3-amino-9-ethylcarbazole (ACE) were from Vector Laboratories (Burlingame, Calif.). Type I and IV collagen were obtained from Becton Dickinson (San Jose, Calif.), fibrin and hydroxyurea from Sigma (St. Louis, Mo.), and [^{35}S]-methionine/cysteine was from ICN (Irvine, Calif.). LY294002, PP2, aprotinin, pansorbin, the fluorescent substrate MCA-PLANvaDpaARNH$_2$, and the antibody against IGF-IR (clone αIR-3) were purchased from Calbiochem (Bad Soden, Germany), ECL Substrate plus, reduced glutathione (GSH) sepharose beads, protein G-sepharose, streptavidin-sepharose from Amersham Pharmacia Biotech (Freiburg, Germany), and Calcein was obtained from Molecular Probes (Eugene, Ore.). EZ Link Sulfo-NHS-LC-Biotin was ordered from Pierce (Rockford, Ill.). Antibodies against MMP-19, β4 integrin (439-9B), and β1 integrin (JB1) were from Chemicon (Temcula, Calif.). Antibodies against α3 integrin (P1B5) were obtained from Santa Cruz (Santa Cruz, CA), secondary antibodies were from Dako (Hamburg, Germany) and Pierce, and the phosphotyrosine-specific antibody 4G10 was from Upstate Biotechnology (Lake Placid, N. Y.). Antibodies against the γ2 subunit of laminin 5 were from Chemicon (D4B5) and Santa Cruz, whereas anti-γ3 (BM165) was a kind gift of M. Aumailley (University of Cologne).

Immunohistochemistry

Tissue specimens of diseased skin were obtained from patients (n=8) undergoing surgery for scc. The study was approved by the ethical committee of the University of Kiel. Tissue specimens were embedded in paraffin and processed as described elsewhere [23]. To detect MMP-19, the monoclonal anti-MMP-19 antibody CK8/4 was used. Laminin 5 was detected with a monoclonal antibody specific for the γ2 chain. Bound antibodies were detected using the Vectastain ABC Elite kit following the manufacturer's instructions. Peroxidase activity was detected using ACE. Sections were counterstained with hematoxylin. The specificity of the staining was proved with an IgG1 control antibody (not shown). Visualization was via an Olympus BX-500 fluorescent microscope and images were processed with the Analysis Soft Imaging system (Lakewood, Calif.).

Cells and culture conditions

The HaCaT keratinocyte cell line was generously provided by N. Fusenig (DKFZ, Heidelberg, Germany). Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 in DMEM supplemented with 10% FBS and 50 units/ml penicillin and 50 µg/ml streptomycin. Generation of the HaCaT cell lines with stable overexpression of wild-type MMP-19 (HaCaT-WT) and a catalytically inactive mutant of MMP-19 harboring an E213A mutation (HaCaT-EA) has been described before [22].

Immunoblotting

Cell lysates were prepared and immunoblotted essentially as described previously [22]. Bound antibodies were detected using peroxidase-conjugated goat anti-rabbit and anti-mouse IgG antibodies and the ECL Substrate plus. Signals were recorded with a Luminescent Image Analyzer (FujiFilm, Tokyo, Japan).

Immunoprecipitation

Keratinocytes were labeled overnight with 50 µCi/ml [^{35}S]-methionine/cysteine. Media were collected, centrifuged to discard cellular debris, and incubated with 50 µl/ml pansorbin for 1 h at 4 °C. After centrifugation, antibodies against MMP-19 were added at a final concentration of 2 µg/ml, followed by overnight incubation at 4 °C. After the addition of protein G-sepharose and a further incubation for 1 hour at 4 °C, the precipitates were washed three times with cold Tris buffer (10 mM Tris, 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100, pH 7.4). Precipitates were separated on 10% SDS-polyacrylamide gels under reducing conditions.

For analysis of tyrosine-phosphorylated proteins, cell lysates were prepared as described above for immunoblotting with the exception that the lysis buffer was further supplemented with 1 mM sodium orthovanadate and 25 mM NaF. Equal protein amounts were subjected to immunoprecipitation by incubating with a monoclonal antibody recognizing tyrosine-phosphorylated proteins (4G10) at 4 °C, followed by the addition of protein G-sepharose. The precipitates were washed three times with cold RIPA buffer and immunoblotted. Membranes were probed with anti-phosphotyrosine antibody as described above.

For detection of secreted laminin 5, conditioned media were immunoprecipitated with monoclonal antibodies directed against the γ2 subunit (D4B5), followed by the addition of protein G-sepharose. The precipitates were washed three times with cold Tris buffer and separated on 5% SDS-polyacrylamide gels under reducing conditions. Afterwards, membranes were probed sequentially with anti-γ2 and anti-α3 antibodies.

Cell surface localization of MMP-19

Flow cytometry analysis was performed using a FACScan device (BD Biosciences, San Jose, Calif.). An aliquot of 10^6 cells/ml was stained for 1 h at 4 °C in PBS containing 0.1% NaN_3 and 2% FCS using the mouse monoclonal antibody CK8/4 against hMMP-19 or an isotype control IgG. The primary antibody was detected with a PE-conjugated goat anti-mouse IgG. Dead cells were excluded by propidium iodide staining.

Cell surface proteins were biotinylated with EZ-Link Sulfo-NHS-LC-Biotin as described elsewhere. Briefly, HaCaT keratinocytes were washed with PBS and incubated for 30 min with 0.25 mM EZ-Link Sulfo-NHS-LC-Biotin in PBS. The reaction was stopped with NH_4Cl and the cells were extensively washed in PBS before lysis. Biotinylated proteins were precipitated using streptavidin-sepharose. Immunoblotting and detection of MMP-19 was conducted as described above.

Expression and purification of recombinant MMP-19

Fusion proteins of glutathione-S-transferase (GST) and MMP-19 were prepared as has been described [22]. GST-MMP-19 was purified from the soluble fraction of *Escherichia coli* [BLR(DE3) strain] with GSH-sepharose beads and activated by incubation for 8 h at 37 °C. Proteolytic activity was detected using the synthetic fluorescent substrate McaPLANvaARNH$_2$. Routine assays were performed at 37 °C at a substrate concentration of 1 mM in TNC buffer. Inhibition of activated GST-MMP-19 by BB94 was demonstrated using the above-mentioned assay.

Cell transmigration assay

HaCaT keratinocytes were labeled with 0.75 µg/10^5 cells Calcein in PBS at 37 °C for 1 h. Nunclon Cell Culture Inserts (Nunc, Wiesbaden, Germany) were coated with 10 µg/cm^2 type IV collagen and 10 µg/cm^2 fibrin. An aliquot of 200 µl fibrin solution, 822 µl DMEM with 2% FCS, and 0.5 U thrombin was added to a Nunclon Cell Culture Insert. The matrix then polymerized for 30 min at room temperature, was overlaid with 500 µl DMEM, 10% FCS and incubated for another 12 h at 37 °C. To assess transmigration, Calcein-labeled HaCaT keratinocytes were seeded at an initial number of 300,000 cells/100 µl serum-free medium into the wells of the type-IV-collagen] and fibrin-coated inserts. The lower chamber contained supernatant of 3T3 fibroblasts. After a 10-h incubation, fluorescence of the upper and lower chamber was measured by Lambda Fluoro 320 (Bio–Tek Instruments, Winooski, Vt.) at 485 and 530 nm. Some experiments were performed in the presence of BB94 (1 µM), aprotinin (2 µg/ml), and anti-IGF antibody αIR-3 (1 µg/ml).

Analysis of cell signaling on different HaCaT-conditioned matrices

The ECM deposited by HaCaT-WT and HaCaT-EA cells was prepared by plating the cells for 48 h onto type-I-collagen-coated culture dishes (10 µg/cm^2). Afterwards, cells were briefly frozen and dislodged from the plates by extensive washing in PBS and a short incubation in 20 mM EDTA. Parental HaCaT that had been serum starved for 2–3 days were seeded at 10^6 cells into the dishes. After incubation for various time points at 37 °C, cells were scraped from the dishes and tyrosine phosphorylation was analyzed. For determining integrin-dependent signaling, the following antibodies were added to parental HaCaT when seeded on the different matrices: β1 (20 µg/ml), α3 (20 µg/ml), β4 integrin (10 µg/ml), and laminin 5 (20 µg/ml, Santa Cruz). The kinase inhibitors PP2 and LY294002 were used at 10 µM final concentration.

Adhesion assay

The ECM deposited by HaCaT-WT and HaCaT-EA cells was obtained as described above. Parental HaCaT were seeded at 5×10^4 cells in the wells of the microtiter plates. After incubation for various time points at 37 °C, nonad-

herent cells were removed by washing with PBS, and the remaining cells were fixed in 4% paraformaldehyde and stained with methylene blue as described elsewhere [25]. The dye was eluted in 1:1 (v/v) ethanol and 0.1 M HCl and absorbance at 650 nm was read in a microplate photometer.

Scratch assay

Keratinocytes were cultured on the ECM of HaCaT-WT and HaCaT-EA cells until they reached confluence. To avoid a proliferative effect, cells were then treated with 100 mM hydroxyurea for 24 h. Media were changed to DMEM with 0.5% FBS and a cell-free area was introduced by scraping the monolayer with a blue pipette tip. After 48 h under standard culture conditions, cells were fixed in 4% paraformaldehyde and stained with methylene blue. Photographs were taken using an inverted phase-contrast microscope (Zeiss, Jena, Germany).

Processing of laminin 5

The intracellular precursor form of laminin 5 was immunoprecipitated from HaCaT lysates with anti-γ2 antibodies as described above. Precipitates were washed three times with cold Tris buffer and twice with the enzyme assay buffer (50 mM Tris, 150 mM NaCl, 10 mM $CaCl_2$, 0.05% Brij 35, 20 µM $ZnCl_2$, pH 7.5). GST-MMP-19 was mixed with the immunoprecipates and incubated at 37 °C for 8 h. At the end of the incubation period, precipitates were boiled in Laemmli buffer and separated on 5% SDS-polyacrylamide gels under reducing conditions. To evaluate degradation of matrix-incorporated laminin 5 by MMP-19, the ECM secreted by HaCaT-EA cells was prepared as described above and incubated with GST-MMP-19 at 37 °C for 2 h. The ECM was then extracted by scraping the culture dish in Laemmli buffer. Detection of the γ2 subunit by immunoblotting was performed as described above.

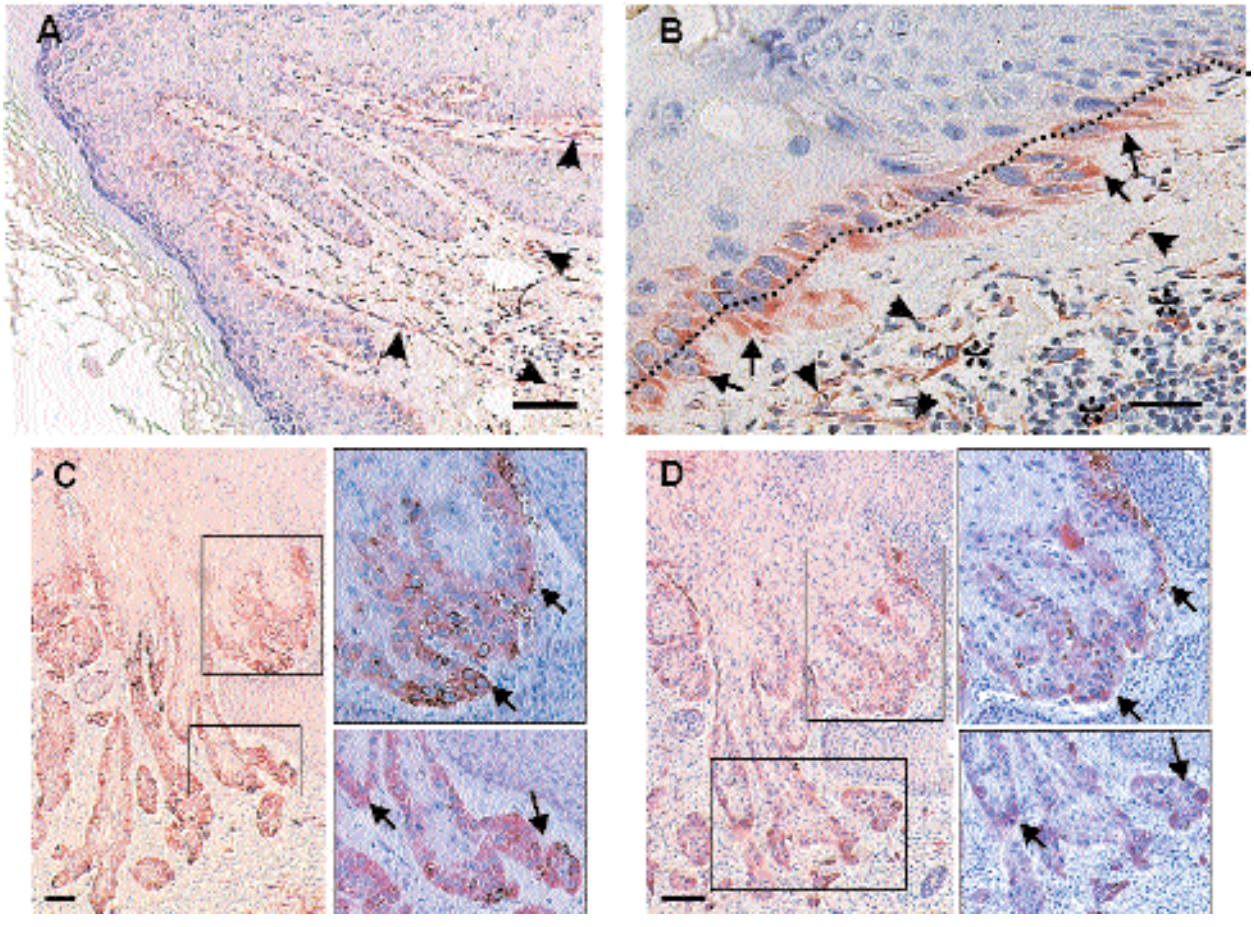

Figure 1. Co-expression of MMP-19 and laminin 5 in SCC. MMP-19 is expressed in the basal invading layer of the tumor tissue of scc. (*A*). The dermal compartment contained few endothelial cells (arrowheads) and inflammatory cells (asterisk) that were also positive for MMP-19 expression (*A*, *B*). An area with invading squamous tumor cells showed an increased number of cells with atypical mitosis that were stained with the anti-MMP-19 antibody CK4/8 (*B*, arrows). The dotted line marks the disrupted basement membrane. Parallel sections of grade II SCC showed co-localization of MMP 19 (*C*) and laminin 5 γ2 chain (*D*, arrows) expression. Bars: 100 µm, *A*; 20 µm, *B*; 50 µm, *C*, *D*.

Results

Co-expression of MMP-19 and laminin 5 in SCC

In the present study we analyzed the expression of MMP-19 in well-differentiated (grade I) and moderately differentiated (grade II) SCC samples with the previously described monoclonal antibody [23]. Intense staining was observed in keratinocytes of the epidermal basal layer of the tumor tissue invading into the dermis (fig. 1A, B). In contrast, a low but constitutive expression was apparent in the suprabasal epidermis of the tumor (fig. 1B). In most of the analyzed patient samples, signals for MMP-19 were absent from tumor cells that had already invaded deeply into the dermis (not shown). The tumor stroma contained only a few MMP-19-positive cells, and mostly endothelial and inflammatory cells (fig. 1A, B).

Moreover, we found significant co-localization of signals for MMP-19 and the γ2 chain of laminin 5 (fig. 1C, D). Intense staining was detected in carcinoma cells along the invasive border and in neoplastic islands. As has been observed before, the staining for laminin 5 was mostly cytoplasmic and was not detected in the ECM [26].

MMP-19 localizes to the cell surface of human keratinocytes

Next, we were interested in the subcellular distribution of MMP-19 in human keratinocytes. As we obtained only a faint signal after immunoprecipitating MMP-19 from the culture medium of HaCaT keratinocytes (data not shown), we analyzed whether MMP-19 expressed by keratinocytes can also localize to the cell surface, as has already been described for monocytes and endothelial cells [27, 28]. Biotinylated cell surface proteins were precipitated with streptavidin-agarose and analyzed by immunoblotting with anti-MMP-19 antibodies. A specific band of 70 kDa was detected in the precipitate of biotinylated proteins, which corresponds to the unprocessed glycosylated form of MMP-19 (fig. 2A) [23]. The association of MMP-19 with the cell surface could be further confirmed by staining cells with monoclonal anti-MMP-19 antibodies directed against the propeptide domain and analysis by flow cytometry (fig. 2B).

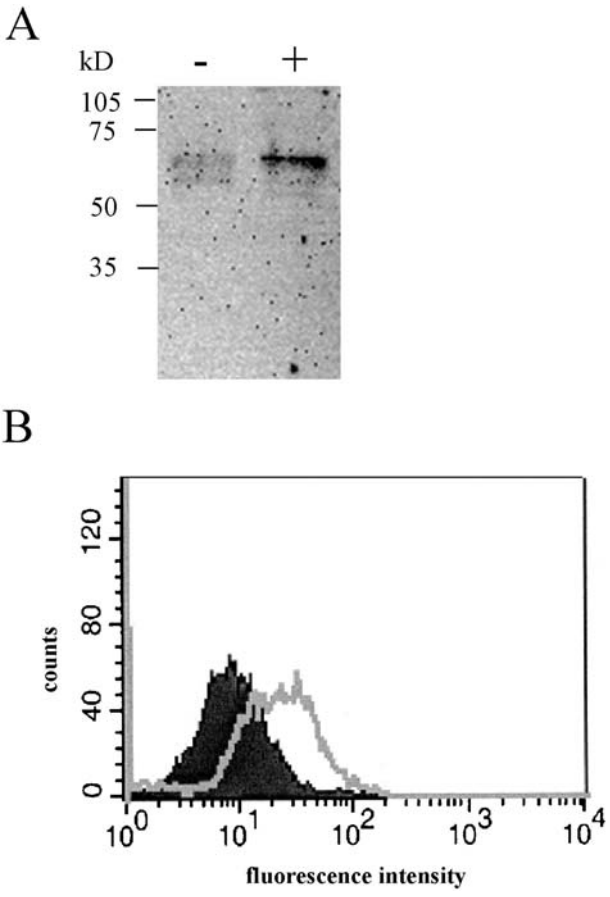

Figure 2. MMP-19 associates with the cell surface of keratinocytes. (*A*) After biotinylation of the cell surface, MMP-19 was precipitated with streptavidin-sepharose and identified using polyclonal antibodies against the hinge region. A specific band of 70 kDa that corresponds to the unprocessed glycosylated form of MMP-19 was detected in the precipitate. +, biotinylation; –, without biotinylation. (*B*) The cell surface of HaCaT cells was stained with the monoclonal antibody CK8/4 directed against the propeptide domain. Only viable cells were used for the analysis. White, staining with anti-MMP-19; black, isotype control staining.

MMP-19 promotes keratinocyte migration through fibrin and type IV collagen

Increased production of MMP-19 by keratinocytes at the invading tumor front (fig. 1) and its localization at the cell surface of keratinocytes suggests that this protease may promote basement membrane degradation and could play a role in the invasion of neoplastic cells into the dermis. As the recombinant catalytic domain of MMP-19 has been previously reported to degrade various basement membrane components [29], we asked whether MMP-19 activity could facilitate transmigration of keratinocytes through ECM substrates. For these experiments we used previously described HaCaT cell lines with stable overexpression of wild-type MMP-19 (HaCaT-WT) and the functionally inactive mutant of MMP-19 (HaCaT-EA) [22]. Measuring transmigration of these cells against supernatants of 3T3 fibroblasts as chemotactic stimulus, we found significantly increased migration of HaCaT-WT through type IV collagen and fibrin matrices, as compared to HaCaT-EA and vector-transfected cells (fig. 3A, B). As we have previously shown that MMP-19 promotes migration of HaCaT on type I collagen by providing free IGF through degradation of IGFBP-3 [22], we also measured transmigration in the presence of a blocking antibody against the IGF-IR (clone αIR-3). However, in contrast to the results obtained with a type I collagen matrix, this treatment did not influence the increased

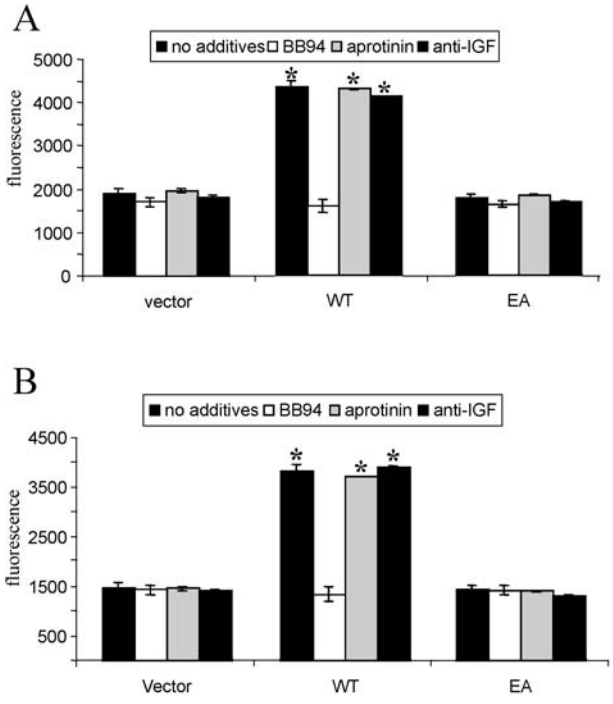

Figure 3. MMP-19 promotes transmigration through type IV collagen and fibrin matrices. To study the effect of MMP-19 on migration of keratinocytes, the HaCaT cell line was stably transfected with wild-type MMP-19 (HaCaT-WT) and the functionally inactive mutant of MMP-19 (HaCaT-EA). Transmigration of Calcein-labeled cells was assessed in a Transwell chamber and fluorescence was measured in the lower compartment (see Materials and methods). HaCaT-WT cells exhibited significantly increased migration through type IV collagen (*A*) as well as fibrin matrix (*B*), compared to HaCaT-EA and vector-transfected cells. The transmigration of HaCaT-WT cells was inhibited by the MMP inhibitor BB94 (1 µM) but not by aprotinin (2 µg/ml) or the anti-IGF antibody αIR-3 (1 µg/ml). Each bar represents the mean ± SE of three independent experiments. *, significantly different with $p<0.05$ (Student's two-tailed t test).

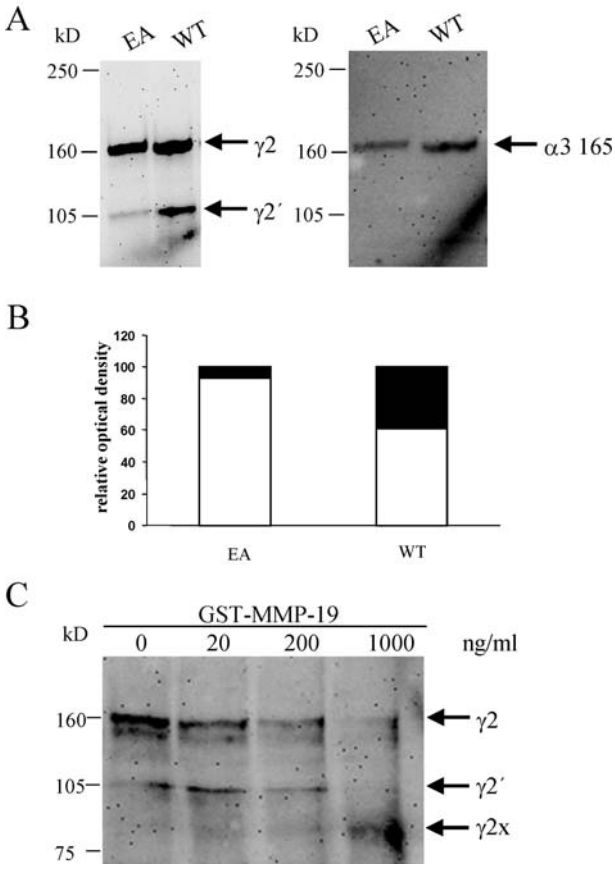

Figure 4. Processing of the laminin 5 γ2 chain by MMP-19. (*A*) Laminin 5 was immunoprecipitated from conditioned media of HaCaT-EA (EA) and HaCaT-WT (WT) cells. After immunoblotting, staining with antibodies directed against the α3 and γ2 chains revealed that medium of HaCaT-MMP19WT cells contained increased amounts of the 105-kDa processed form of the γ2 chain compared to that from HaCaT-MMP19EA cells (*A*). (*B*) Comparison of the relative optical density of the bands corresponding to the 155-kDa and 105-kDa forms of the γ2 chain between MMP-19WT- and MMP19EA-overexpressing cells. White, unprocessed γ2; black, processed γ2. (*C*) Direct involvement of MMP-19 in processing of the γ2 subunit of laminin 5. Precursor laminin 5 was immunoprecipitated from cell lysates of HaCaT and incubated with increasing amounts of a purified GST-MMP-19. The immunoblot was stained with an anti-γ2 antibody.

migration of HaCaT-WT cells. The migratory response of HaCaT-WT could be blocked by the broad-spectrum MMP inhibitor BB94, but was insensitive to the serine protease inhibitor aprotinin.

Cleavage of laminin 5 in keratinocytes overexpressing MMP-19

Taking into account the co-expression of MMP-19 and laminin 5 in SCC, we were interested whether MMP-19 is involved in the proteolytic processing of laminin 5. Immunoprecipitation of laminin 5 from conditioned media followed by immunoblotting with antibodies directed against the α3 and γ2 chains revealed that medium of HaCaT-WT cells contained increased amounts of the 105-kDa processed form of the γ2 chain compared to that from HaCaT-EA cells (fig. 4A). Estimation of the relative optical density of the bands corresponding to the 155-kDa and 105-kDa forms of the γ2 chain showed that in supernatants of MMP-19WT-overexpressing cells, approximately 40% of the total amount of the γ2 chain was in its processed form, whereas this form constituted only approximately 10% in medium of cells overexpressing the inactive mutant (fig. 4B). Conversely, no differences were observed for processing of the α3 chain, which was present in media of both cell lines as the 165-kDa processed form (fig. 4A).

To confirm that MMP-19 was directly involved in processing of the γ2 subunit of laminin 5, precursor laminin 5 was immunoprecipitated from cell lysates of HaCaT, incubated with increasing amounts of a purified MMP-19-GST fusion protein and immunoblotted with an

anti-γ2 antibody. Figure 4C shows a progressive loss of the 155-kDa form with increasing concentrations of GST-MMP-19 and the appearance of proteolytic fragments of 105 and 80 kDa in size, apparently corresponding to the two previously described processed forms of the γ2 subunit, γ2′ and γ2× [17].

The matrix of keratinocytes overexpressing MMP-19 has different signaling properties

Processing of laminin 5 has been described as leading to changes in the engagement of integrin receptors [30]. Therefore, we were interested whether differences in cell signaling could be detected after contact of parental HaCaT with the matrices of HaCaT-WT and HaCaT-EA cells, which differed markedly in their content of proteolytic fragments of the laminin 5 γ2 chain (fig. 5A). Parental HaCaT cells were serum starved for 3 days and seeded in serum-free medium onto ECMs prepared from HaCaT-WT and HaCaT-EA cells. After 30 and 120 min, cell lysates were analyzed for protein tyrosine phosphorylation. Altogether four main tyrosine-phosphorylated proteins were observed in the cells incubated on the HaCaT-EA matrix that were less phosphorylated in cells incubated on the HaCaT-WT matrix (fig. 5B, nos 1–4). A major difference was found for a protein species around 75 kDa (fig. 5B, no. 2).

To confirm that MMP-19-dependent processing of matrix components was responsible for the observed differences in cell signaling, we treated the matrix of HaCaT-EA cells with recombinant MMP-19 prior to seeding parental HaCaT. As observed before, this treatment led to dephosphorylation of the protein around 75 kDa (fig. 5C). In addition, this treatment also resulted in increased degradation of laminin 5 γ2 (fig. 5D).

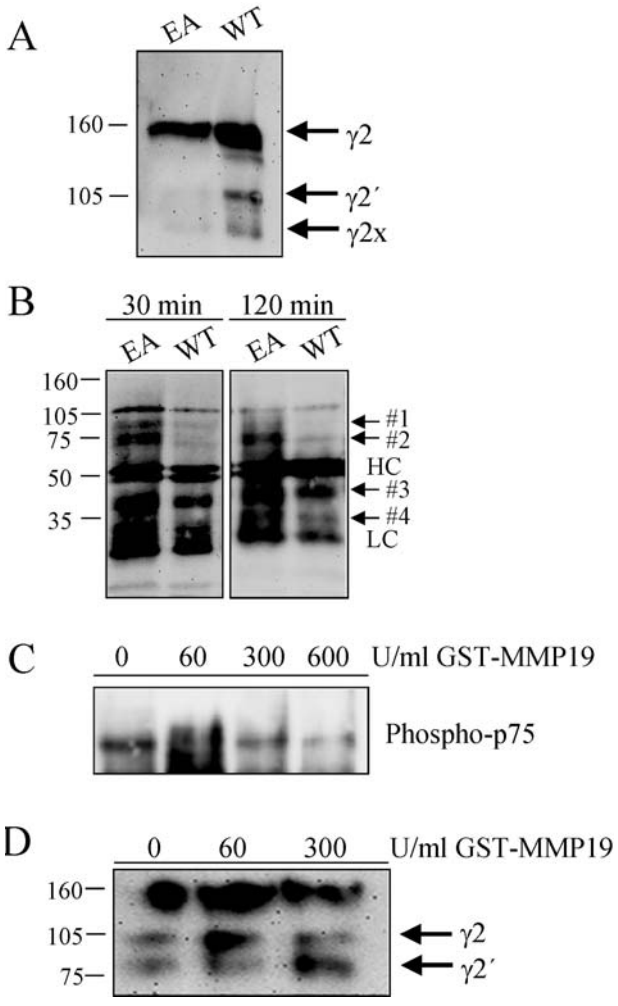

Figure 5. Processing of the ECM by MMP-19 alters cell signaling. (*A*) To study differences in cell signaling, the matrices of HaCaT-WT (WT) and HaCaT-EA (EA) cells were prepared as described in Materials and methods and analyzed by immunoblotting for the content of proteolytic fragments of the laminin 5 γ2 chain. (*B*) Parental HaCaT cells were seeded in serum-free medium onto ECM matrices from the HaCaT-WT and HaCaT-EA cells for 30 and 120 min. Tyrosine-phosphorylated proteins were precipitated from cell lysates and immunoblotted with an anti-phosphotyrosine antibody. Arrows denote the four major tyrosine-phosphorylated proteins that were less phosphorylated in cells incubated on the HaCaT-WT matrix (nos 1–4). HC, antibody heavy chain; LC, antibody light chain. (*C*) The matrix of HaCaT-EA cells was treated with recombinant MMP-19 that led to the dephosphorylation of the protein around 75 kDa. (*D*) The treatment of the HaCaT-EA matrix also resulted in increased degradation of laminin 5 γ2.

Activity of MMP-19 switches ligand-binding properties of the ECM

We next asked whether the distinct tyrosine phosphorylation pattern in parental HaCaT cells adhered to the matrices of HaCaT-WT and HaCaT-EA was mediated through ligation of different integrin receptors. For the processing of the γ2 chain of laminin 5, we were especially interested in those intergrins that mediated binding to laminin 5, i.e. α3β1 and α6β4. HaCaT cells were preincubated with blocking antibodies against the β1 (JB1a) and α3 (P1B5) integrin subunit and the stimulating antibody against the β4 integrin (439-9B) followed by 30 min incubation on the matrices of HaCaT-WT and HaCaT-EA cells. Whereas the antibody against β1 did not prevent the differences in phosphorylation of the 75-kDa protein at the chosen concentration, blockade of α3 and stimulation of β4 both resulted in increased phosphorylation of the 75-kDa protein on the matrix of HaCaT-WT cells (fig. 6A). Furthermore, the presence of polyclonal antibodies directed against the C terminus of the γ2 chain prevented phosphorylation of the 75-kDa protein on the matrix of HaCaT-EA cells. Previous reports have indicated that α6β4 signaling is mediated by Src family kinases [31]. In addition, β4 activates phosphatidylinositol-3 kinase (PI-3K) [32]. Using the src-kinase-specific inhibitor PP2 and the PI-3K-specific inhibitor LY294002, we tried to evaluate which of these kinases was responsible for phosphorylation of the 75-kDa protein. As shown in

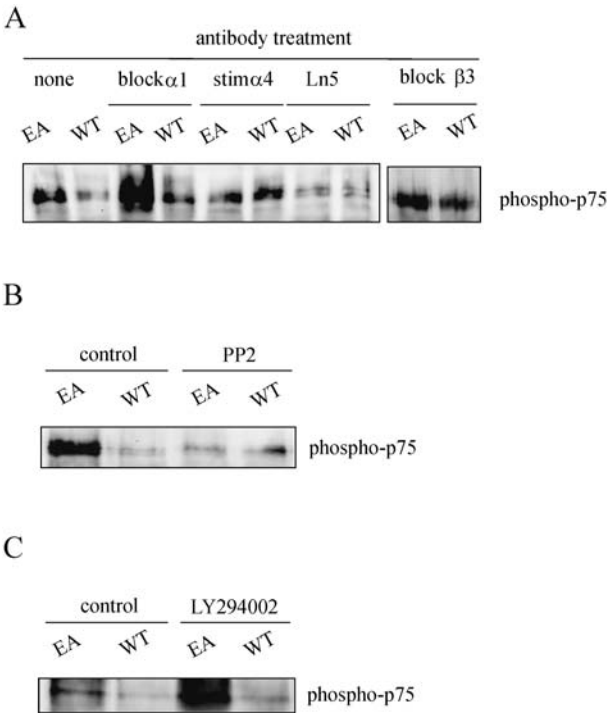

Figure 6. Altered integrin ligand binding and signaling on the matrix processed by MMP-19. (*A*) HaCaT cells were seeded on the matrices of HaCaT-WT (WT) and HaCaT-EA (EA) cells in the presence of blocking antibodies against the α1 (JB1a) and β3 (P1B5) integrin subunit, the stimulating antibody against the β4 integrin (439-9B) and an antibody against the γ2 chain of laminin 5. Tyrosine phosphorylation was analyzed after adhesion for 30 min. The blocking antibody against β1 did not prevent the differences in phosphorylation of the 75-kDa protein under the conditions used. Both the blocking and stimulating antibody against α3 and β4, respectively, resulted in increased phosphorylation of the 75-kDa protein on the matrix of HaCaT-WT cells. (*B*) Tyrosine phosphorylation of the 75-kDa protein after inhibition of kinases involved in β4 signaling. Incubation of HaCaT cells on the matrices of HaCaT-WT (WT) and HaCaT-EA (EA) cells in the presence of the src-kinase-specific inhibitor PP2 (10 μM) prevented the phosphorylation of the 75-kDa protein. (*C*) No effect was observed after inhibition of PI-3K by the specific inhibitor LY294002 (10 μM).

figure 6B, we could completely block the increased phosphorylation of the 75-kDa protein on the EA matrix with PP2, whereas the PI-3K inhibitor LY294002 was without effect (fig. 6C).

ECM processed by MMP-19 changes the migratory behavior of keratinocytes

After defining the ligand properties of the ECM processed by MMP-19, we studied whether differences in cell signaling observed on the matrices conditioned by HaCaT-WT and HaCaT-EA cells could result in altered cell behavior. As cleavage of laminin 5 by MT1-MMP and MMP-2 has been shown to induce motility in epithelial cells [33, 34] we examined whether the ECM laid down by HaCaT-WT and HaCaT-EA cells influences the migration of parental HaCaT cells during wound healing in a scratch assay. The scratch closure was nearly completed after 48 h in HaCaT grown on the ECM produced by HaCaT-WT cells whereas those cells grown on the matrix produced by HaCaT-EA cells could not recover the denuded area within this time (fig. 7A). Conversely, parental HaCaT adhered only slightly better to the HaCaT-WT matrix as compared to HaCaT-EA matrix (fig. 7B).

Discussion

MMPs facilitate the metastasis of tumor cells by degrading ECM and enhancing tumor-induced angiogenesis. In previous studies, a link between the expression of MMP-19 in vascular smooth muscle and endothelial cells and the manifestations of acute inflammatory conditions, which is suggestive of an involvement in angiogenic processes, was proposed. Conversely, not known is whether MMP-19 participates in the degradation of basal membrane components during neoplastic transformation.

In the present study we found overexpression of MMP-19 in epithelial cells at the invasive front of early tumor stages. Interestingly, this expression pattern appears to change with tumor progression, as in a recent study on grade II and III tumors [24], MMP-19 was only detected in hyperproliferative epithelium covering or bordering SCC. In contrast, the collagenases MMP-1 and MMP-13 as well as MT1-MMP localize to the cells at the invading front in advanced stages, where they might be needed to degrade interstitial collagen [11, 35]. Moreover, MMP-7, -9, -13 and MT1-MMP have been detected exclusively in malignantly transformed SCC cells but not in premalignant lesions [11, 12, 36]. Together with our results, these observations illustrate the fact that during tumor progression, a strict spatially and temporally regulated expression of individual MMPs is established.

MMP-19 activity might be needed to enable cells to transmigrate through type IV collagen, which represents a major barrier component to tumor outgrowth into the dermis. Conversely, overexpression of MMP-19 was not sufficient to drive transmigration through a type I collagen matrix (data not shown). This is interesting in view of our observation that tumor cells that invaded the dermis, where they mainly encounter interstitial collagen as matrix barrier, lose MMP-19. In contrast to our previous finding that MMP-19 can drive keratinocyte migration by providing bioavailable IGF through degradation of IGFBP-3 [22], transmigration through type IV collagen and fibrin was not dependent on IGF signaling. Moreover, type IV collagen and fibrin matrix can be directly degraded by MMP-19 while type I collagen can not.

We observed significant co-localization of laminin 5 and MMP-19 at the invasive front of early stage SCC. Our in

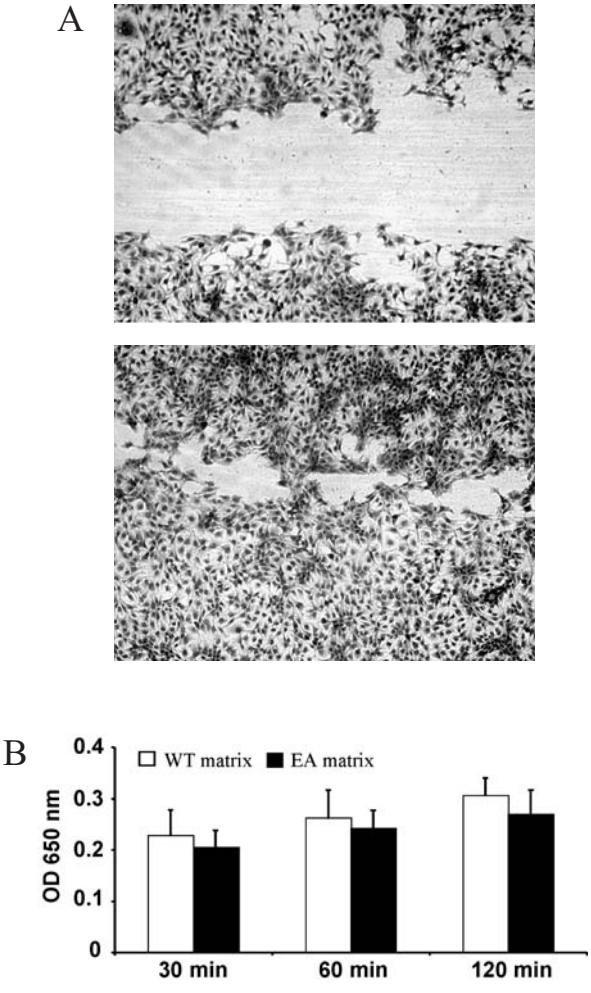

Figure 7. ECM processed by MMP-19 changes the migratory behavior of keratinocytes. (*A*) Parental HaCaT cells were seeded onto the ECM laid down by HaCaT-WT (WT) and HaCaT-EA (EA) cells. At confluence, a cell-free area was introduced and migration was evaluated after 48 h. Scratch closure was nearly completed after 48 h in HaCaT grown on the ECM produced by HaCaT-WT cells. The cells grown on the matrix produced by HaCaT-EA cells could not recover the denuded area. (*B*) Impact of MMP-19 on the cell adhesion. Parental HaCaT were seeded onto the ECM laid down by HaCaT-WT (WT) and HaCaT-EA (EA) cells and were allowed to adhere for 2 h. Nonadherent cells were then washed away and attached cells were quantified as described in Materials and methods. Each bar represents the mean + SE of three independent experiments.

vitro findings showed that overexpression of MMP-19 increased degradation of cell-derived laminin 5 γ2 chain and that recombinant MMP-19 was able to cleave the γ2 chain in a cell-free assay suggesting that this enzyme is critically involved in the processing of this protein. In vivo this might extend to the activation of other laminin-degrading proteases. However, not known at present is whether MMP-19 can activate protease zymogens by limited proteolysis.

Previous studies have yielded conflicting results regarding the ability of other MMPs to cleave the human laminin 5 γ2 chain. MMP-2 and MT1-MMP have been reported to process the γ2 chain to a form which triggers epithelial migration [17, 18]. However, these results were obtained using rat laminin 5, whereas in two recent studies examining the processing of laminin 5 of human origin, these MMPs were unable to process this protein [20, 33]. In contrast Gilles et al. [37] demonstrated that the catalytic domain of human MT1-MMP cleaves the γ2 chain of laminin 5 purified from human squamous-carcinoma-SCC25-cell-conditioned medium. In yet another study, MMP-3, -12, -13, and -20 also processed rat laminin 5 γ2 to an 80-kDa fragment [38]. Clearly, the contribution of these MMPs to processing of human laminin 5 γ2 deserves further investigation.

Processing of laminin 5 has been suggested to result in altered integrin engagement and subsequently in different cell behavior [30]. We approached this issue by determining the pattern of tyrosine-phoshorylated proteins in parental HaCaT after incubation on the matrices of HaCaT-WT and HaCaT-EA cells. Indeed, we observed remarkable differences in the tyrosine phosphorylation pattern in HaCaT cells incubated on the two matrices with a major difference in the phosphorylation of a protein around 75-kDa, which was only found after adhesion of parental HaCaT to the EA matrix. Our results obtained with blocking and stimulating integrin antibodies suggest that the phosphorylation of this protein on the HaCaT-EA matrix is $\alpha 6\beta 4$ dependent and is prevented on the HaCaT-WT matrix by a shift toward $\alpha 3$-integrin-dependent signaling. Of note, $\alpha 6\beta 4$-dependent phosphorylation of a similarly sized 80-kDa protein has been previously described as an early event responsive to and possibly required for anchorage to laminin 5 by keratinocytes [39]. At the moment we cannot exclude that processing of matrix proteins other than laminin 5 by MMP-19 also contributed to these differences in cell signaling. However, as laminin 5 is abundant in the matrix of keratinocytes and $\alpha 6\beta 4$ and $\alpha 3\beta 1$ integrins are the main receptors for this protein, these differences are likely mainly due to increased MMP-19-dependent processing of the γ2 chain to the mature 105-kDa form. In addition, we tested if src kinases, which physically interact with $\alpha 6\beta 4$ in HaCaT keratinocytes and are known to be involved in $\beta 4$ signaling [31], affect phosphorylation of the 75-kDa protein. Inhibition of src kinases was sufficient to prevent phosphorylation of this protein. Conversely, inhibition of PI-3K that could be activated in HaCaT cells by ligation of $\alpha 6\beta 4$ [40] was not effective. Thus, phosphorylation of the 75-kDa protein might be an early event after ligation of $\alpha 6\beta 4$ by laminin 5. Unfortunately, we did not succeed in identifying the nature of this protein. However, by using specific antibodies we could exclude that the 75-kDa protein represents known src kinase substrates like cortactin [41], PKCδ [42], or a degradation product of the focal adhesion kinase [43], all

of which have a molecular weight around 75 kDa (data not shown).

The physiological relevance of MMP-19-mediated cleavage of the laminin 5 γ2 chain was demonstrated by applying a scratch assay in which cells exhibited increased migration on the matrix of HaCaT overexpressing MMP-19. In accordance with these results, migration on laminin 5 cleaved by MMP-2 could be inhibited with antibodies against α3β1 integrin [17, 34]. Furthermore, haptotactic migration of HaCaT on laminin 5 was negatively affected by α6β4 signaling [40], illustrating the importance for a shift in integrin engagement by laminin 5 to induce migration of epithelial cells [44, 45].

In addition to the hypothesis that γ2 might serve as a migratory substrate in neoplasia, increased synthesis and secretion of this protein could also represent an attempt of the cells to reconstitute stable cell-substratum adhesion. The short arm of the γ2 chain, especially might sustain interactions with specific basement membrane components and cell receptors and participate in basement membrane assembly [46]. Thus, Impola et al. [24] concluded from their studies on MMP-19 expression in SCC that this protein might be involved in repair attempts. Although our results do not support the idea that processing of the γ2 chain influences adhesion, we cannot exclude that the co-localization of MMP-19 and laminin 5 in the initial stages of SCC might reflect repair attempts of epithelial cells undergoing malignant transformation.

Taken together, we have identified the γ2 chain of laminin 5 as a new substrate of MMP-19 and showed that MMP-19-dependent processing of the γ2 chains leads to the integrin switch favoring epithelial migration. We further propose that MMP-19 actively participates in the early stages of SCC invasion.

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