Role of Cell Surface Metalloprotease MT1-MMP in Epithelial Cell Migration over Laminin-5

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Abstract. Laminin-5 (Ln-5) is an extracellular matrix substrate for cell adhesion and migration, which is found in many epithelial basement membranes. Mechanisms eliciting migration on Ln-5 need to be elucidated because of their relevance to tissue remodeling and cancer metastasis. We showed that exogenous addition of activated matrix metalloprotease (MMP) 2 stimulates migration onto Ln-5 in breast epithelial cells via cleavage of the α2 subunit. To investigate the biological scope of this proteolytic mechanism, we tested a panel of cells, including colon and breast carcinomas, hepatomas, and immortalized hepatocytes, selected because they migrated or scattered constitutively in the presence of Ln-5. We found that constitutive migration was inhibited by BB94 or TIMPs, known inhibitors of MMPs. Limited profiling by gelatin zymography and Western blotting indicated that the ability to constitutively migrate on Ln-5 correlated with expression of plasma membrane bound MT1-MMP metalloprotease, rather than secretion of MMP2, since MMP2 was not produced by three cell lines (one breast and two colon carcinomas) that constitutively migrated on Ln-5. Moreover, migration on Ln-5 was reduced by MT1-MMP antisense oligonucleotides both in MMP2+ and MMP2− cells lines. MT1-MMP directly cleaved Ln-5, with a pattern similar to that of MMP2. The hemopexin-like domain of MMP2, which interferes with MMP2 activation, reduced Ln-5 migration in MT1-MMP +, MMP2+ cells, but not in MT1-MMP +, MMP2− cells. These results suggest a model whereby expression of MT1-MMP is the primary trigger for migration over Ln-5, whereas MMP2, which is activated by MT1-MMP, may play an ancillary role, perhaps by amplifying the MT1-MMP effects. Codistribution of MT1-MMP with Ln-5 in colon and breast cancer tissue specimens suggested a role for this mechanism in invasion. Thus, Ln-5 cleavage by MMPs may be a widespread mechanism that triggers migration in cells contacting epithelial basement membranes.

Key words: migration • extracellular matrix • epithelial cell • invasion

Introduction

Cell motility is a determinant of epithelial morphogenesis and regeneration (Thiery, 1984). An important issue is to define the molecular nature of spatial cues in the environment surrounding epithelial cells, which may signal initiation of migration during processes such as tissue remodeling or wound healing. Finding and characterizing these cues should make it possible to understand and manipulate epithelial tissue organization and pathological conditions such as metastasis.

The extracellular matrix (ECM) of the basement membrane (BM) is a likely structural site for motility cues since the BM is a critical interface between epithelial cells and the rest of the body. Laminin-5 (Ln-5), an ECM glycoprotein found in the BM, is a strong candidate for playing a major role in epithelial cell motility (Miyazaki et al., 1993b; Giannelli et al., 1997). We hypothesized that it may act not only as a passive ECM substrate (Roskelley et al., 1995; Malinda and Kleinman, 1996), but may actively participate in the regulatory aspects of motility.

Ln-5 is a recognized ligand for integrins α6β4 and α3β1 (Carter et al., 1990; Jones et al., 1991; Niessen et al., 1994;
The interaction of Ln-5 with α6β4 leads to the assembly of hemidesmosomes, which are static adhesive devices that anchor epithelial cells to the underlying BM (Baker et al., 1996). Interestingly, though, Ln-5 was also shown to promote vigorous cell scattering when added to the medium of epithelial cell cultures (Miyazaki et al., 1993b; Giannelli et al., 1997). These apparently opposing functions of Ln-5 (i.e., the ability to induce either static adhesion via hemidesmosomes [Jones et al., 1991] or cell motility [Miyazaki et al., 1993b; Giannelli et al., 1997]) may reflect physiological mechanisms to maintain tissue integrity: in quiescent tissues, Ln-5 may be predominantly a static adhesive substrate, whereas during regenerative or wound healing responses, it may deliver migratory stimuli. In support of this hypothesis, primary breast epithelial cell cultures, and the immortalized breast cell line MCF-10A, become migratory on Ln-5 upon addition of activated MMP2, an ECM metalloprotease (Giannelli et al., 1997). An indication that this mechanism may have physiological significance is that Ln-5 γ2 fragments, corresponding in size to those generated by MMP2 cleavage, are detectable in remodeling, but not in quiescent mammary glands (Giannelli et al., 1999) and in epithelial tumor tissue specimens. Thus, MMP2 cleavage of Ln-5 may play a mechanistic role in epithelial cell invasion during tissue remodeling as well as in cancer invasion and metastasis. However, these processes require some degree of spatial definition. Since MMP2 is secreted in the extracellular space and it is soluble, it is not clear how Ln-5 cleavage by MMP2 may be spatially directed to discrete BM sites.

Several MMPs have been associated with the remodeling of epithelial tissues as well as metastasis (Birkedal-Hansen, 1995), but the molecular mechanisms remain poorly understood (Werb et al., 1996). One critical issue is MMP activation and its spatial restrictions. In the breast epithelial cells we studied (primary cultures and MCF-10A), exogenous addition of chemically activated MMP2 was an absolute requirement for Ln-5 cleavage and consequent migration, raising the possibility of alternative, though not mutually exclusive, scenarios including the following: (1) epithelial cells may rely on another cell, e.g., mesenchymal or inflammatory, to activate pro-MMP2, cleave Ln-5, and migrate; (2) in cell cultures, MMP2 mimics the activity of other physiological proteases, which may be secreted and/or activated by epithelial cells; and (3) epithelial cells, upon responding to appropriate stimuli, may secrete MMP2, express the MMP2 activation apparatus, and thereby regulate migration on Ln-5 in an autonomous fashion.

To investigate these possibilities, we characterized the role of MMP during Ln-5 migration in a panel of cell lines of distinct histologic derivation. These cell lines present one critical difference with respect to MCF-10A and primary mammary epithelial cells: they do not require exogenous addition and/or activation of MMPs, but rather they migrate on Ln-5 constitutively. Here, we show that this constitutive Ln-5 migration depends upon expression of surface membrane type 1 (MT1-MMP) and mouse mAb against human type 1 (BTM1-MMP) and mouse mAb against human tissue inhibitor of metalloprotease-2 (TIMP-2) were obtained from MZ-1 and HLF, hepatoma; and HT-1080, fibrosarcoma. BRL, MCF-10A, MDA-MB-231, DLD-1, HT-29, and HT-1080 cell lines were obtained from the American Type Culture Collection. HLE and HLF were obtained from the Japanese Cancer Resources Bank. The 804G cell line was described previously (Falk-M arziller et al., 1998). Each cell line, except for MCF-10A, was maintained in DMEM (GIBCO BRL) plus 10% (vol/vol) FCS (Irvine Scientific), penicillin, and streptomycin. MCF-10A was maintained in DMEM, penicillin, and streptomycin. MCF-10A was maintained in DFC-1 plus 1% (vol/vol) FCS as described previously (Plopper et al., 1998).

The purification of Rat Ln-5 from 804G Serum-free Conditioned Medium (CM)

Serum-free DMEM CM of 804G cells was prepared in roller bottles, concentrated ~100-fold by ammonium sulfate at 80% saturation and dialyzed against 20 mM Tris-HCl (pH 7.5)/0.5M NaCl/0.005% Brij-35 (TNB buffer). Nonfunctional Ln-5 mouse mAb b TR-1 (Plopper et al., 1996) was chemically conjugated to protein A–Sepharose 4B as previously reported (Koshikawa et al., 1992). Concentrated CM was passed over the TR-1 antibody column (0.8 × 4.0 cm) (Bio-Rad Laboratories), previously equilibrated with TNB buffer at a flow rate of 15 ml/h. After washing with TNB, absorbed Ln-5 was eluted with 10 ml of 0.05% trifluoroacetic acid (TFA), pH 2.5. Each eluted fraction (1 ml) was quickly neutralized by 300 µl of 1 M Tris-HCl, pH 8.0, and then 1% of CHAPS (wt/vol) was added to each fraction.

Gelatin Zymography and Immunoblotting

Gelatin zymography was performed as described (Koshikawa et al., 1992). Serum-free CM was prepared from confluent cultures of each cell line incubated for 48 h in serum-free basal media. The CMs were concentrated ~30-fold by ammonium sulfate at 80% saturation and dialyzed against TNA buffer (Koshikawa et al., 1992). Crude plasma membranes were prepared from confluent cultures of each cell line incubated for 48 h in serum-free basal medium. Cells were scraped in 0.25 M sucrose/10 mM Hepes, pH 7.5, and then collected by centrifugation at 3,000 rpm for 5 min, the supernatant was centrifuged at 15,000 rpm for 30 min, and the crude plasma membrane fraction was recovered as pellets. Immunoblotting was performed with rabbit polyclonal antibodies against human membrane type 1 MMP (MT1-MMP) and mouse mAb against human tissue inhibitor of metalloprotease-2 (TIMP-2) by reported methods (Miyazaki et al., 1993a), except that the membrane was detected by the enhanced chemiluminescence method with a NEN Life Science Products kit.

Preparation of MMP2 and MMP Inhibitors

Human TIMP-2-free M2P2 was purified from serum-free CM of human glioblastoma T98G cells (Miyazaki et al., 1993a). TIMP-1 and TIMP-2 were purified from serum-free CM of human bladder carcinoma EJ-1 cells (Miyazaki et al., 1993a). Hemopexin-like domain (HLD) of MMP2 was purified from human TIMP-2-free M2P2 treated with neutrophil elastase, by Reactive-Red agarose affinity chromatography as described before (Strongin et al., 1993). Rice and Banda, (1995). BM94 (Batimastat) was a gift from British Biotechnology Ltd.

Bacterial Expression and Purification of Recombinant Rat MT1-MMP

Rat smooth muscle cell–derived MT1-MMP cDNA encoding amino acids Ile128-Glu228 was amplified by a PCR using 5'- and 3'-primers with addi-

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tional Ndel and EcoRI sites at the ends, respectively. The PCR products were digested with these enzymes and subcloned into the P E T-30α vector (Novagen, Inc.), modified to express the FLAG peptide (Kinosita et al., 1998) fused to the COOH terminus of rat MT1-MMP protein in E. coli. Other experimental methods were performed as previously reported (Kinosita et al., 1998).

Cleavage of Ln-5 by MMP2 or MT1-MMP

5 g of purified Ln-5 was incubated with 3.2 mglm of p-aminophenyl mercuric acetate-activated MMP2 for 2 h at 37°C in 50 mM Tris, pH 7.5, 0.005% Brij-35, 10 mM CaCl2. In some cases, purified Ln-5 (1 g) was adsorbed onto a 96-well plate well, and then incubated with recombinant MT1-MMP (0.2–2 mglm) for 16 h at 37°C in 50 mM Tris, pH 7.5, 0.005% Brij-35, 10 mM CaCl2. A fer incubation, each reaction mixture was electrophoresed on 6% SDS-PAGE under reducing conditions, and then analyzed by Western blotting with a rabbit polyclonal antibody against rat Ln-5 β2 chain antibody (1963).

Cell Scattering Assay

BRL cells were placed into 24-well plates containing 0.5 ml of DMEM plus 1% FCS at 7000 cells per well. Purified Ln-5 (80 ng/ml) was added. A fer 6–24 h, cells were fixed in 100% methanol for 10 min and stained with 0.5% crystal violet/20% methanol. Scattering was judged by microscopic observation.

Cell Migration Assay

Cell migration assays were performed in Transwell chambers as reported (Mizushima et al., 1997). Cells were resuspended in DMEM plus 0.1% (w/v) BSA and seeded at 20,000 cells/well for BRL, MDA-MB-231, DLD-1, and HT-29; 10,000 cells for HLF; and 5000 cells for HLE. Ln-5 was added to the lower chamber at 400 ng/ml (cancer cells) or 200 ng/ml (BRL). In some cases, MMP2-cleaved, MMP inhibitors, antibodies, HLD of MMP2, or oligonucleotides were also added, at indicated concentrations. A fer incubation (16 h for BRL, DLD-1, and HT-29; 6 h for MDA-MB-231 and HLE; 3.5 h for HLF), cells that migrated onto the lower surface of the filters were stained with 0.5% crystal violet/20% methanol and counted (Giannelli et al., 1997).

Antisense Oligonucleotides

Rat and human MT1-MMP antisense (AS) oligonucleotide sequences and their scrambled control oligonucleotides were designed by computer program (A advanced G ene C omputing T echnologies). The following phosphorothioate oligodeoxyribonucleotides were synthesized: AS oligonucleotide, 5'-CTCGTGGTTCAAGCTGG-3'; control oligonucleotide, 5'-TCCGAGTTCTCGAGG-3' for rat MT1-MMP, or AS oligonucleotide, 5'-GCGCTAATATCTTCG-3'; and control oligonucleotide, 5'-ATCTCGGATCTCAGACT-3' for human MT1-MMP. These oligonucleotides were freshly dissolved in PBS and added to BRL or HT-29 cells, respectively, at 10 m. A fer 2 of pretreatment with test or control oligonucleotides, cells were tested in migration assays. Oligonucleotides were added to the lower chamber of Transwells at 10 m.

Confocal Microscopy

Double immunofluorescence and confocal microscopic analyses were performed on 8-mm cryostat sections of human colon and breast carcinomas (The Cooperative Tissue Network), fixed in freshly made 4% formaldehyde (from paraformaldehyde) for 20 min at 4°C, permeabilized in 0.1% Triton X-100 for 10 min at room temperature, and then incubated in 50 mM glycine in PBS to saturate reactive groups formed by formaldehyde fixation. Non-specific binding was blocked by incubation in PBS containing 2% donkey anti–mouse IgGs (5 mglm) (preadsorbed on bovine, chicken, goat, guinea pig, hamster, horse, human, mouse, rat, and sheep serum proteins); indodicarbocyanine (Cy5)-conjugated affinity-purified donkey anti–mouse IgGs (5 mglm) (preadsorbed on serum proteins from species above). A fer washing in PBS-D-S, sections were mounted in slow-fade medium (Molecular Probes), and viewed on a Zeiss Axiovert 200M microscope equipped with a laser scanning confocal attachment (MRC-1024; Bio-Rad Laboratories), using a 40×1.3 NA objective lens. Fluorescent images were collected using the 488 (for FITC) and 647 nm (for Cy5) excitation lines from an argon/krypton mixed gas laser. Color composite images were generated using A dobe Photoshop 4.0 by attributing the green and red color to either FITC- or Cy5-specific fluorochrome spectra depending on experimental conditions. Images were printed with a Fuji Pictography 3000 color printer. Each experimental condition was performed in triplicate. A number of 27 slides from human breast carcinoma and 11 slides for colon carcinoma were immunostained, with at least 20 microscopic fields scored per slide.

Determination of Protein Concentration

Protein concentration was determined by dye methods with a Bio-Rad Laboratories assay kit with BSA as the standard.

Reagents

mA bs against T IM P-2 (D 52) (Shofuda et al., 1998), hum an M MP 2 (2-22) (K aw ano et al., 1997), human L n-5 β 2 chain (D 4B 5) (Mizushima et al., 1998), and rat L n-5 (M 1G-1 and T R-1) (Plopper et al., 1996), and polyclonal antibody against rat Ln-5 β2 chain (1963) were generated in our laboratories. Transwells were purchased from Corning-Costar; polyclonal antibody against human MT1-MMP (A B 815) were purchased from Chemicon International, Inc.; protein A-Sepharose 4B was purchased from Pharmacia Biotech Sverige.

Results

We previously showed that, in breast epithelial cells, migration over Ln-5 is triggered by MMP2 (Giannelli et al., 1997). In that system, though, MMP2 must be activated and added exogenously, raising the question as to how general the mechanism might be, particularly in cell types that display constitutive migration over Ln-5. To address this question, we tested the effects of BB94, a hydroxamate compound known to broadly inhibit MMPs, in migration assays with a panel of epithelial cells that constitutively migrate on Ln-5 (Miyazaki et al., 1993b). In every case, migration on Ln-5-coated Transwell filters was inhibited by BB94 in a dose-dependent manner (Fig. 1). Similar levels of inhibition were achieved with TIMP-1 and TIMP-2, specific inhibitors of MMPs that are naturally occurring in tissues (Fig. 2A). These data were a first indication that MMPs may be involved in the mechanism that elicits the constitutive migration of those cells on Ln-5.

In both migration (not shown) and scattering (Fig. 2 B) assays, the inhibitory activity of BB94 was abolished if Ln-5 had been cleaved with MMP2 before exposure to cells. Furthermore, the anti–Ln-5 antibody, M1G-1, inhibited scattering (Fig. 3 A) and blocked migration (Fig. 3 B) on Ln-5. These results further supported the possibility that constitutive migration on Ln-5 (i.e., without exogenous addition of activated MMPs) depends on cleaving Ln-5 with endogenously produced and activated MMPs.

To investigate this possibility further, we monitored MMP production by zymography in these cell lines. In Fig. 4, we show representative results with the cell line BRL, compared with HT1080 cells, known to secrete and activate pro-MMP2 (Shofuda et al., 1998; Stanton et al., 1998), and MCF-10A, a breast cell line that instead requires endogenous activation of MMP2 to cleave and migrate on Ln-5 (Giannelli et al., 1997). Gelatin zymography of condi-
tioned medium demonstrated that all three cell lines secrete a 72-kD gelatinolytic activity corresponding to pro-MMP2 (Fig. 4 A). However, the 62-kD–activated MMP2 could be detected only in HT1080, not in BRL or MCF-10A conditioned media (Fig. 4 A). Since MMP2 activation occurs at the cell surface, we next prepared plasma membranes from these cells and tested them by zymography. Two gelatinolytic bands at 72 and 62 kD, corresponding to the latent and activated form of MMP2, respectively, were detected in plasma membrane preparations from BRL and HT1080, but not MCF-10A cells (Fig. 4 B).

Cell surface activation of MMP2 may occur via a complex of pro-MMP2 with the tissue MMP inhibitor, TIMP-2, and the membrane-bound metalloprotease, MT1-MMP, as described previously (Strongin et al., 1995; Stanton et al., 1998). To confirm that BRL cells activate MMP2 via this mechanism, we tested for the presence of plasma membrane–associated TIMP-2 and MT1-MMP. By Western blotting, TIMP-2 was detectable in the conditioned media of all three cell lines. In contrast, only plasma membrane preparations from BRL and HT1080 cells, not MCF-10A cells (Fig. 4 C) contained TIMP-2. Similarly, membrane-associated MT1-MMP was clearly detectable in BRL and HT1080, and was very faint in MCF-10A cells (Fig. 4 D).

MT1-MMP [Stanton et al., 1998] was detected in the membrane of HT-1080 cells, [Fig. 4 D, asterisk.] These results suggested that constitutive migration on Ln-5 may depend upon secretion of MMP2 and expression of MT1-MMP and TIMP-2, which are required to activate MMP2. Surprisingly, however, MMP profiling of the cell line panel from Fig. 1 showed that three of these cell lines do not secrete MMP2 (Table I), even though they constitutively migrate on Ln-5. Every migratory cell, however,
positive for MT1-MMP, suggesting that this cell surface MMP may be directly involved in cleaving Ln-5 and promoting migration. Therefore, purified Ln-5 was incubated with recombinant, soluble MT1-MMP. This recombinant MT1-MMP preparation, produced in a bacterial expression system, was functional since it activated pro-MMP2 to the intermediate MMP2 form in vitro, as expected, and displayed proteolytic activity by gelatin zymography (data not shown). As shown in Fig. 5A, recombinant MT1-MMP cleaved the γ2 subunit of Ln-5 in a dose-dependent manner. The cleavage products have the same molecular size of γ2⁺ (Marinkovich et al., 1992) and γ2x (Giannelli et al., 1997), respectively, two previously described proteolytically processed forms of the γ2 subunit (Fig. 5, B and C). No changes were observed in the size of the other two subunits of Ln-5, α3, and β3 (not shown). A detailed account of Ln-5 cleavage by MT1-MMP will be published elsewhere.

Taken together, these results suggested a model whereby constitutive migration on Ln-5 may be achieved in two manners: (1) by MMP2 secretion in conjunction with expression of MT1-MMP, which activates pro-MMP2 and leads to Ln-5 cleavage (Fig. 6A); and (2) by expression of MT1-MMP alone, with no MMP2 secretion, since MT1-MMP can directly cleave Ln-5 and presumably cause mi-
gration (Fig. 6 B). To test this model, we diminished the expression of MT1-MMP in BRL and HT-29 cells by treatment with rat or human MT1-MMP antisense oligonucleotides, respectively.

In cells treated with MT1-MMP antisense, but not with control, scrambled oligonucleotides, expression of surface MT1-MMP was reduced by 67% in HT-29 cells and 40% in BRL cells (compared with β-actin internal controls), as determined by Western blotting followed by scanning densitometry on a Molecular Dynamics FluorImager (not shown). Importantly, in the antisense-treated cells, there was 50% inhibition of constitutive Ln-5 migration (Fig. 7 A), as well as scattering (not shown).

As expected, inhibition was observed both in the MT1-MMP+-, MMP2+-, and in the MT1-MMP+, MMP2− cell lines. Furthermore, addition of MMP2-cleaved, rather than intact Ln-5, to antisense-treated cells circumvented the inhibition of constitutive Ln-5 migration (not shown) or scattering (Fig. 7 B), supporting a direct relationship between MT1-MMP expression and Ln-5 cleavage.

In a further test, we used the MMP2 hemopexin-like domain (HLD), which inhibits activation of pro-MMP2 by competitively binding to TIMP-2 and preventing formation of the activating complex with MT1-MMP (Strongin et al., 1995). However, HLD is not known to interfere with MT1-MMP enzymatic activity. HLD significantly inhibited Ln-5 migration of BRL cells (Fig. 8). In contrast, no inhibition was observed for HT-29 cell migration on Ln-5 (Fig. 8). These results are in agreement with our working model (Fig. 6) that constitutive Ln-5 migration depends on both MT1-MMP and MMP2 in BRL cells, whereas, in HT-29, cells are dependent on MT1-MMP only.

To evaluate the possible relevance of these mechanisms in vivo, we immunostained with antibodies to Ln-5, MT1-MMP, and MMP2 sections of human breast (not shown) or colon cancer tissues (Fig. 9). Double immunofluorescence and confocal microscopy indicated that Ln-5, which was expressed predominantly at the outer edge of cancer cell nests, colocalized with either MT1-MMP or MMP2 at many locations (Fig. 9, arrows). These results are consistent with the possibility that Ln-5 cleavage, and consequent induction of migration, occurs at defined locations corresponding to areas of the cell surface where either MT1-MMP or activated MMP2 are expressed.

Discussion

We report that Ln-5 cleavage by MMPs may be a widespread mechanism that triggers cell migration. This conclusion is based on the following findings: (1) in several cell lines that migrate (or scatter) constitutively on Ln-5, migration (or scattering) on Ln-5 was blocked by inhibitors of MMPs, both naturally occurring, like TIMPs, or synthetic, like BB94; (2) in these cell lines, the ability to
migrate constitutively on Ln-5 correlated with expression of membrane bound MT1-MMP; (3) decreasing expression of MT1-MMP via antisense oligonucleotides inhibited migration; and (4) purified Ln-5 itself was cleaved in vitro by MT1-MMP with a pattern similar to MMP2, thus, providing a mechanism for induction of migration (Giannelli et al., 1997).

These findings significantly extend the physiological implications of our previous report that cleavage of Ln-5 by MMP2 may induce migration (Giannelli et al., 1997). In that report, we had shown that adding exogenously activated MMP2 to purified Ln-5 changed the latter into a substrate that triggered cell migration. The only indication that the mechanism may operate in vivo was that Ln-5 fragments, similar to those generated by MMP2, were detectable in remodeling, but not in quiescent tissues. Here we show that, in fact, cells that have the constitutive ability to migrate on Ln-5 do so by using an MMP-dependent mechanism. MMP2 was secreted and activated by several of the constitutive migratory cells. This MMP also contributed to stimulating migration, since its HLD fragment, which blocks its activation, inhibited migration. However, inhibition was only partial in MT1-MMP+ cells, and nondetectable in MT1-MMP− cells. Together, these results suggest a model whereby one preferred mode for stimulating migration on Ln-5 is via MMP cleavage of Ln-5 itself. Cleavage may be carried out by MT1-MMP alone or in concert with MMP2. Since MT1-MMP is required to activate MMP2, it is likely that MT1-MMP plays an essential role in this mechanism, whereas MMP2 may represent a potentiation loop. An attractive feature of this model is that, whether Ln-5 is cleaved by MT1-MMP alone or by MT1-MMP–activated MMP2, the proteolytic components are anchored onto the cell surface because MT1-MMP is a transmembrane protein. Thus, spatially directed cleavage of Ln-5 may occur, perhaps coincident with hot spots for migration.

By incubating recombinant MT1-MMP with purified Ln-5, we showed, for the first time, that Ln-5 is a proteolytic substrate for MT1-MMP (Fig. 5). While the details of this cleavage are being currently characterized and will be published elsewhere, it is clear that only the γ2 subunit of Ln-5 is cleaved by MT1-MMP, whereas the α3 and β3 subunits remain intact. The Ln-5 γ2 subunit is synthesized as a 135-kD polypeptide which is proteolytically processed (Vailly et al., 1994) by removal of 434 NH2-terminal amino acids (γ2a). The enzyme that carries out this processing is not known, but our preliminary results indicated that it may be MT1-MMP. This would be consistent with the pattern generated by digestion of purified Ln-5, showing a time-dependent increase in γ2 (Fig. 5). MMP2 can cleave both γ2 and γ2′, yielding an 80-kD γ2x chain (Giannelli et al., 1997). It is clear that MT1-MMP can also produce the γ2x fragment (Fig. 5). Thus, we predict two MT1-MMP cleavage sites on Ln-5 (Fig. 5, B and C).
Miyazaki and colleagues described ladsin, a soluble protein in the spent media of several epithelial cell lines, that induced scattering and migration within hours upon addition to cultured cells (Miyazaki et al., 1993b). Ladsin turned out to be identical to Ln-5 (Mizushima et al., 1996), raising the following apparent conflict: in epithelial tissues and in certain culture systems, Ln-5 promotes static adhesion of epithelial cells via formation of hemidesmosomes; in contrast, ladsin promotes an opposite effect, scattering. Our results provide an explanation for this apparent conflict and reconcile the data in the literature. Thus, MT1-MMP+ cell lines are capable of cleaving Ln-5 into its migratory form, directly and via activation of MMP2, if present. In contrast, MT1-MMP− cells leave Ln-5 intact and use it for static adhesion.

To date, MT1-MMP was only known to digest collagens I, II, and III. Our results add new perspective in at least two respects. First, the substrate Ln-5 is located in the BM, in direct contact with epithelial cells, which are anchored to it via receptor integrins (α3β1 or α6β4). In contrast, epithelial cells do not express receptors for collagen IV (integrin α1β1) and, as far as we are aware, there is no report of MT1-MMP cleaving collagen IV or of promigratory activity by collagen fragments. Thus, it is possible that, in the context of the BM, the Ln-5 substrate may play a prominent role in mediating MT1-MMP effects on epithelial cells. Second, because the Ln-5 cleavage mechanism may work in tissue remodeling and repair (Giannelli et al., 1997, 1999), it is expected to be spatially constrained. This requirement may be fulfilled by MT1-MMP, which is transmembrane-anchored and presumably does not diffuse freely in the extracellular space, so that it may be targeted at discrete BM sites by cell surface contact. In addition, MT1-MMP may also constrain spatially MMP2, which is secreted extracellularly, by recruitment via TIMP-2 (see below).

A II evidence indicates that upon addition to constitutive migratory cell types, Ln-5 is cleaved by MMPs. However, in spite of extensive efforts, we were not able to detect cleaved Ln-5 in these cultures. This is not entirely surprising, in view of the fact that relatively small amounts of Ln-5 molecules may gain access to relevant cell surface sites, and nonetheless exert their biological effect when MMP cleaved. More sensitive detection methods may shed light on this point. Previously, it was reported that cleavage of the Ln-5 α3 chain by plasmin correlated with nucleation of hemidesmosomes (Goldfinger et al., 1998), presumably inhibiting cell migration. Therefore, it is possible that plasmin and metalloproteases act coordinately to regulate epithelial cell migration on Ln-5.

MMPs are involved in tissue remodeling under various physiological and pathological conditions such as morphogenesis, angiogenesis, inflammation, tissue repair, and tumor invasion (Matrisian, 1992; Stetler-Stevenson et al., 1993). In particular, inflammatory macrophage and malignant cancer cells secrete MMP2 to degrade BM and connective tissue ECM to invade. Deryugina et al. (1997) have shown that MMP2 activation and integrin αvβ3 modulate glioma cell migration on ECM. MMP2 also modulates melanoma cell attachment and facilitates migration and invasion (Ray and Stetler-Stevenson, 1995). Ln-5 has also been associated with carcinoma cell invasion. In particular, the γ2 chain of Ln-5 was detected at the leading edge of invasive colon and breast cancer tissue (Pyke et al., 1995; Sordat et al., 1998). In our study, migration of hepatoma, breast and colon carcinoma cells was induced by Ln-5, via the MMP cleavage mechanism. Furthermore, colocalization of Ln-5 with MT1-MMP and MMP2 was detected in breast and colon cancer tissue. Thus, our results suggest a mechanistic framework for the observed association between MMPs and invasion, or Ln-5 and invasion. We propose that MT1-MMP cleavage of Ln-5 is a candidate to play a role in the early phases of tissue invasion, e.g., when carcinoma in situ may still be dependent on external factors to initiate local invasion. These issues are deserving of further investigation because of their obvious relevance to cancer progression.

Our results raise several questions concerning the regu-
lation of motility of epithelial cells in contact with Ln-5. In particular, it is important to determine how MMPs may be deployed. A n attractive possibility is that epithelial cells may be stimulated to synthesize MT1-MMP by environmental signals and factors in situations that require remodeling, e.g., steroid-induced branching morphogenesis (Y et al., 1997; Kado no et al., 1998). Aternatively, MMPs may be delivered to remodeling sites by third party cells, stromal or inflammatory. These mechanisms may also be exploited, inappropriately, by invading carcinoma cells. A nother important issue concerns the nature of the cellular interactions with cleaved Ln-5, which trigger migration. Integrins are likely to play an important role because they can mediate adhesion and migration. A dditional studies are now necessary to further our understanding of these molecular mechanisms of invasion, both in normal and neoplastic tissues.

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