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13. ABSTRACT *(Maximum 200 Words)*

The overall goal of our project is to identify novel treatments for breast cancer invasion and metastasis. We view invasion and metastasis as a breakdown of mechanisms that specify tissue organization. We postulated that metastasis is initiated by molecular cues that improperly stimulate cancer cell motility. Therefore, our approach is to block metastasis by understanding, and then interfering with, molecular and cellular mechanisms that regulate cell motility. We found that, in the mammary gland, several of these mechanisms revolve around the interaction of matrix metalloproteinases (MMP) with laminin-5 (Ln-5), an extracellular matrix macromolecule of the breast gland basal lamina. Our major findings are as follows: 1) identification of the Ln-5 site onto which cells adhere and migrate; 2) mapping, relative to this cell adhesion site, of a docking site for antibodies that block cell motility; 3) mapping of Ln-5 sites that are cleaved by MMPs; 4) identifying the composition of the Ln-5 fragments resulting from this proteolytic MMP activity. This information should aid the design of reagents for in vivo animal experiments in which to test the ability of antibodies to Ln-5, or Ln-5 fragments, to hopefully block breast cancer cell motility and metastasis.

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Introduction:

Our goal is identifying novel treatments that effectively prevent and/or interfere with breast cancer invasion and metastasis. The innovative aspect of our work is that we view cancer invasion and metastasis as consequences of a breakdown in the organization of tumor tissue. Based on this premise, we propose that cancer treatments should take into account the whole of the mammary gland, not just individual cells that may have become cancerous. Our focus is on the molecular mechanisms that segregate breast epithelial cells, both normal and cancerous, on the luminal side of the basal lamina. Invasive breast cancer arises precisely from these cells, as they become unable to recognize the basal lamina as a functional and physical barrier. It is not until the basal lamina is transgressed that the gate becomes open for invasion and metastasis. We identified a molecular mechanism (1, 2) that specifies whether normal or cancer breast cells may cross the basal lamina. This mechanism requires the interaction of laminin-5, a major extracellular matrix macromolecule of basal lamina, with matrix metalloproteases and integrins. In this proposal we determined that this mechanism plays a primary role in regulating migratory versus stationary behavior of breast epithelial cells. Furthermore, we identified intriguing clues for drug targeting this process, thus interfering with cancer invasion.

Body:

AIM 1. To inhibit mammary epithelial cell motility in vitro and cell metastasis in vivo by blocking the migratory site of laminin-5 (Ln-5).

We mapped the binding site on the Ln-5 molecule of antibody MIG-1, which blocks migration of cancer cells on Ln-5 cleaved by metalloproteinases (MMPs). The epitope is located on the LG2 domain of the Ln-5 α3 subunit. By site-directed mutagenesis, we showed that the epitope is restricted to a stretch of 6 amino acids towards the center of the LG2 domain. Recently, the three-dimensional structure of the Ln-2 α2LG5 domain was solved. This domain is structurally homologous to α3LG2. Therefore, we were able to model the primary sequence of LG2 (using the structure of LG5 as a template). This comparison predicted that the MIG-1 epitope would be located in a loop of the LG2 domain which is exposed to the aqueous environment, corroborating our mapping results. To prove that the LG2 domain is involved in cell adhesion and migration, we also mapped the integrin binding site of Ln-5. Unexpectedly, the integrin binding site maps not to LG2, but rather to the LG3 domain, which is spatially close to LG2. This result suggest a mechanism whereby the LG2 and LG3 domain interact with each other and with integrins in order to support adhesion and migration.

AIM 2. To inhibit mammary epithelial cell motility in vitro and cell metastasis in vivo by inhibiting the cleavage of Ln-5 by MMP2.

During last year, we found that MMP2 is not the only protease to cleave Ln-5. MT1-MMP, a surface bound MMP, cleaves Ln-5 at the same site as MMP2, as well as to another site, approximately 150 amino acids upstream (1). The interesting fact is that the combined actions of MMP2 and MT1-MMP liberate a fragment of the Ln-5 γ2 chain, whose structure resembles that of EGF (epidermal growth factor). EGF and EGF-like ligands are well known to display mitogenic and motogenic activity. We have made recombinant proteins spanning the cleavage sites of these MMPs, as well as the liberated fragment. By using these recombinant fragments with appropriate tags, we have confirmed that both MMP2 and MT1-MMP cleave them in vitro. Furthermore, some of these fragments interfered with cell migration in in vitro assays. An

intriguing result is that the liberated fragment binds to the cell surface and stimulated tyrosine phosphorylation, suggesting it may bind to a receptor. These results strongly encourage testing these recombinant fragments in vivo, which we have initiated. We are still in the process of defining optimal protocols and establishing suitable end-points for these in vivo experiments. We should be able to complete them, under separate funding, in the near future.

Aim 3. To produce monoclonal antibodies that react with MMP2-cleaved Ln-5 and not with intact Ln-5, and to use them in immunohistological assays for correlating the location of cleaved Ln-5 with breast cancer cell invasion sites.

Using purified Ln-5 for this purpose has proven not feasible, because both intact and cleaved Ln-5 are present in purified preparations and are difficult to separate. We plan to change approach and use instead the recombinant fragments mentioned in the Aim above. We have now scaled up production of these fragments, and have produced LG1, LG2, LG3, LG4 and LG5 domains in the tens of milligrams range. This task proved somewhat more challenging than anticipated. In the end, the appropriate combination of vector and bacteria solved the problem. The LG3 and LG2 fragments have been injected in rabbits, and as soon as we obtain positive antisera, we will proceed to manufacturing monoclonal antibodies.

Key Research Accomplishments:

- Mapped integrin binding site on Laminin-5 to domain LG3, first time this was done on any laminins
- Mapped epitope for antibody MIG-1, which blocks cell migration, to Laminin-5 domain LG2
- Discovered that MT1-MMP is another proteolytic enzyme, besides MMP2, which cleaves Laminin-5
- Defined the boundaries of a Laminin-5 fragment that is proteolytically cleaved out by the action of MMPs
- Produced recombinant Laminin-5 MMP fragments

Reportable Outcomes:

- Manuscripts, abstracts, presentations
  Two manuscripts in print, see References below
  Presentations: 2000 Gordon Conference "Basement Membranes"
  2001 Gordon Conference "Mammary Gland Biology"
- Patents and licenses
  None
- Degrees obtained
  None
- Development of reagents
  Recombinant Domain III from Laminin-5
  Recombinant LG1-5 domains from Laminin-5
- Informatics
  None
- Funding applied for
  NIH grant application RO1-GM46902 "Molecular Regulation of Integrin Functions by Laminin Domains", PI Vito Quaranta, based on results from Aims 1 and 2 of this grant.
- Employment
  None

Conclusions:

Overall, our results support our original view that "normal" mechanisms of cell migration may be responsible for breast cancer invasion, when activated at the wrong place and/or the wrong time. This conclusion is mostly based on the fact that breast cancer cells respond to Ln-5 cues for migration, just like normal breast epithelial cells. Therefore, it may well be that invasion is not so much an intrinsic property of breast cancer cells, but may depend upon the environment, e.g., the tumor stroma.

Our major accomplishments led to the following specific conclusions, as anticipated last year. We mapped the Ln-5 binding site for antibodies that block cell adhesion and/or migration. In addition, we identified the domain of Ln-5 that binds integrins and supports cell adhesion and migration. Surprisingly, this domain is close to, but does not overlap with the binding site of the blocking antibodies. Nonetheless, this is an important insight towards our goal of designing reagents that inhibit the migratory site of Ln-5 and therefore may interfere with metastasis.

We have overcome limitations imposed by protein purification methods, and set up methods to produce monoclonal antibodies to cleaved Ln-5 by using recombinant fragments as immunogens.

We further defined Ln-5 fragments that are the targets of, or result from MMP cleavage, and are closer to test them in vivo for possible inhibitory effects on metastasis. This has been a long-standing question in the Ln-5 field, and therefore we expect our results to have significant impact. In spite of the fact that Ln-5 is clearly involved in metastasis, absence of structural details on its adhesion/migration domains has frustrated efforts to interfere with cancer invasion. By comparison, in the case of fibrinogen, another extracellular matrix molecule involved in blood clotting, knowledge of its adhesion site for platelets has led to the development of clotting pharmaceuticals that are already available to the public.

Similar considerations are applicable to our studies on the MMP2 cleavage site. MMP inhibitors are widely considered strong candidates as anti-metastasis drugs (2). In Aim 2, we have shown that a fragment of Ln-5 is cleaved by MMPs. This result was not obviously predictable, and puts us in a position to eventually use the Ln-5 fragment as a basis for the design of MMP inhibitors.

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2. Quaranta, V. Cell migration through extracellular matrix: membrane-type metalloproteinases make the way. J. Cell Biol. *149:*1167-1170, 2000.

3. Schenk, S., Hintermann, E., Bilban, M., and Quaranta, V. A fragment of laminin-5 excisable by matrix metallopreteinases is an EGF receptor ligand that stimulates cancer cell migration. J. Cell Biol. Submitted.

Appendices:

Three publications.

A fragment of laminin-5 excisable by matrix metalloproteinases is an EGF-receptor ligand that stimulates cancer cell migration

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Running title

Proteolytic fragment of Ln-5 is a ligand for EGFR

Keywords

Laminin-5 (Ln-5), extracellular matrix (ECM), basement membrane (BM), proteolytic fragment, laminin-type EGF-like (LE) repeat, epidermal growth factor receptor (EGFR), mitogen-activated protein kinase (MAPK), breast cancer, cell migration, MMP gene expression, microarray

SUMMARY

Extracellular matrix (ECM) fragments and sites unmasked by proteinases have been postulated to stimulate tumor invasion. However, the identity of such fragments has remained elusive. We provide direct evidence that an epidermal growth factor (EGF)-like fragment, generated by proteolytic cleavage of the ECM macromolecule Laminin-5 (Ln-5), is an active ligand for EGF-receptor (EGFR) tyrosine kinase and stimulates downstream signaling and breast cancer cell migration. These findings explain cell migration triggered upon cleavage of Ln-5 by matrix metalloproteinases (MMPs), and clarify mechanisms linking proteolytic degradation of ECM to cancer invasion.

INTRODUCTION

The ability of cancer cells to invade surrounding tissue is primarily responsible for cancer mortality. Invasion is a complex, multi-step process. In the case of epithelia-derived carcinomas, which represent the majority of human cancers, the landmark of invasion is the acquired ability of carcinoma cells to infiltrate the basement membrane (BM), a thin layer of specialized extracellular matrix (ECM) that separates epithelial cells from connective tissue. The molecular mechanisms whereby cancer cells gain their migratory properties are under intensive study.

Disruption of the BM and ECM architecture via degradation of their macromolecules (e.g. collagens and laminins) by proteolytic enzymes, such as metalloproteinases, plays a crucial role in cancer invasion. Not only does this proteolysis create a migratory path, but it frees signaling molecules, including growth factors latently immobilized within the matrix, and likely generates neo-epitopes and BM/ECM fragments with biological activities related to migration[1-5]. It is therefore of great interest to determine the structural nature of these ECM moieties liberated by proteolytic action, and to define their biological effects on cells at the molecular level.

A major component of epithelial BM that is implicated in tumor invasion is laminin-5 (Ln-5). Ln-5 has a cruciform structure with one long and three short arms. The coiled-coil structure of the long arm is formed by three chains ($\alpha 3$, $\beta 3$, $\gamma 2$), which are covalently linked via interchain disulfide bonds. The rod-like regions in the short arms are composed of EGF-like repeats intercalated with globular domains.

Several laboratories have reported enhanced expression of Ln-5 and especially of its γ2 subunit, at sites of tumor cell penetration in histological specimens of for instance, breast, colon, and gastric cancers[6,7]. Ln-5 could facilitate tumor invasion by promoting at least two relevant functions, i.e. cell adhesion and cell migration.

We have recently shown that binding of the globular LG3 domain (α3-chain) of Ln-5 to integrin α3β1 mediates cell adhesion and to some extent cell migration[8]. Adhesion may also be promoted by the Ln-5 receptor α6β4 integrin, which is involved in formation of hemidesmosomes[9].

Robust cell migration on Ln-5 has been shown to be triggered by cleavage of its γ2-chain by MMP-2[10] and MT1-MMP[11]. The suggested cleavage sites imply the liberation of the entire Ln-5 γ2 short arm (domains III, IV, and V) as well as of domains IV and V, or just domain III.

A fragment comprising domains IV and V has recently been found to mediate integration of Ln-5 into the ECM and to promote cell adhesion[12].

Domain III is comprised of EGF-like repeats and for this reason seems especially interesting. Not only is its structure reminiscent of EGF-like ligands, but several studies with laminin-1 (Ln-1) fragments, which contain arrays of EGF-like repeats, demonstrated biological activities, some of them related to cell migration[13,14], and some possibly engaging EGFR[15]. Given the multi-domain architecture of Ln-5, it seems conceivable that other cellular receptors in addition to integrins interact with one of its many potential ligand sites to mediate its diverse cellular functions.

In this paper, we address the hypothesis that domain III of the Ln-5 γ2-chain is a cryptic migratory signal liberated by MMP action. To this end we produced recombinant domain III (rDIII) and attempted to assign the migratory activity of cleaved Ln-5 to this specific fragment and to investigate a possible engagement of EGFR.

RESULTS

Expression and characterization of recombinant γ2 subunit DIII (rDIII).

We previously reported suggestive evidence that the membrane-bound metalloproteinase MT1-MMP cleaves the γ2 subunit of Ln-5[11]. The exact positions of the MT1-MMP cleavage sites have now been identified and are indicated in Fig 1A (N. Koshikawa & V. Quaranta, 2001 personal communication).

Since γ2-chain domain III (DIII) is effectively bracketed by MMP cleavage sites, and its structure after cleavage is reminiscent of EGF receptor ligands, we tested the hypothesis that MMP proteolytic fragments corresponding to DIII may have biological activity. To this end, we produced a recombinant γ2 domain III (rDIII) in baculovirus. The N-terminal boundary of rDIII (D^{414} E^{415} N^{416}) coincides with the MT1-MMP cleavage site (YSG⇓DEN), while the C-terminus is 17 residues downstream from the MMP-2 cleavage site (AAA⇓LTS)[10], which is also used by MT1-MMP (Fig.1A). A His_6-tag sequence was included at the C-terminus for the purpose of purification and antibody recognition.

Mass spectrometry (MALDI) revealed a molecular weight (MW) of 21,531 Da for purified rDIII, in excellent agreement with its calculated MW (21,525 Da). However, by SDS-PAGE rDIII resolved as a major 30 kDa band under reducing conditions, and as a 23 kDa band under non-reducing conditions (Fig.1B). This anomalous migration in SDS-PAGE agrees with previous findings for Ln-1 fragments containing EGF-like repeats[16].

By Western blotting of reducing PAGE, both the DIII-specific polyclonal antibody 2778 and an anti-His-tag monoclonal antibody recognized the 30 kDa band. In addition both antibodies reacted with a minor approximately 50 kDa band, likely a dimerized form of rDIII. To further characterize the 30 kDa band, its N-terminal sequence was determined by automated Edman degradation, and its first seven amino acids perfectly matched the expected sequence of the cloned rDIII. Taken together, these results confirm the identity of the recombinant protein rDIII.

Cell surface binding of rDIII.

To determine whether rDIII binds to the cell surface, MDA-MB-231 breast carcinoma cells were incubated with rDIII, followed by excess anti-DIII antibody 2778 and fluorescently labeled anti-IgG secondary antibody. Flow cytometry showed dose-dependent staining of rDIII treated cells. At a 2 μM rDIII concentration, fluorescence was approximately 7-fold higher than the control, while at 4.5 μM it was 12-fold higher (Fig. 2A). In several experiments, detectable fluorescence required a concentration of at least 1 μM rDIII. Concentrations of up to 45 μM did not significantly increase the maximum fluorescence intensity above that seen at 4.5 μM, indicating that saturation of rDIII-cell surface interactions occurred close to 4.5 μM. Detection of rDIII with monoclonal anti-His-tag antibody gave similar results (not shown). A His-tagged protein, rDIII-V, was recombinantly expressed in baculovirus in a similar fashion to rDIII and was used in

control experiments. We could not detect any binding of this protein to MDA-MB-231 cell surfaces (not shown).

rDIII binding to Epidermal Growth Factor Receptor (EGFR).

To investigate binding to a specific cell surface receptor, rDIII was biotinylated and incubated with MDA-MB-231 cells in the presence of the cross-linker bis(sulfosuccinimidyl)-suberate (BS^3). Cells were then washed, detergent solubilized, and the lysates were allowed to react with streptavidin-coated beads. Since rDIII is EGF-like, binding to EGFR was tested. To this end, the material adsorbed to the streptavidin beads was separated by SDS-PAGE and identified by Western blotting using EGFR-specific antibody (Fig. 2B). A band with a MW consistent with that of EGFR (175 kDa) was detected (lane 3). As a control, parallel incubations were performed with biotinylated authentic mouse EGF, resulting in a band of the same MW (lane 2). In the absence of ligand, no band was detectable (lane 1). Blotting with anti-insulin β-receptor antibodies as an additional control was negative for samples containing streptavidin-precipitated material (Fig. 2B, lower panel, lanes 1-3), whereas total lysate revealed the presence of the insulin receptor (lane 4).

In a complementary experiment biotin-tagged EGF and rDIII were cross-linked as described above and EGFR was immunoprecipitated directly with a monoclonal antibody. Western blotting with horseradish-peroxidase (HRP) conjugated streptavidin (Fig. 2C) revealed a band at 175 kDa in the ligand-containing samples (lanes 2-4), but not in the

control sample (lane 1). Stripping the blot and re-probing with anti-EGFR antibody demonstrated the presence of EGFR in all four lanes, indicating that the biotin-tagged ligands had bound to EGFR.

EGFR autophosphorylation upon cell treatment with EGF and rDIII.

Western blots containing cross-linked material (Fig. 2C) were stripped and re-probed with anti-phosphotyrosine antibody (anti-p-Tyr, Fig. 3A). Significant EGFR phosphorylation was detected in samples containing EGF (lane 2) or rDIII (lanes 3 and 4), whereas control samples devoid of ligand failed to show EGFR phosphorylation (lane 1). Enhanced tyrosine phosphorylation of EGFR was observed when the concentration of rDIII was increased (lanes 3 and 4).

As additional controls, in parallel experiments, treatment with BS^3 cross-linker alone, in the absence of ligand (Fig. 3B, lane 2), as well as omission of both ligand and cross-linking agent BS^3 (lane 1) did not result in EGFR phosphorylation. Taken together, these data show that rDIII stimulates EGFR phosphorylation significantly over controls.

Competition receptor binding between rDIII and EGF.

To test specificity of rDIII binding to EGFR, MDA-MB-231 cells were incubated with rDIII in the presence of increasing concentrations of mouse EGF, and analyzed by flow cytometry. As shown in Fig. 4A, EGF gradually decreased the fluorescence signal of bound rDIII, in a dose-dependent fashion. This competitive displacement of rDIII by EGF

strongly supports the specificity of rDIII binding to EGFR indicated by cross-linking experiments (Fig.3).

Specificity of rDIII binding was further tested in a reverse setting, by competitively inhibiting binding of ^{125}I-EGF with cold rDIII. Initially, we determined the ^{125}I-EGF concentration where about 50 % specific binding to MDA-MB-231 was seen (Fig. 4B, upper panel, inset). For competition binding, MDA-MB-231 cells were incubated with ^{125}I-EGF at approximately half-maximal saturating concentrations ($\approx$ 0.5 nM $\approx$ 0.15 μCi) and increasing concentrations of either cold EGF (Fig. 4B, upper panel), or rDIII (lower panel). At a concentration of approximately 1.5 nM (3-fold excess), unlabeled EGF competed with ^{125}I-EGF for 50 % of the receptor binding sites (IC_{50}). Five nM unlabeled EGF (10-fold excess) competed successfully for 60 % of the binding sites on MDA-MB-231 cells over ^{125}I-EGF and rDIII exhibited about 28 % competition at about the same concentration. In the presence of a 100-fold excess of unlabeled EGF (50 nM), ^{125}I-EGF binding was reduced by about 95 %. An approximately 100-fold excess of rDIII over ^{125}I-EGF showed almost 40 % competition with ^{125}I-EGF binding, indicating that displacement of ^{125}I-EGF occurred in a dose-dependent fashion.

These results suggest that rDIII specifically interacts with the same receptor as EGF on MDA-MB-231 cells, implying that rDIII has a lower affinity than EGF for this receptor, which presumably is EGFR.

Induction of mitogen-activated protein kinase (MAPK) phosphorylation by rDIII.

Growth factor receptors like EGFR stimulate downstream signaling events leading to MAPK activity. Since our data indicated that rDIII binds to EGFR, we investigated phosphorylation of the MAPK extracellular signal-regulated kinase (ERK) in MCF-7 and MDA-MB-231 breast cancer cells incubated with rDIII, and using EGF as a positive control. Activation of the MAPKs ERK1/2 was detected by reactivity with antibodies specific for dually phosphorylated ERK1/ERK2.

In MCF-7 cells (Fig. 5A, upper panel), ERK1 (p44) and ERK2 (p42) were rapidly phosphorylated. ERK1/2 phosphorylation levels peaked at 5 min upon stimulation with rDIII, were maintained for 10 to 20 min post-stimulation, and returned to background level after 30 min. Stimulation of ERK1/2 by EGF had similar kinetics, with a pronounced peak in the phosphorylation level after 5 min (Fig. 5C). In control experiments, rDIII-V was tested for its ability to stimulate ERK1/2 (Fig. 5D). In contrast with rDIII (lane 3) and EGF (lane 4) no signal over background level (lane 1) could be observed for rDIII-V (lane 2). In MDA-MB-231 cells (Fig. 5B), phosphorylated forms of ERK1/2 were detected upon rDIII or EGF stimulation similar to the results of previous experiments with MCF-7 cells (Fig 5A), but with the following distinctive features. There was a more prominent phospho-ERK2 band (upper panel), consistent with a higher expression of total ERK2 compared to ERK1 (lower panel, loading control), and constitutive levels of phosphorylated ERK1/2 in non-stimulated MDA-MB-231 cells were higher than in MCF-7 cells, as reported[17].

In order to confirm our assumption that rDIII-induced ERK1/2 phosphorylation is dependent on EGFR, MCF-7 cells were incubated with both rDIII and either Tyrphostin

(AG1478), which selectively prevents EGFR phosphorylation by inhibiting EGFR tyrosine kinase activity, or antibodies (528), which block EGFR by competing for EGFR ligand binding sites. As shown in Fig. 5E, ERK phosphorylation above constitutive levels (lane 1) was induced by rDIII (lane 5), but was completely blocked by AG1478 (lane 6) as well as by EGFR blocking antibody 528 (lane 7). As a control, in the presence of the same inhibitors, EGF gave similar results (lanes 2-4).

These data strongly support the notion that rDIII induces ERK1/2 activation via binding to EGFR.

Stimulation of EGFR, but not ERK1/2 phosphorylation, by intact Ln-5.

Since the accessibility of DIII for cell surface receptor binding from within intact Ln-5 was not known, we tested whether incubation of cells with Ln-5 affects EGFR signaling. MDA-MB-231 cells were incubated with intact purified Ln-5 in suspension under conditions similar to hose used for rDIII, with the exception that BS^3 was omitted. After 10 minutes, EGFR phosphorylation was stimulated by EGF (Fig. 6, left panels) and to a lower extent by Ln-5. However, at 90 minutes post addition (Fig. 6, right panels) more enhanced receptor phosphorylation was observed with Ln-5 than with EGF.

However, Ln-5 did not stimulate MAPK phosphorylation at any time point investigated (5, 20, 30 and 90 min) in either MDA-MB-231 or MCF-7 cells and this result was independent of whether the cells were kept in suspension or were adherent (data not shown).

Thus, purified Ln-5 can stimulate phosphorylation of EGFR, but with slower kinetics than rDIII.

Gene expression changes by rDIII treatment in breast cancer cell lines.

In our study we demonstrated that rDIII readily stimulates ERK1/2. Activated ERKs are capable of phosphorylating downstream transcription factors that regulate gene expression[18]. We therefore investigated a possible effect of rDIII on gene expression using cDNA microarrays. Total RNA from MCF-7 cells cultured in the presence or absence of rDIII was isolated, amplified by *in vitro* transcription (IVT), and hybridized to a cDNA microarray ('metastasis chip'), which carries probes for members of the integrin and protein tyrosine kinase families, MMPs, MMP inhibitors and ECM molecules[19] (in press). Using spiking experiments with exogenously added control RNAs we established a 2.0 fold change as an adequate threshold for statistically significant induction or repression of gene expression. In two independent experiments (two chips) we found that MMP-2, MMP-9 and PI3Kinase gene expression are up-regulated upon rDIII treatment, whereas urokinase plasminogen activator (uPA) expression is down-regulated (Fig. 7A). Enhanced expression of MMP-2 by rDIII treatment was confirmed by quantitative RT-PCR (Fig.7B). Induction of MMP-2 gene expression can also be observed with EGF, but not with intact Ln-5.

These results are independent evidence that rDIII interacts with cell surface receptors and triggers downstream signaling, resulting in altered gene expression.

Stimulation of cell migration by rDIII via EGFR binding.

Having shown that rDIII binds to cell surface receptor EGFR and activates downstream MAPK, both of which are known to have motogenic properties[20,21], we then tested rDIII for its ability to affect cell motility. In Transwell migration assays with MDA-MB-231 cells on coated Ln-5, addition of rDIII to the upper or lower chamber resulted in an increase in the migratory activity by approximately 1.5-2 fold (Fig. 8A, panels a-c). Though the increase in migration was modest, it was highly reproducible in multiple independent experiments. More pronounced stimulation of migration on Ln-5 substrate was observed in MCF-7 cells (Fig. 8A, panels d-f), when rDIII was added to the upper chamber.

In order to determine whether enhancement of migration by rDIII depended upon binding to EGFR and activation of ERK1/2, Transwell assays were performed in the presence of the EGFR blocking antibody LA1 (5-10 μg), AG1478 (0.5-2 μM) or the ERK-kinase (MEK) specific inhibitor PD98059 (50 μM) (Fig. 8B). Neither of these compounds affected constitutive MDA-MB-231 cell migration on coated Ln-5 (Fig 8B, left panel). However, in their presence, no enhanced effect with rDIII could be observed (Fig 8B, right panel).

These results support the conclusion that rDIII may promote cell migration in breast cancer cells by engaging EGFR and downstream ERK1/2.

DISCUSSION

In this paper we investigated the molecular mechanisms that are responsible for stimulation of cancer cell migration upon MMP cleavage of Ln-5 ECM[10,11]. Our data indicate that these mechanisms revolve around an EGF-like domain III (DIII) of the Ln-5 γ2 subunit. This conclusion is based on the following evidence: 1.) The transmembrane proteinase MT1-MMP cleaves Ln-5 at two sites that flank the γ2 subunit domain III (DIII) thus excising a fragment that encompasses three EGF-like repeats[11], N. Koshikawa & V. Quaranta 2001, personal communication); 2.) Recombinant protein DIII (rDIII) binds EGFR specifically; 3.) rDIII activates downstream signaling, including EGFR and MAPK phosphorylation; 4.) rDIII stimulates biological effects, such as changes in gene expression and increased cell motility; 5.) Intact Ln-5 also promotes phosphorylation of EGFR.

These molecular interactions explain how MMPs may turn Ln-5 into a migratory substrate for cancer cells, and point to several elements of novelty. First, they show that the action of MMPs on ECM macromolecules can initiate precise sequences of molecular events, including ligand-receptor binding, signal transduction and changes in cell behavior. Second, they indicate that EGF-like ligands to receptor tyrosine kinases (RTK) may be solid-phase, i.e., contained within the structure of the ECM itself, or may be released as small diffusible factors. Third, they imply that laminins are multi-ligand macromolecules, exerting their diverse functions by interaction with distinct receptor families, such as RTKs, in addition to integrins[22] and dystroglycan[23].

Rather than a maturation step *per se*, proteolytic processing of the Ln-5 γ2-chain appears to be related to conveying cell behavioral cues in the context of tissues and physiological conditions. Meneguzzi and co-workers recently reported that processing within a conserved region of the γ2 short arm relates to incorporation of Ln-5 into the ECM, and may modify cell adhesion[12]. One enzyme that has been found to be involved in this processing is bone morphogenetic protein-1 (BMP-1)[24]. Cleavage of Ln-5 γ2-chain by MMP-2 and MT1-MMP however, is associated with cell motility, possibly by exposing a cryptic migratory signal[10,11]. Meneguzzi speculates that "BMP-1 governs proteolysis of Ln-5 in a physiologic context, whereas MT1-MMP cleaves Ln-5 and activates cell migration in pathologic circumstances". In this context it is interesting to note that γ2-chain and Ln-5 have been repeatedly associated with sites of tumor invasion (see introduction). Direct evidence that they deliver cell motility enhancing cues at these sites is technically difficult to obtain, but our results justify further experimentation along these lines.

rDIII was modeled after Ln-5 γ2 DIII and unequivocally binds the RTK EGFR, as supported by three independent lines of experimentation.

First, by flow cytometry, rDIII but not rDIII-V bound to the cell surface. The specificity of this interaction was suggested by the fact that binding of rDIII was saturable and dose-dependent, and that competitive displacement of rDIII by EGF occurred in a dose-dependent fashion as well.

Second, cross-linking of rDIII to the cell surface resulted in formation of a complex between rDIII and EGFR. Cross-linking is a well-known, reliable approach for the identification of receptor-ligand pairs, provided non-specific effects are ruled out and proper controls are performed. In our case, it is unlikely that the rDIII-EGFR complex was the result of non-specific interactions, because it was possible to isolate this complex both with reagents that recognize EGFR (a monoclonal antibody) and with streptavidin, which reacts with biotinylated-rDIII. Antibodies to unrelated receptors were instead negative, further supporting the specificity of the rDIII-EGFR complex.

Third, rDIII specifically displaced EGF bound to the cell surface. EGF is the best known ligand for EGFR and the prototypical member of the EGF-like family of growth factors, which also includes transforming growth factor alpha (TGF-α), heparin-binding-EGF-like growth factor (HB-EGF), betacellulin (BTC), amphiregulin (AR), and epiregulin (ER). Binding of rDIII to EGFR was weaker than that of authentic EGF itself as monitored in ^{125}I-EGF competition binding assays, but resembled kinetics observed for AR[25] and ER[26]. Another feature of EGF-like ligands is that they bind distinct heterodimers formed by pairing of various members of the EGFR family (i.e., ErbB-2, -3 and -4). Whether rDIII binds to such heterodimers remains to be investigated. Nonetheless, it is important to stress that, judging from displacement experiments (Fig. 4B), the affinity of rDIII binding to EGFR is lower than that of authentic EGF but similar to that of other known EGFR ligands triggering biological effects.

Two additional lines of evidence substantiate rDIII binding to EGFR: 1.) stimulation of EGFR, and 2.) ERK1/2 phosphorylation. EGFR becomes activated by a mechanism of autophosphorylation, which is triggered by ligand-induced dimerization[27]. As a consequence thereof, several protein kinase cascades are activated, including the well-characterized ERK (MAPK) pathway[28]. In our system, EGFR phosphorylation and ERK activation occur with kinetics that parallel those triggered by authentic EGF[29], further substantiating the specificity of rDIII interaction with EGFR.

The EGFR binding property of rDIII is corroborated by its structure, comprised of three EGF-like repeats, one of which respects the canonical fold of EGF, while the other two contain an extra loop and are referred to as laminin-type EGF repeats (LE repeats)[30].

Both EGF and LE repeats are highly conserved structures that are commonly found in growth factors and different classes of multi-domain proteins, in particular ECM proteins[31]. Whether they possess receptor binding activity is a critical issue that requires experimental testing in each case. Nevertheless, there are precedents for biological activities associated with LE domains found in laminins. For instance, LE repeats of laminin γ2- and γ3-chains stimulate neurite outgrowth[14]. Antibodies to DIII of Ln-5 γ2-chain inhibited migration of squamous cell carcinoma cells by approximately 50 %[13]. A proteolytic fragment of Ln-1, encompassing multiple LE domains, stimulated MMP synthesis[32], as well as epidermal cell growth[33], and induced EGFR phosphorylation and

S6 kinase activity[34]. Though such effects were absent in EGFR-deficient cells, EGFR competition binding between the laminin fragment and EGF could not be demonstrated[15]. Our observations are novel because we were able to connect the biological activities of rDIII to receptor binding and downstream signaling activation.

A mechanistic issue that remains to be addressed is whether or not DIII can bind EGFR in the context of intact γ2-chain, or whether DIII binding requires MMP cleavage, complete or partial. To begin addressing this issue, we tested whether intact Ln-5 stimulated signal transduction similar to rDIII, and found that EGFR, but not ERK phosphorylation is induced by intact Ln-5. These results suggest that intact Ln-5 is a ligand for EGFR, likely via the γ2 DIII domain, whereas induced downstream signaling seems to be different than that triggered by soluble diffusible DIII. Although originally thought to exert their functions exclusively as part of large multi-domain proteins, the list of EGF-like molecules, proteolytically liberated as small diffusible ligands, is increasing[14,35,36]. It would be interesting to determine whether MMPs can modify EGFR-mediated signaling in response to intact Ln-5.

Another intriguing aspect of EGFR activation by intact Ln-5 is its delayed kinetics. In our hands, maximum phosphorylation occurred at 90 minutes, i.e., slower than EGF or rDIII (5-10 minutes), and is reminiscent of the activation kinetics of the discoidin domain receptor (DDR) RTKs, which bind fibrillar collagens[37]. It is tempting to speculate that

RTKs interacting with ECM macromolecular structural ligands may display slow kinetics, and that these kinetics may be accelerated by the intervention of MMPs.

While Ln-5-EGFR interactions remain to be detailed, it is possible to speculate at this point that Ln-5 may be a multi-ligand structure for cell surface receptors. This possibility is well in line with recent findings that a surface proteoglycan, dystroglycan, is an important receptor for Ln-1 and Ln-2[23]. It has been proposed that dystroglycan may bind to other laminins, including Ln-5, because of its wide tissue distribution in cells contacting BMs. The best-established receptors for laminins are integrins, which are both adhesion and signaling receptors[22,38]. Recently, we showed that the integrin $\alpha 3\beta 1$ binds to the LG3 domain of Ln-5 $\alpha 3$ subunit[8]. Dystroglycan instead, binds to the LG4/5 domain of Ln-1/2 $\alpha 1/2$ subunit[39]. Thus, EGFR is the first surface receptor for a ligand site located on the laminin γ subunit.

In Ln-1, it has been shown that domain III of the $\gamma 1$ subunit is occupied by nidogen[16]. Whether $\gamma 1$ domain III may also bind surface receptors remains to be seen. On the other hand, despite the high degree of overall sequence homology between the laminin $\gamma 1$ and Ln-5 $\gamma 2$-chains, two residues that are critical for nidogen binding in $\gamma 1$ are absent from $\gamma 2$[40], indicating that both isoforms may have diverged in their functional properties. Importantly, $\gamma 2$ but not $\gamma 1$ expression is associated with cancer invasion and upregulated cell motility.

Since EGF binding to EGFR controls cell growth, we tested whether rDIII has a similar effect. However, our results were inconclusive and need to be investigated in more detail. On the other hand we were able to demonstrate that rDIII causes reproducible effects on cell motility and on gene expression. This is similar to the action of other EGF-like ligands. Cell migration and enhanced invasive potential have been repeatedly reported to coincide with induction and up-regulation of MMPs upon engagement of EGFR by EGF and other EGFR ligands[41-43].

Stimulation of migration by rDIII required the presence of Ln-5 substrate. This is not surprising, since migration on Ln-5 is known to occur via integrins[44,45] and integrin-binding sites are located on the α subunit of laminins[8].

Changes in gene expression were detected with a small-scale cDNA array, containing about 100 selected genes known to be cancer invasion-related. Of the genes altered in their expression level by rDIII, MMP-2, MMP-9 are particularly interesting because they have stood out in numerous other studies[2,4] and were found to be upregulated in this study. Moreover, the enhanced expression of the MMP-2 gene by rDIII is suggestive of positive feedback loops regulating tightly coordinated ECM degradation and associated migratory responses.

Both MMPs and ECM have been recognized as key players in cancer invasion and metastasis[1-5]. MMPs are a family of zinc-dependent endopeptidases whose substrates are mainly ECM macromolecules, such as collagen and laminins. Invading cancer cells secrete

increased levels of MMPs, albeit the majority of proteinases are made by stromal and inflammatory cells, especially in the proximity of invading cancer cells[4]. The mechanisms whereby MMPs may aid cancer invasion are of great interest, because they may lead to the discovery of invasion blocking compounds[3,5]. Several mechanisms for MMP action have been identified. In addition to their contribution in removing physical barriers, fracture of the ECM liberates and increases the bio-availability of soluble growth factors embedded within the ECM, as well as unveiling cryptic ECM epitopes[1-5]. It should be noted that these mechanisms are, in essence borrowed from normal cells, as exemplified by MMP-1 dependent keratinocyte migration across the dermal matrix in wound healing, MMP-2 enhancement of neurite extension in peripheral nerve section[2] and MMP driven remodeling in the mammary gland[46,47]. Angiostatin, a fragment of plasminogen released by MMP-12, endostatin[48] and restin, fragments of collagens XVIII and XV, respectively, are also excellent examples of MMP dependent mechanisms that alter endothelial cell apoptosis and proliferation, strongly suppressing neo-vascularisation[4,46].

In this study we were able to show that an ECM fragment interacts with a specific cell surface receptor, thereby transmitting new or modulated biological information. The molecular interactions we describe here are an important step forward in understanding the complex interplay between MMPs and the ECM, and although many details remain to be clarified, they promise to shed light on the mechanisms underlying cancer invasion.

MATERIALS AND METHODS

Cell lines and culture conditions

All cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and routinely propagated in Dulbecco's Modified Eagle Medium (DMEM) containing 10 % (v/v) fetal calf serum (FCS), 1x gentamycin-penicillin-streptomycin and 10 mM Hepes. Insect cells *Sf9* (*Spodoptera frugiperda*) and High Five™ were grown at 27 °C in the absence of CO_2 in EX-Cell™ 401, containing 5 % FCS and EX-Cell™ 400 (JRH Bioscience, Lenexa KS) respectively, and supplemented with 1 × gentamycin and 1 × Fungibac (Gemini Bio-Products, Inc, CA).

Construction and expression of rDIII in Baculovirus

DIII cDNA was amplified by PCR using a template, which spans the 3' 2900 bp of the Ln-5 γ2-chain gene. The forward primer (FPRIIIBHI, 5' to 3': CGC GGA TCC GAC GAG AAT CCT GAC ATT GAG) contained the *Bam*HI site and the reverse primer encoded both an *Eco*RI site and a C-terminal His_6-tag (RPRIIIHIS+ERI, 5' to3': CGG GAA TTC TCA GTG ATG ATG ATG ATG ATG CTG GTC CAT CTG AGT CTT CAC). The fragment obtained had the expected size of about 720 bp and was cloned directly into the appropriate sites (*Bam*HI/*Eco*RI), five amino acids downstream of the gp67 (acidic glycoprotein gp67) secretion signal sequence cleavage site, provided by the baculovirus transfer vector pACGP67-B (PharMingen, San Diego, CA). Recombinant rDIII/pACGP67-B was sequenced and together with BaculoGold® DNA (PharMingen, San Diego, CA) co-

transfected into *Sf*9 insect cells. After 3 rounds of virus amplification, high-titer recombinant Baculovirus stock solution was used to infect High Five™ insect cells. After growth at 27 °C for 3 days, supernatants were harvested and secreted recombinant protein III (rDIII) was purified using Ni-NTA agarose (Qiagen, Valencia, CA). The purity of the rDIII was judged by Coomassie blue staining.

Western blotting

Western Blots were performed according to the method described by Towbin *et al.*[49]. rDIII was routinely detected with monoclonal anti-His-tag antibody (Qiagen) or polyclonal anti-domain III Ln-5 γ2 antibody 2778 for 2 h at room temperature, washed (6 × 5 min) with PBST (phosphate buffered saline (PBS) containing 0.1 % (v/v) Tween 20) and incubated for additional 2 h with horseradish peroxidase (HRP) conjugated donkey anti-mouse or anti-rabbit secondary antibody (Amersham Pharmacia Biotech, Inc., Uppsala, Sweden). After washing with PBST (5 × 5 min) and a final wash with PBS (5 min), rDIII was visualized using enhanced chemiluminescence (ECL; NEN® Life Science Products, Boston, MA).

Flow Cytometry

Cells were trypsinized and washed twice in 1 × Hanks' Balanced salt Solution (HBSS), pH 7.2, containing 0.02 % (w/v) sodium azide and 0.1 % (w/v) bovine serum albumin (BSA). Approximately 0.5-1x10^6 cells were re-suspended in 50 µl HBSS containing 1 to 45 µM of

rDIII or rDIII-V. After incubation on ice for 1 h, cells were washed twice with HBSS. Bound rDIII was stained with anti-His-tag antibody (anti-His) or specific anti-domain III antibody (2778) for 1 h on ice. Cells were washed twice (HBSS) and then stained with fluorescently labeled anti-mouse or anti-rabbit secondary antibody (Alexa 488, goat anti-mouse IgG (H+L) $F(ab')_2$, Molecular Probes, Eugene, OR). Finally, cells were washed in PBS, re-suspended in 500 µl FACS Fix (PBS, 1 % (v/v) HCHO) and analyzed for their fluorescence intensity with a Becton Dickinson FACScan.

Immunoprecipitation and chemical cross-linking

EGF and rDIII were labeled with biotin (EZ-Link™ Sulfo-NHS-LC-Biotin) according to the manufacturer's instructions (Pierce, Rockford, IL). After incubation on ice for 2 h, biotinylated proteins were transferred into a microconcentrator (Microsep, PALL Gelman, Ann Arbor, MI) and overlaid with 10 × volume of PBS to remove unbound biotin. Ligand binding analyses were performed with $2x10^7$ MDA-MB-231 cells. Cells were washed 4 × with binding buffer (DMEM, 0.2 % (w/v) BSA, 1mM glutamine) and incubated on ice for 2 h with biotinylated ligands. As a negative control, all ligands were omitted. The chemical cross-linking reagent bis(sulfosuccinimidyl)-suberate (BS^3, Pierce) was added to 1 mM final concentration and after an additional 2 h incubation on ice, the reaction was quenched with 20 mM Tris, pH 7.5 for 15 min at room temperature. Cells were pelleted by centrifugation, washed with PBS, and subsequently detergent extracted using lysis-buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 % (v/v) Triton X-100, 1 mM EDTA, 1 mM

phenylmethylsulfonyl fluoride (PMSF), 1 μg ml^{-1} Aprotinin, 1 μg ml^{-1} Pepstatin, 1 μg ml^{-1} Leupeptin) for 45 min on ice. Lysates were cleared by centrifugation (15,500 × *g*, 4 °C, 15 min) and immunoprecipitated with monoclonal anti-EGFR antibody (clone EGFR.1, PharMingen, San Diego, CA) at 4 °C. After a further 2 h incubation with 50 μl of protein G sepharose beads (Boehringer Mannheim, Mannheim, Germany), immune complexes were washed 4 × with lysis buffer, harvested (2400 × *g*, 4 °C), and re-suspended in 30 μl of 4 × SDS-PAGE sample buffer. Samples were separated by SDS-PAGE (4-12 % gradient, NOVEX) under reducing conditions and analyzed by Western blotting. Bound ligands were detected with Streptavidin-HRP (PIERCE, Rockford, Ill) at a working dilution of 2 μg ml^{-1} and subsequently visualized using ECL.

Precipitation with magnetic beads was done as described above with the following modifications. MDA-MB-231 lysates were incubated with Dynabeads® M-280 Streptavidin-coated beads (Dynal, Oslo, Norway). The beads were collected using a magnetic device and washed 4 × with lysis buffer. Proteins were eluted by heating the beads with 4 × SDS-sample buffer (5 min, 95 °C) and the supernatant was subjected to SDS-PAGE and Western blotting. Detection was performed with a polyclonal anti-EGFR antibody (sco3, Santa Cruz Biotechnology) and HRP conjugated anti-rabbit secondary antibody at 1:300 and 1:10,000 dilutions, respectively.

EGFR autophosphorylation

Serum-starved MDA-MB-231 cells were detached with 10 mM EDTA/PBS, washed (3 x) and re-suspended in binding buffer. Cells (1x 10^7) were kept in suspension at 37 °C for 30 min, then EGF (10 ng ml^{-1} = 1.6 nM) or rDIII (4 μg ml^{-1} = 180 nM) was added and cells were incubated at 37 °C for 5 min. Negative controls (± BS^3) were included with the ligands omitted. Cross-linking was performed as previously described. Cells were lysed for 1 h on ice in lysis buffer to which 100 mM NaF and 1 mM Na_3VO_4 was added. Lysates were immunoprecipitated with monoclonal anti-EGFR antibody as described and detection was performed with monoclonal anti-phosphotyrosine antibody (PY-20, Transduction Laboratories, Lexington, KY) at a 1:2000 dilution, followed by HRP-conjugated goat anti-mouse IgG and subsequent ECL. To confirm equal loading amounts in each lane, blots were stripped (65 mM Tris pH 6.7, 2 % SDS, 100 mM β-mercaptoethanol, 55 °C, 45 min) and re-probed with polyclonal anti-EGFR (sc-03, Santa Cruz Biotechnology) or monoclonal antibody Ab-12 (Cocktail R19/48), (Neomarker Inc., Fremont, CA).

I^{125}-EGF displacement assay

MDA-MB-231 cells were trypsinized, washed 3 × with a buffer containing DMEM, 25 mM Hepes, 0.2 % (w/v) BSA, 0.3 mM 1,10 phenanthroline, 0.16 mM PMSF, and diluted to 2 × 10^6 ml^{-1}. 500 μl cells (1 × 10^6) were transferred into BSA-coated reaction tubes and an unlabeled competitor molecule (EGF or rDIII) added. I^{125}-EGF (100 μCi μg^{-1}, Amersham Pharmacia) was mixed with cells and incubated on ice for 3 h. To terminate ligand binding, triplicate 150 μl aliquots of the cell-label mixture were layered onto 300 μl of a mixture of two cold silicon oils (1013 kg cm^{-3} density), where the latter was prepared by mixing equal

volumes of AR-20 and Addid-200 oil (Wacker Chemie, Munich, Germany). Unbound radioactivity was removed by centrifugation for 1 min at 500 × *g*, followed by removal of the tube tip at the height of the oil layer. The portion of the tube containing the cell pellet was subjected to γ-counting (cpm).

MAP Kinase phosphorylation

Cells were grown to 60 % confluency and were serum-starved for 15 h. Fresh serum-free medium containing 250 nM AG1478 or 10 μg ml^{-1} anti-EGFR antibody 528 was added where indicated. Detached cells were held for 30 min at 37°C. rDIII (4 μg ml^{-1}), Ln-5 (20-400 ng ml^{-1}) or EGF (1 ng ml^{-1}) was then added and cells were incubated for the indicated times at 37°C. Cells were washed in PBS and lysed for 1 h on ice in 0.5 ml lysis buffer containing 1 tablet per 50 ml of complete protease inhibitor cocktail (Roche Diagnostics Corporation, Indianapolis, IN). Immunoprecipitation was performed with rabbit anti-ERK1/2 IgG (Santa Cruz Biotechnology, Santa Cruz, CA) and immune complexes were bound to protein G sepharose and subjected to SDS-PAGE as before. Each sample was divided in half and analyzed for total ERK1/2 content (rabbit anti-ERK1/2 IgG) or for phosphorylation (mAb to phosphorylated ERK1/2, New England Biolabs, Beverly, MA) by Western blotting as described.

Cell Migration Assay

Migration experiments were performed using Transwell Chambers (tissue culture treated, 8 μm pores, Corning Costar Corp., Cambridge, MA). The undersides of the polycarbonate membranes were coated with 0.25 μg ml^{-1} Ln-5 for MDA-MB-231 cells, and with 1 μg ml^{-1} for MCF-7 cells at 4 °C overnight. The filters were washed (2 × 300 μl PBST), blocked with 300 μl BlottoT (5 % (w/v) skim milk, PBST, 0.02 % (w/v) azide) for 2 h at room temperature, and finally washed again (2 × 300 μl PBST, 1 × PBS). Membranes were placed into the lower chamber containing 600 μl migration medium (serum-free DMEM). Cells were trypsinized, washed 3 × in migration medium and plated onto the filters (0.6-1 × 10^5 cells per well). In some cases, rDIII (0.02-1.5 μg ml^{-1}), EGF (1-100 ng ml^{-1}) or protein kinase inhibitors were added to the lower and/or upper chamber. Cells were allowed to migrate to the underside of the filter for 6 or 18 h at 37 °C, 5 % CO_2. Non-migratory cells remained on the uncoated, upper membrane surface and were removed with a cotton swab. Filters were fixed and stained using the HEMA3 Stain Set (Biochemical Sciences, Swedesboro, NJ). To quantitate cell migration, stained cells were counted in four to eight random fields of vision per well (× 20 or × 40 objective). Each assay was performed in duplicate and repeated at least three times.

RNA labeling and cDNA microarray hybridization

MCF-7 cells were grown to 60 % confluency, serum-starved for 12 h and kept for a further 24 h under serum-free conditions in the presence or absence of rDIII. Cells were lysed and total RNA was isolated using the RNeasy mini kit (Qiagen, Valencia, CA). 1 μg of total

RNA was amplified by *in vitro* transcription (IVT)[50]. To make labeled cDNA by reverse transcription, 5 μg amplified RNA was incubated in a cocktail containing 20 μg random octamers, Cy3 or Cy5-dCTP (Amersham, Piscataway, NJ), and SuperScript II RT (Life Technologies, Inc.) for 2 h at 37 °C. Fluorescent cDNAs were combined along with 10 μg human COT-1 DNA, 10 μg poly(A)-DNA, 3 μl 20 × SSC and 1.2 μl 2 % SDS in a final volume of 12 μl. After denaturation, labeled samples were hybridized to a cDNA microarray chip ('metastasis chip') for 10-16 hours at 65 °C in a humidified chamber. Before spin-drying, slides were washed for 5 min each in 2 × SSC, 0.2 % SDS, 2 × SSC, and finally in 0.2 × SSC. Fluorescent images were captured using a Scanarray5000 (GSI Lumonics, Watertown, MA).

Quantitative RT-PCR

Total RNA of MCF-7 cells was obtained as described for the cDNA microarray experiment. MMP-2 gene expression was assessed by RT-PCR using the One-step RT-PCR™ kit (Qiagen), total MCF-7 RNA (2 μg) and the primer pair (5'-TCAGATCCGTGGTGAGATCTT-3') and (5'-GCTCTTCAGACTTTGGTTCTC-3'). After 26 cycles (94 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min) the PCR reaction was finished with a final incubation at 72 °C for 10 min. As a control, GAPDH was amplified using the primers 5'-TGAAGGTCGGAGTCAACGGAT-3' and 5'-GTCATGAGTCCTTCCACGATA-3' in 22 cycles using the same profile as for MMP-2. MMP-2 (504 bp) and GAPDH (516 bp) cDNAs were resolved by agarose gel electrophoresis and visualized by ethidium bromide staining.

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Our apologies to those whose work was not cited due to space limitations.

FIGURE LEGEND

Figure 1. Characterization of Domain III (DIII) of Ln-5 γ2-chain.

(A) Schematic depiction of domain III, shown within the MMP cleavage boundaries of Ln-5. Recombinant domain III (rDIII) encompasses the most C-terminal 3.5 EGF-like repeats (γ2III5, 4, 3, C-terminal part of 2) of Ln-5 γ2-chain Domain III. Its N-terminus coincides with the MT1-MMP cleavage site, and its C-terminus was chosen to be 17 amino acids downstream of the indicated MMP-2 & MT1-MMP cleavage site. In addition, rDIII includes an N-terminal stretch of five amino acids (ADLGS) derived from the Baculovirus vector, and a C-terminal His_6-tag. Within the shown amino-acid sequence of rDIII, cysteines have been represented as filled circles in order to highlight the EGF-like domain signature, characterized by 6 cysteines (-x(4)-C1-x(0,48)-C2-x(3,12)-C3-x(1,70)-C4-x(1,6)-C5-x(2)-G-a-x(0,21)-G-x(2)-C6-x-), and the Laminin-type EGF (LE) repeat signature, characterized by 8 cysteines and an additional loop (-C4-x(1,2)-C5-x(5)-G-x(2)-C6-x(2)-C7-x(3,4)-[FYW]-x(3,15)-C8-). The position of rDIII within the Ln-5 cruciform structure and the products of MMP cleavage are shown below.

(B) Characterization of rDIII using SDS-PAGE and Western blotting. In SDS-PAGE (two left panels), rDIII resolved as a single band of about 23 and 30 kDa, under non-reducing and reducing conditions, respectively. Purified rDIII was judged to be better than 95 % homogenous by Coomassie blue staining. In Western blots of reducing SDS-PAGE (two right panels), the 30 kDa band of rDIII was recognized by both rDIII-specific antibody (2778) and anti-His-tag antibody (anti-His), as expected. A much fainter, higher molecular

weight band at around 50 kDa was also visible, which is likely dimerized rDIII. The apparent molecular weight was calculated based on the pre-stained molecular weight standard SeeBlue® (Invitrogen, Carlsbad, CA).

Figure 2. Binding of rDIII to EGFR.

(A) rDIII binding to cell surfaces as detected by flow cytometry. MDA-MB-231 cells were incubated with 4.5 μM rDIII (open black histogram), 2 μM rDIII (open gray histogram), or control rabbit IgG (filled histogram), and then with anti-rDIII polyclonal antibody 2778, followed by appropriate Alexa-conjugated secondary antibody. Cells were stained by rDIII in a dose-dependent manner.

(B) Recovery of biotin-rDIII-EGFR complexes with streptavidin-coated beads. Biotinylated rDIII or EGF was incubated with MDA-MB-231 cells, followed by cross-linking with BS^3. After detergent solubilization, cell lysates were precipitated with streptavidin-coated beads. Western blotting of adsorbed material with polyclonal anti-EGFR antibody (upper panel) detected a distinct band of 175 kDa in samples containing rDIII (lane 3) or EGF (lane 2), but not in control samples (no ligand, lane 1). To control for EGFR expression and specificity of cross-linking to EGFR, total MDA-MB-231 cell lysates were loaded in lane 4 and stripped blots were treated with anti-insulin receptor β antibody (lower panel), respectively.

(C) Immunoprecipitation of biotin-rDIII-EGFR complexes with antibodies to EGFR. Cells were treated with biotinylated rDIII or EGF and a cross-linker (BS^3), but detergent-

extracted cell lysates were immunoprecipitated with monoclonal anti-EGFR antibody instead. Western blots were detected with Streptavidin-HRP and ECL. A distinct band at 175 kDa was visible for samples containing EGF (0.65 μM, lane 2) or rDIII (0.9 μM, lane 3 and 1.6 μM, lane 4) (upper panel). There is no corresponding band in the control lane (lane 1). To assure equal loading in each lane, the filter was stripped and re-probed with polyclonal anti-EGFR antibody (lower panel).

Figure 3. Induction of EGFR tyrosine phosphorylation by rDIII.

(A) The Western blot shown in Fig.2C was stripped and re-probed with monoclonal anti-phosphotyrosine antibody (PY-20). Incubation with either EGF (lane 2) or rDIII (lane 3 and 4) stimulated phosphorylation of EGFR, compared to the control (lane 1).

(B) To exclude non-specific effects due to cross-linking, MDA-MB-231 cells were exposed to BS^3 in the absence of ligands (upper panel, lane 2). No increase in phosphorylation was detectable compared to the control (lane 1), while EGFR phosphorylation readily occurred when cells were stimulated with rDIII (lane 3). To assure equal amounts of EGFR protein, blots were stripped and re-probed with polyclonal anti-EGFR antibody (middle panel). Quantification of relative EGFR phosphorylation was performed with a STORM fluorimeter (lower panel).

Figure 4. Competitive binding of rDIII and EGF to MDA-MB-231 cells.

(A) Flow cytometry. MDA-MB-231 cells were incubated with purified rDIII (2.00 μM), in the presence of increasing concentrations of EGF (0.45, 0.85, 1.25, 2.00 μM). rDIII binding to the cell surface was detected with anti-His-tag antibody and fluorescently labeled goat anti-mouse IgG (H+L) $F(ab')_2$ antibody. Flow cytometry analyses revealed that the fluorescence intensity for rDIII gradually decreases with increasing EGF concentrations.
(B) Displacement of cell surface-bound I^{125}EGF by rDIII. MDA-MB-231 cells were incubated with I^{125}-EGF (0.5 nM) and increasing concentrations of cold EGF (upper panel) or rDIII (lower panel). The 0.5 nM (≈ 0.15 μCi) working concentration of I^{125}-EGF was determined by calculating the specific binding of I^{125}EGF ('specific') based on total and non-specific binding of I^{125}EGF to MDA-MB-231 cells (inset in upper panel). To this end, cells were incubated with increasing concentrations of I^{125}-EGF (0.5 nM) in the absence (total binding, 'total') or presence of an excess amount (330 nM) of unlabeled EGF (non-specific binding, 'non-specific').

Figure 5. Stimulation of ERK1/2 phosphorylation by rDIII.

Time course of ERK1/2 activation following exposure to rDIII. MCF-7 (A) or MDA-MB-231 (B) cells were treated with rDIII for the indicated time periods at 37°C, detergent lysed and immunoprecipitated with polyclonal anti-ERK1/2 antibody. The precipitated material was divided into two and analyzed for phosphorylation (upper panels) and total ERK1/2 content (middle panels) on separate gels. ERK phosphorylation intensities in relation to the total amount of loaded ERK1/2 were determined using a STORM 860 fluorimeter (lower

panels). Determinations were performed with two distinct purified preparations of rDIII protein (Prep. A, and Prep. B). Control cells were treated identically but the ligand was omitted.

ERK1/2 activation induced by EGF (C) but not by control protein rDIII-V (D). MCF-7 cells were stimulated with EGF (C) for up to 30 min and phosphorylated MAPK detected as described in the legend to (A). As compared to rDIII (D, lane 3) and EGF (lane 4) no phosphorylation signal above control level (lane 1) was seen with rDIII-V (lane 2).

(E) Dependency of ERK1/2 activation on EGFR. MCF-7 cells were stimulated for 5 min with either rDIII or EGF as in (A), except that either EGFR phosphorylation inhibitor AG1478 or EGFR blocking antibody 528 were also present. Both inhibitors prevent activation of ERK1/2 (upper panels) by rDIII or EGF. The identities of the additional upper bands in lane 4 (EGF + 528) and 7 (rDIII + 528) are unknown. The total amount of loaded ERK1/2 protein is shown in the lower panel.

Figure 6. Induction of EGFR tyrosine phosphorylation by purified intact Ln-5.

Treatment of MDA-MB-231 cells with EGF for 10 min at 37 °C results in significant phosphorylation of 175 kDa EGFR (left panels, lane 2) over control (no ligand, lane 1), whereas purified Ln-5 causes only weak EGFR phosphorylation (lane 3). However, induction of cells for 90 min at 37 °C (right panels) with Ln-5 (lane 3) show an EGFR phosphorylation signal that is still maintained well above control (lane 1). In contrast, incubation of cells for 90 min in the presence of EGF (lane 2) decreases the signal back towards background level (lane 1).

Figure 7. Effects of rDIII on the expression of genes that are related to migration.

(A) cDNA Microarray hybridization. MCF-7 cells were cultured in the presence or absence of rDIII. In two independent experiments cells were lysed, total RNA was isolated and amplified by IVT (*in vitro* transcription) as described in Materials & Methods. Amplified RNA prepared from MCF-7 cells cultured in the presence (cy3) or absence (cy5) of rDIII was co-hybridized to the 'metastasis-chip' containing 83 human cDNAs related to cancer cell metastasis (filled histograms). The open histograms represent RNA isolates where the cy3 and cy5 labels were switched. Ratios below 1.0 were inverted and multiplied by –1 to aid their interpretation. Gene expression signals that exceeded a 2-fold change threshold independently of the dye label orientation are shown. Results are expressed as mean ± standard deviation of six fluorescence signal ratios (PI3K = p110α catalytic subunit; uPA = Plasminogen activator, urokinase type).

(B) Quantitative RT-PCR. Total RNA was isolated from MCF-7 cells, which were cultured in the presence or absence of rDIII, Ln-5 or EGF. RT-PCR was carried out to assess changes in MMP-2 expression upon the influence of each of these ligands. As a control for normalization, GAPDH was amplified similarly to MMP-2. Amplified cDNAs of MMP-2 (504 bp) and GAPDH (516 bp) were resolved by agarose gel electrophoresis and visualized by ethidium bromide staining.

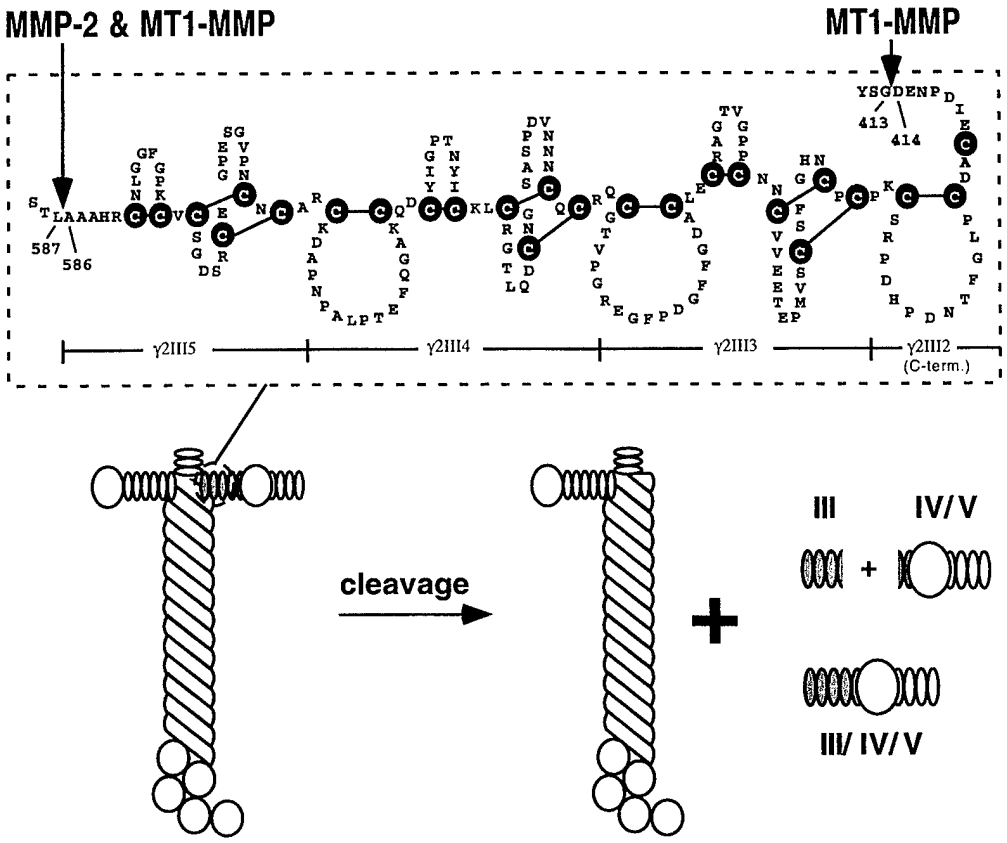
Figure 8. Breast cancer cell migration stimulated by rDIII.

(A) Micrographs of the lower surfaces of Transwell filters after migration of MDA-MB-231 (a-c) or MCF-7 (d-f) cells. rDIII was added to the lower (MDA-MB-231) or upper chamber (MCF-7) and cells were allowed to migrate on coated Ln-5 for 6 and 16 h respectively. Migrated cells were fixed, stained, and eight microscopic fields counted. The results are summarized in the corresponding bar graphs (a-f). All assays were done in duplicate and repeated at least 3 times.

(B) Migration of MDA-MB-231 cells on coated Ln-5 in the presence of rDIII (right panel) was inhibited by the EGFR phosphorylation inhibitors AG1478 or PD1530, as well as by anti-EGFR blocking antibody LA1 and by MAPK kinase inhibitor (MEK) PD98059. In contrast, neither of these inhibitors, nor the carrier DMSO has any effect on constitutive migration on Ln-5 (that is, in the absence of rDIII, left panel).

Figure 1

A


MMP-2 & MT1-MMP
MT1-MMP
587
586
413
414
γ2III5
γ2III4
γ2III3
γ2III2
(C-term.)
cleavage
III
IV/V
III/ IV/V

B

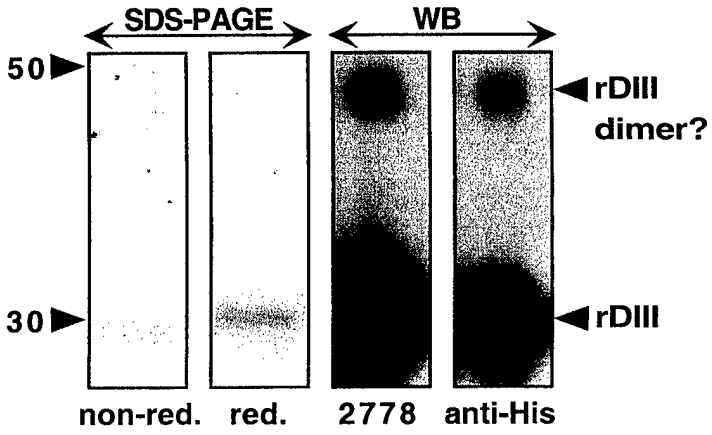
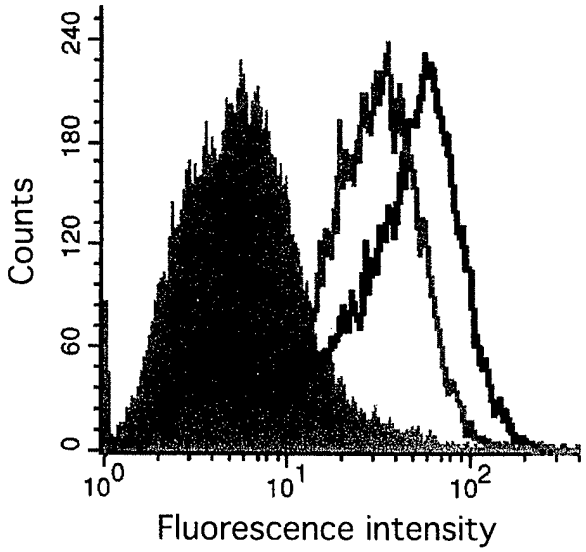
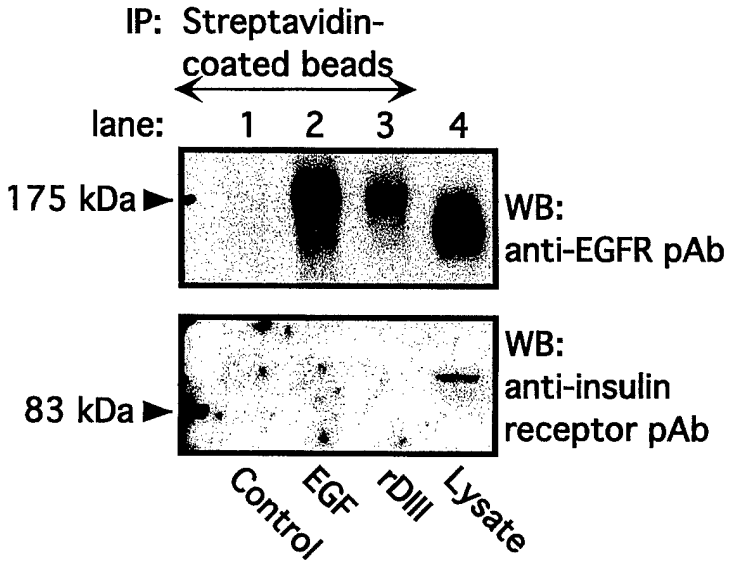

SDS-PAGE
WB
50
30
rDIII
dimer?
rDIII
non-red.
red.
2778
anti-His

Figure 2

A


Counts
0
60
120
180
240
10^0
10^1
10^2
Fluorescence intensity

B


IP: Streptavidin-coated beads
lane: 1 2 3 4
175 kDa
WB: anti-EGFR pAb
83 kDa
WB: anti-insulin receptor pAb
Control
EGF
rDIII
Lysate

C

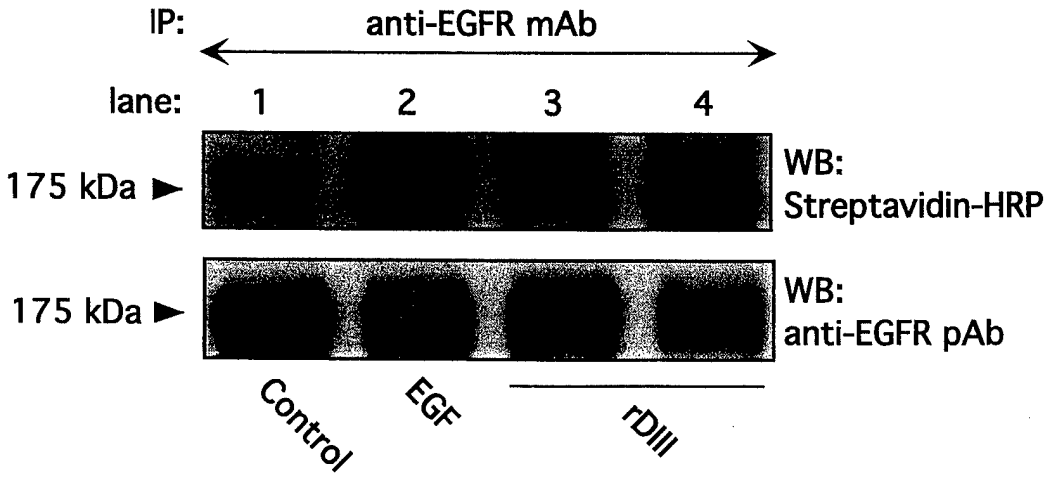

IP: anti-EGFR mAb
lane: 1 2 3 4
175 kDa
WB: Streptavidin-HRP
175 kDa
WB: anti-EGFR pAb
Control
EGF
rDIII

Figure 3

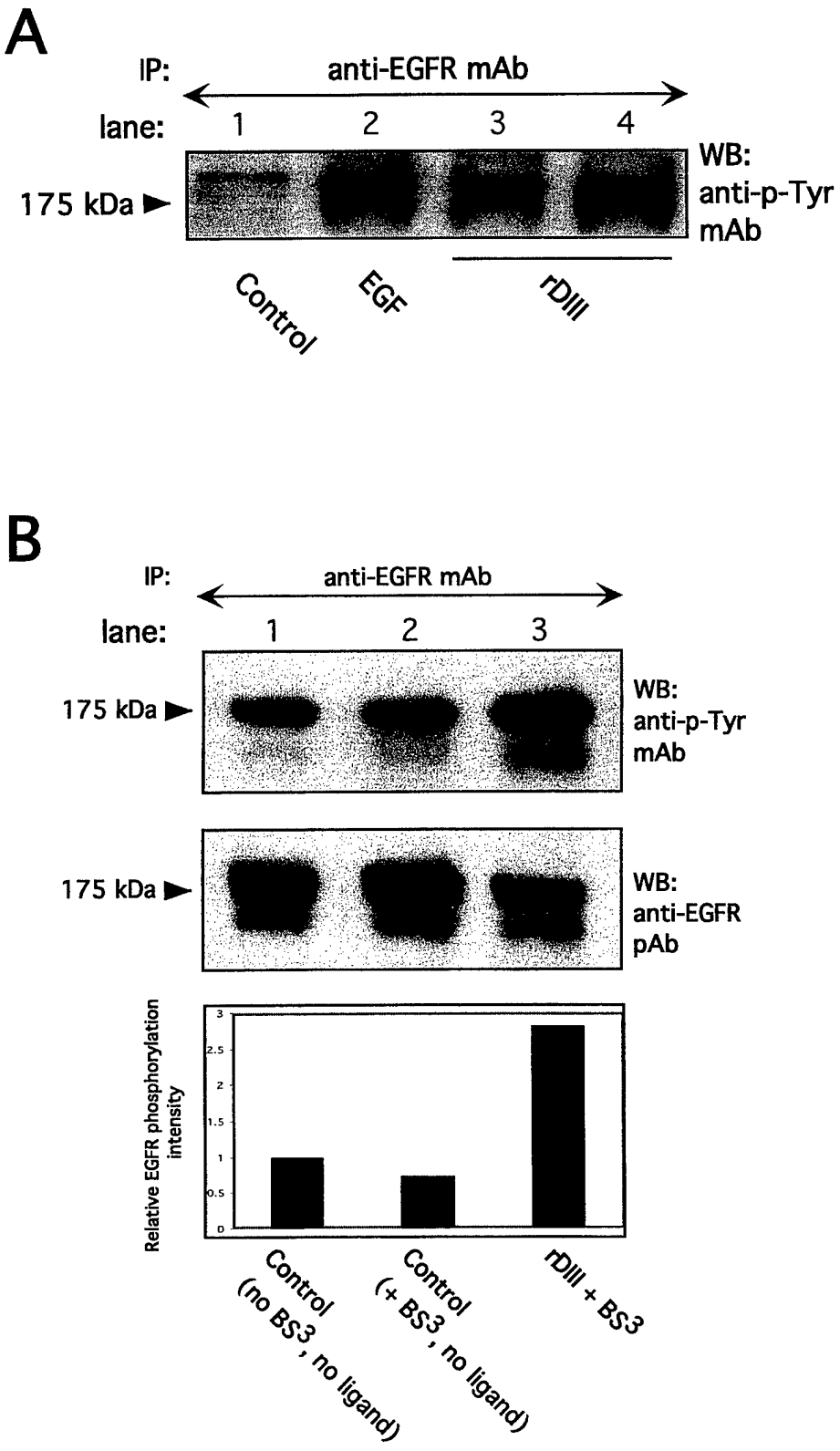
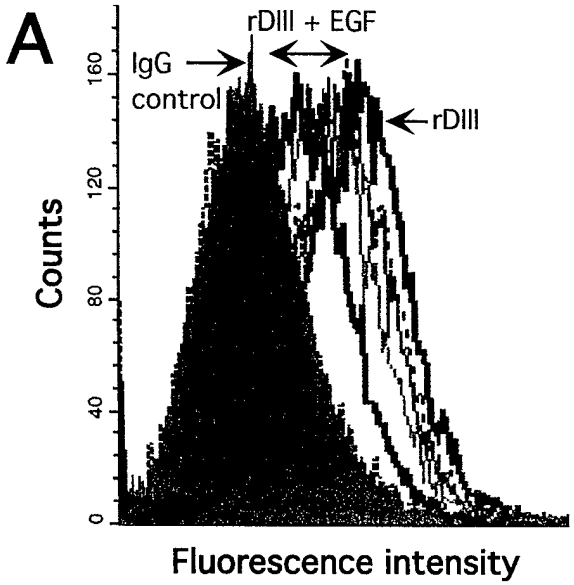
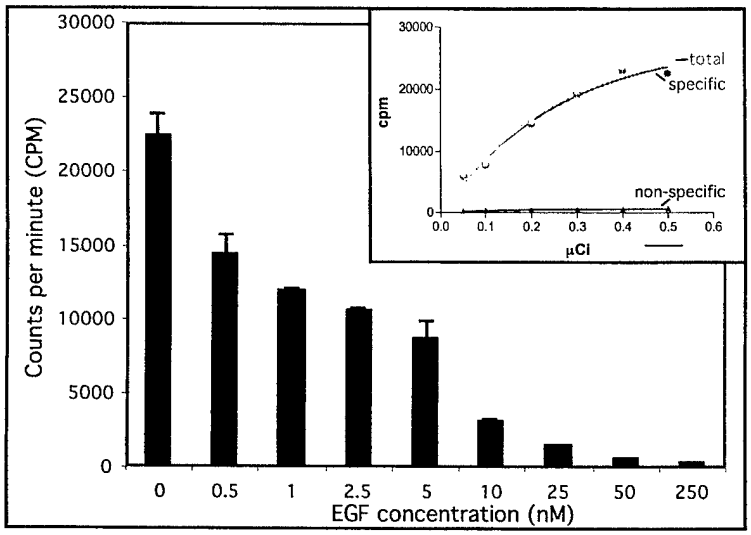

A
IP:
anti-EGFR mAb
lane:
1
2
3
4
175 kDa
WB:
anti-p-Tyr
mAb
Control
EGF
rDIII
B
IP:
anti-EGFR mAb
lane:
1
2
3
175 kDa
WB:
anti-p-Tyr
mAb
175 kDa
WB:
anti-EGFR
pAb
Relative EGFR phosphorylation intensity
3
2.5
2
1.5
1
0.5
0
Control (no BS3, no ligand)
Control (+ BS3, no ligand)
rDIII + BS3

Figure 4


A
rDIII + EGF
IgG
control
rDIII
Counts
0
40
80
120
160
Fluorescence intensity


B
Counts per minute (CPM)
30000
25000
20000
15000
10000
5000
0
0
0.5
1
2.5
5
10
25
50
250
EGF concentration (nM)
cpm
total
specific
non-specific
µCi

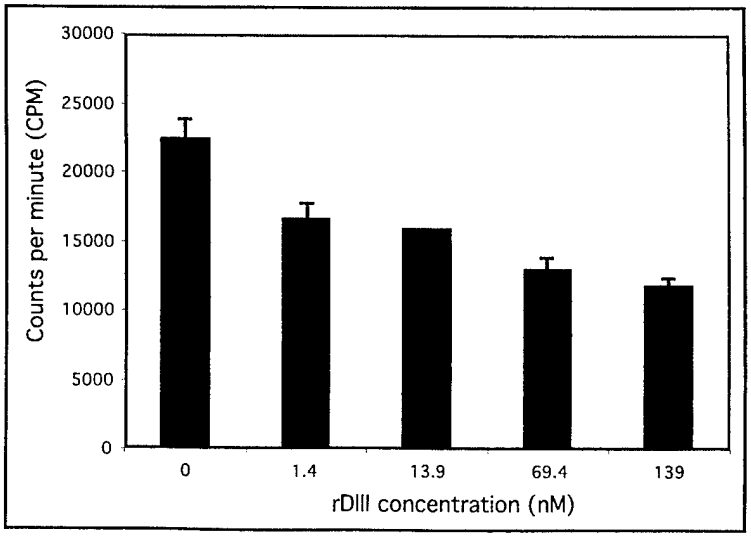

Counts per minute (CPM)
30000
25000
20000
15000
10000
5000
0
0
1.4
13.9
69.4
139
rDIII concentration (nM)

Figure 5

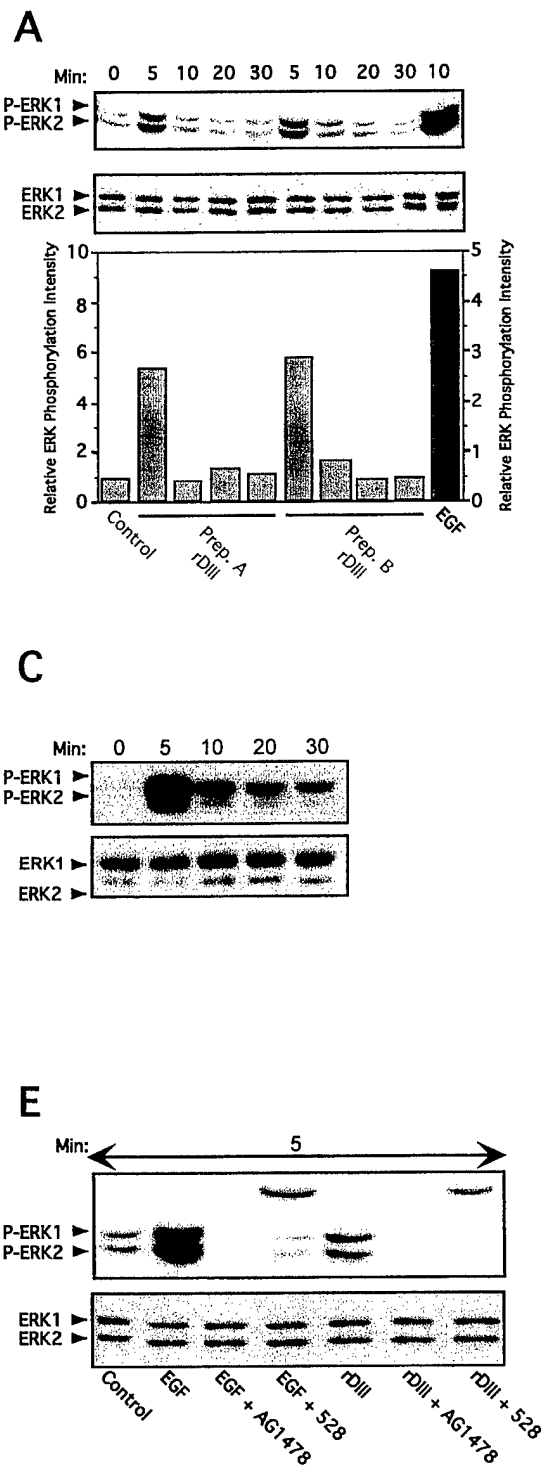

A
Min: 0 5 10 20 30 5 10 20 30 10
P-ERK1
P-ERK2
ERK1
ERK2
Relative ERK Phosphorylation Intensity
Control
Prep. A rDIII
Prep. B rDIII
EGF
B
Min: 0 5 5 10
P-ERK1
P-ERK2
ERK1
ERK2
Relative ERK Phosphorylation Intensity
Control
EGF
rDIII
C
Min: 0 5 10 20 30
P-ERK1
P-ERK2
ERK1
ERK2
D
Min: 5
P-ERK1
P-ERK2
ERK1
ERK2
Relative ERK Phosphorylation Intensity
Control
rDIII-V
rDIII
EGF
E
Min: 5
P-ERK1
P-ERK2
ERK1
ERK2
Control
EGF
EGF + AG1478
EGF + 528
rDIII
rDIII + AG1478
rDIII + 528

Figure 6

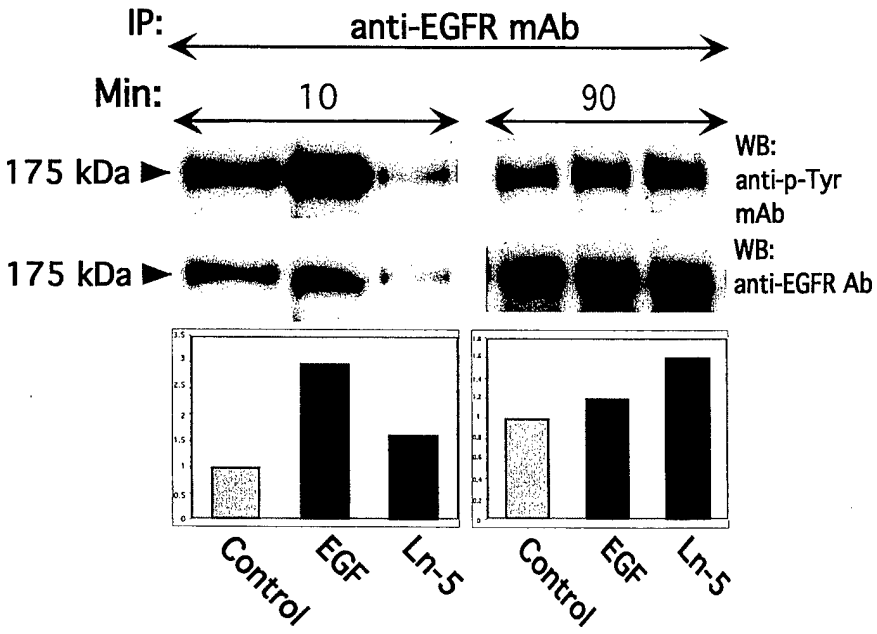
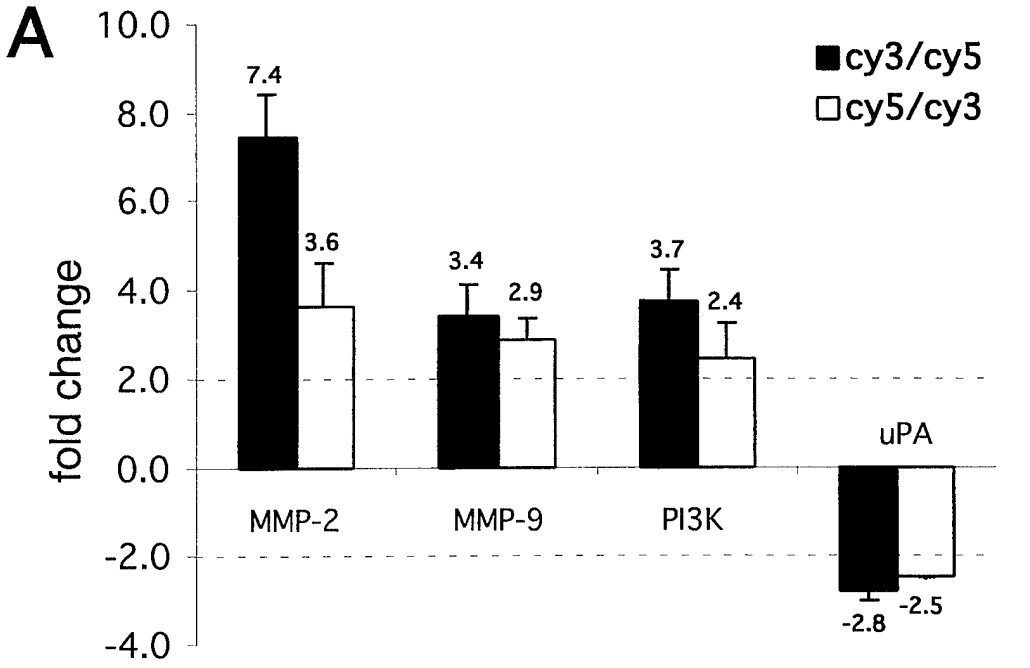

IP:
anti-EGFR mAb
Min:
10
90
175 kDa
WB:
anti-p-Tyr
mAb
175 kDa
WB:
anti-EGFR Ab
Control
EGF
Ln-5
Control
EGF
Ln-5

Figure 7


A
10.0
8.0
6.0
4.0
2.0
0.0
-2.0
-4.0
fold change
cy3/cy5
cy5/cy3
7.4
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-2.5
MMP-2
MMP-9
PI3K
uPA

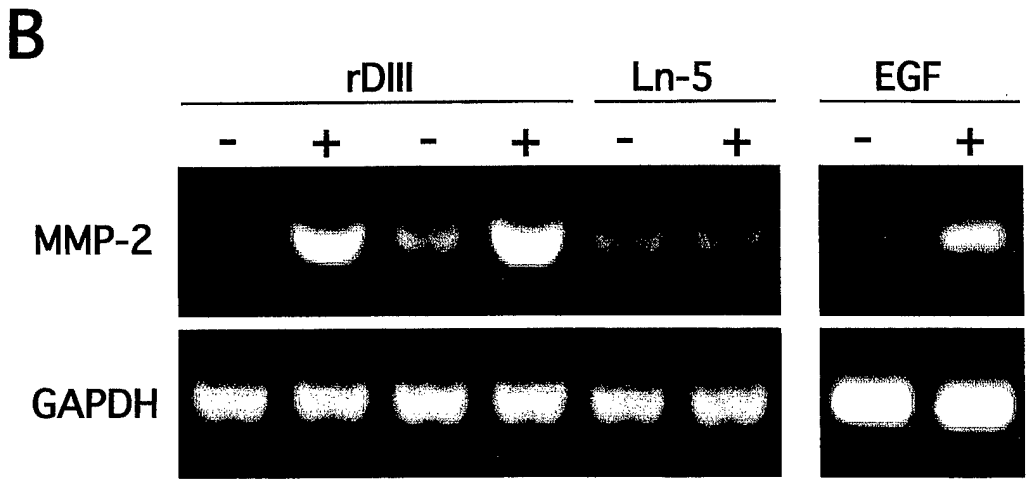
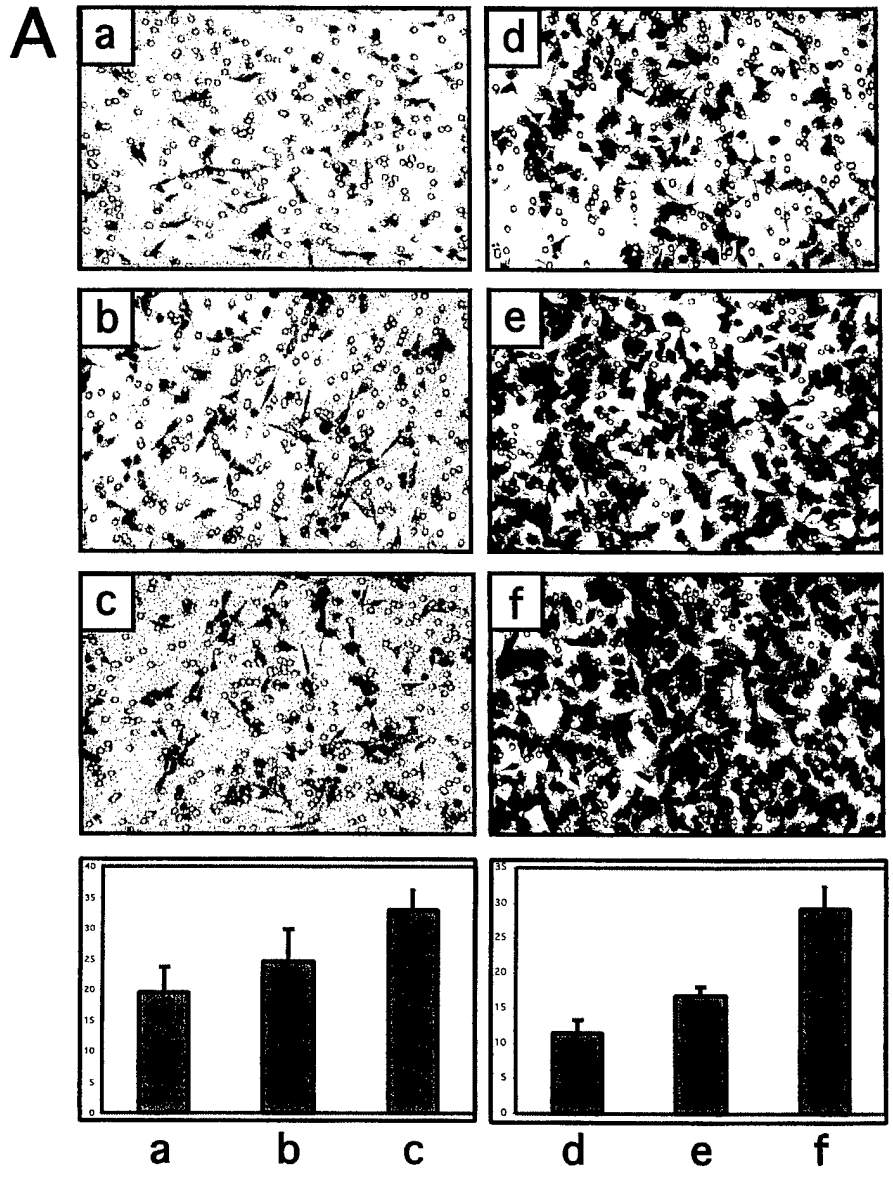
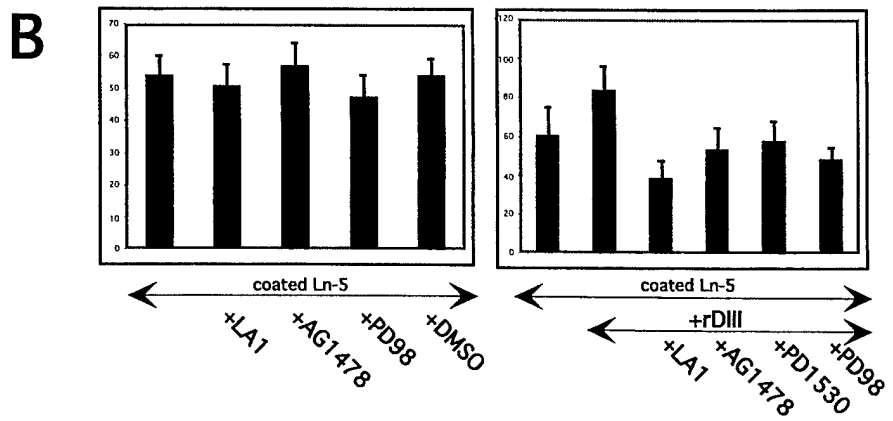

B
rDIII
Ln-5
EGF
- + - + - + - +
MMP-2
GAPDH

Figure 8


A
a
d
b
e
c
f
a
b
c
d
e
f


B
coated Ln-5
+LA1
+AG1478
+PD98
+DMSO
coated Ln-5
+rDIII
+LA1
+AG1478
+PD1530
+PD98

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 γ2 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− cell 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)[1] of the basement membranes (BMs) 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;

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[1]*Abbreviations used in this paper:* AS, antisense oligonucleotide; BM, basement membrane; CM, conditioned medium; ECM, extracellular matrix; HLD, hemopexin-like domain; Ln-5, laminin-5; MMP, matrix metalloprotease; MT1-MMP, membrane type 1-MMP; TIMP, tissue inhibitor of metalloprotease.

Ryan et al., 1994). The interaction of Ln-5 with $\alpha 6\beta 4$ 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 $\gamma 2$ 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, which can both directly cleave Ln-5 and activate MMP2, if present, for further cleavage and migration. These data indicate that the MMP cleavage mechanism for induction of migration over Ln-5 may be of general importance in epithelial cells contacting the BM. Furthermore, because MT1-MMP is cell surface anchored, they provide a mechanism for spatially defined formation of promigratory cues along the BM.

Materials and Methods

Cell Lines and Culture Conditions

BRL is a nontumorigenic liver epithelial cell line from Buffalo rat (Nissley et al., 1977). MCF-10A is a spontaneously immortalized human mammary epithelium cell line, and 804G is a rat bladder carcinoma. Types of human cancer cell lines are as follows: MDA-MB-231, breast carcinoma; DLD-1 and HT-29, colon carcinoma; HLE and HLF, hepatoma; and HT-1080, fibrosarcoma. BRL, MCF-10A, MDA-MB-231, DLD-1, HT29, and HT-1080 cell lines were obtained from American Type Culture Collection. HLE and HLF were obtained from the Japanese Cancer Resources Bank. The 804G cell line was described previously (Falk-Marzillier et al., 1998). Each cell line, except for MCF-10A, was maintained in DME (GIBCO BRL) plus 10% (vol/vol) FCS (Irvine Scientific), penicillin, and streptomycin. MCF-10A was maintained in DFC-1 plus 1% (vol/vol) FCS as described previously (Plopper et al., 1998).

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

Serum-free DME 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 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 reported (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 TNB 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 in Eppendorf tubes. The cell suspension was homogenized at 4°C, nuclei removed 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 antigen was detected by the enhanced chemiluminescence method with a NEN Life Science Products kit.

Preparation of MMP2 and MMP Inhibitors

Human TIMP-2-free MMP2 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 MMP2 treated with neutrophil elastase, by Reactive-Red agarose affinity chromatography as described before (Strongin et al., 1993; Rice and Banda, 1995). BB94 (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 Ile^{114}-Glu^{528} was amplified by a PCR using 5′- and 3′-primers with addi-

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

Cleavage of Ln-5 by MMP2 or MT1-MMP

5 μg of purified Ln-5 was incubated with 3.2 μg/ml 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 $CaCl_2$. In some cases, purified Ln-5 (1 μg) was adsorbed onto a 96-well plate well, and then incubated with recombinant MT1-MMP (0.2–2 μg/ml) for 16 h at 37°C in 50 mM Tris, pH 7.5, 0.005% Brij-35, 10 mM $CaCl_2$. After 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 γ2 chain antibody (1963).

Cell Scattering Assay

BRL cells were placed into 24-well plates containing 0.5 ml of DME plus 1% FCS at 7,000 cells per well. Purified Ln-5 (80 ng/ml) was added. After 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 DME plus 0.1% (wt/vol) BSA and seeded at 20,000 cells/well for BRL, MDA-MB-231, DLD-1, and HT-29; 10,000 cells for HLF; and 5,000 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. After 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 a computer program (Advanced Gene Computing Technologies). The following phosphorothioate oligodeoxyribonucleotides were synthesized: AS oligonucleotide, 5′-TCGTGTCCGAATCGG-3′; control oligonucleotide, 5′-TCCGAGTTCTCGAGG-3′ for rat MT1-MMP, or AS oligonucleotide, 5′-GCCGTAAAACTTCTG-3′; and control oligonucleotide, 5′-ATCTCGGATCAGACT-3′ for human MT1-MMP. These oligonucleotides were freshly dissolved in PBS and added to BRL or HT-29 cells, respectively, at 10 μM. After 2 d of pretreatment with test or control oligonucleotides, cells were tested in migration assays. Oligonucleotides were added to the lower chamber of Transwells at 10 μM.

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 generated by formaldehyde fixation. Nonspecific binding was blocked by incubation in PBS containing 2% donkey serum (DS) (Jackson ImmunoResearch Laboratories, Inc.) and 1% BSA (Sigma Chemical Co.) for 1 h at room temperature. After extensive washes in PBS-DS (0.2% donkey serum, 0.1% BSA, 5 mM glycine), sections were incubated for 1 h at room temperature with a mixture of primary antibodies (5 μg/ml), or a mixture of normal mouse and rabbit IgGs as a control, followed by washing in PBS-DS, and incubation for 1 h at room temperature with a cocktail of $F(ab')_2$ secondary antibodies (Jackson ImmunoResearch Laboratories, Inc.): FITC-conjugated affinity-purified donkey anti-rabbit IgGs (5 μg/ml) (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 μg/ml) (preadsorbed on serum proteins from species above). After washing in PBS-DS, sections were mounted in slow-fade medium (Molecular Probes), and viewed on a Zeiss Axiovert 35M 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 by 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 Adobe Photoshop 4.0 by attributing the green and red color to either FITC- or Cy5-specific fluorophore spectra depending on experimental conditions. Images were printed with a Fujix Pictrography 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

mAbs against TIMP-2 (D52) (Shofuda et al., 1998), human MMP2 (2-22) (Kawano et al., 1997), human Ln-5 γ2 chain (D4B5) (Mizushima et al., 1998), and rat Ln-5 (MIG-1 and TR-1) (Plopper et al., 1996), and polyclonal antibody against rat Ln-5 γ2 chain (1963) were generated in our laboratories. Transwells were purchased from Corning-Costar; polyclonal antibody against human MT1-MMP (AB815) 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. 2 A). 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, MIG-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 exogenously activated MMP2 to cleave and migrate on Ln-5 (Giannelli et al., 1997). Gelatin zymography of condi-

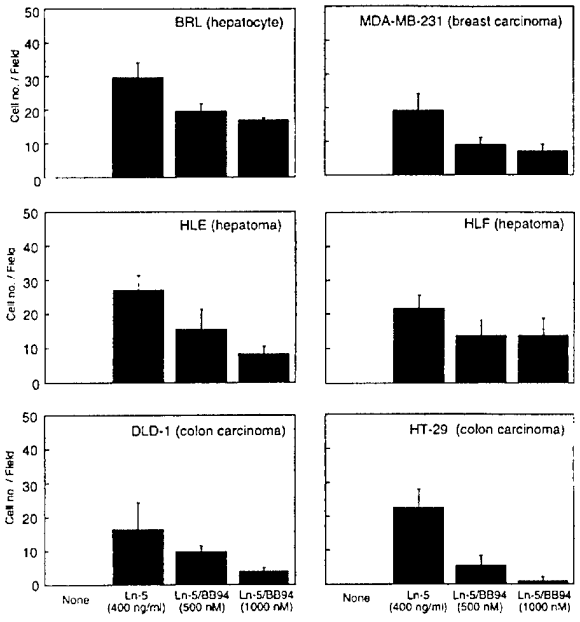

Figure 1. Effects of the hydroxamate MMP inhibitor BB94 on constitutive cell migration over Ln-5. Indicated cell lines were incubated under optimized conditions (see Materials and Methods for experimental details) in the upper compartment of Transwell chambers, which contained purified Ln-5 (200 or 400 ng/ml) in the lower compartment. Constitutive migration occurred in all cases. Likewise, BB94 blocked migration in a dose-dependent fashion. Each bar represents the mean + SD of three wells. The same results were obtained by coating the lower surface of Transwell filters with Ln-5, rather than adding soluble Ln-5 to the lower chamber.

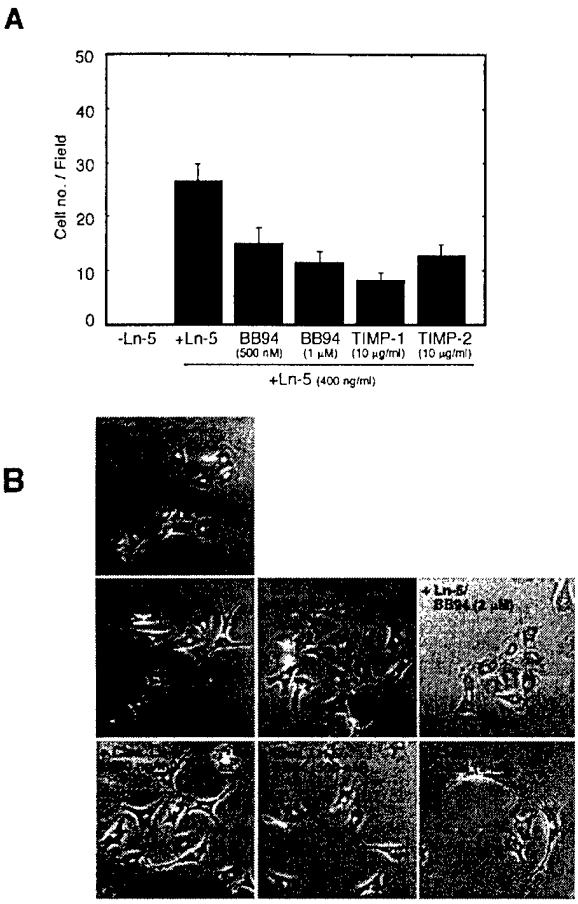

Figure 2. (A) Effect of TIMPs on BRL migration over Ln-5. BRL cells were incubated in Transwell chambers, in control medium or in the presence of Ln-5 (200 ng/ml) and the indicated concentrations of MMP inhibitors in the lower chamber. In the presence of Ln-5, BRL cells migrate constitutively. Both TIMP-1 and TIMP-2 inhibit migration, to levels comparable to BB94. Each bar represents the mean + SD for cell migration of two wells. (B) Lack of inhibition by BB94 of scattering induced by MMP2-cleaved Ln-5. BRL cells were incubated in 24-well culture plates in control medium (top), Ln-5 (80 ng/ml) (middle) or MMP2-cleaved Ln-5 (80 ng/ml) (bottom), in the presence of BB94, 500 nM or 2 μM. Cell morphology was examined under a phase-contrast microscope after incubation for 16 h. Scattering occurs with both intact Ln-5 and cleaved Ln-5. However, BB94 inhibits scattering induced by Ln-5, not by cleaved Ln-5, further supporting involvement of MMPs in the constitutive scattering of BRL cells.

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 cells. 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). (Note that, in addition, a 43-kD self-processed form of 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, was

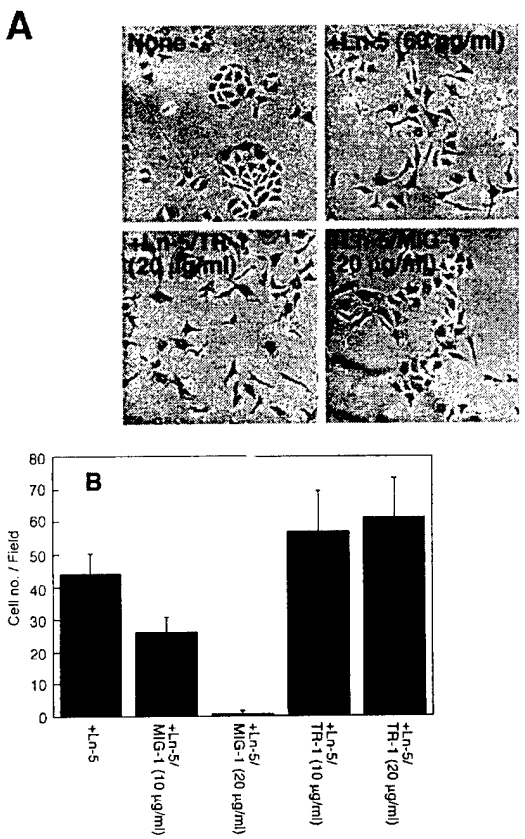

Figure 3. Inhibitory effects of mAb MIG-1 on Ln-5 BRL cell scattering and migration. (A) BRL cells were incubated in control medium (None) or Ln-5 (60 ng/ml) in the presence of mAbs to Ln5 TR-1 (control) or MIG-1 (20 μg/ml), which blocks migration induced by MMP2-cleaved Ln-5. Cell morphology was examined under a phase-contrast microscope after incubation for 16 h. MIG-1, not TR-1, inhibits scattering induced by Ln-5. (B) Transwell chamber migration of BRL cells, stimulated by Ln-5 (200 ng/ml), is blocked by MIG-1, not TR-1 control, in a dose-dependent fashion. Each bar represents the mean + SD of three wells.

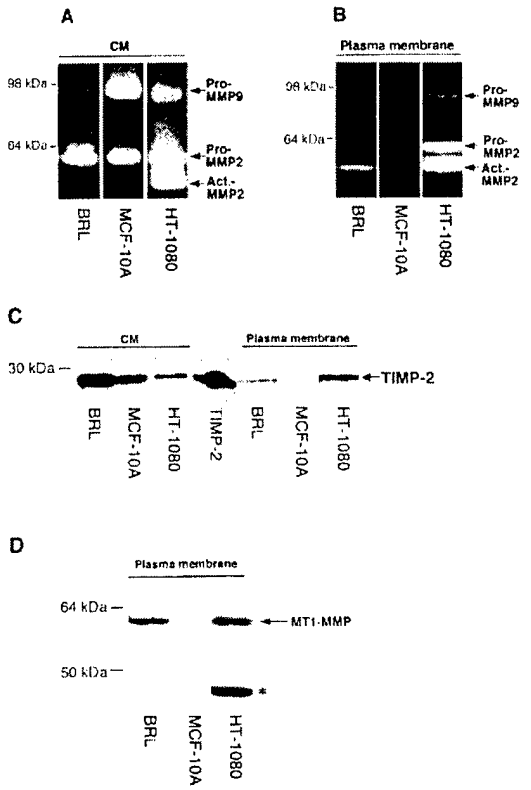

Figure 4. (A and B) Gelatin zymography of (A) conditioned media (CM) (60 μl/lane) or (B) crude plasma membrane fractions (2 μg/lane) from BRL, MCF-10A, and HT-1080 cells. The positions of pro-MMP9 (92 kD), pro-MMP2 (72 kD), and activated MMP2 (62 kD) are indicated. Note the presence of activated MMP2 in BRL, but not MCF-10A plasma membrane fractions. (C and D) Western blotting of BRL, MCF-10A, or HT-1080 conditioned media (CM) (300 μl/each lane) or crude plasma membrane fractions (2 μg/each lane) with antibodies to TIMP-2 (C) or MT1-MMP (D). In C, purified human TIMP-2 (50 ng) was run in the indicated lane. The respective positions of TIMP-2 and MT1-MMP are indicated to the right of gels. (asterisk) Processed form of MT1-MMP (see text).

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. 5 A, 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 where 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. 6 A); and (2) by expression of MT1-MMP alone, with no MMP2 secretion, since MT1-MMP can directly cleave Ln-5 and presumably cause mi-

Table I. Expression of MMPs and TIMP-2 in Plasma Membrane of Normal and Cancer Cells

	BRL	MCF-10A	MDA-MB-231	HLE	HLF	DLD-1	HT-29	HT-1080
MT1-MMP	++	±	++	++	+	+	+	+++
Pro-MMP2	++	−	−	+	+	−	−	+
Active MMP2	+	−	−	±	±	−	−	+++
TIMP-2	+	−	NT	+	+	NT	NT	++

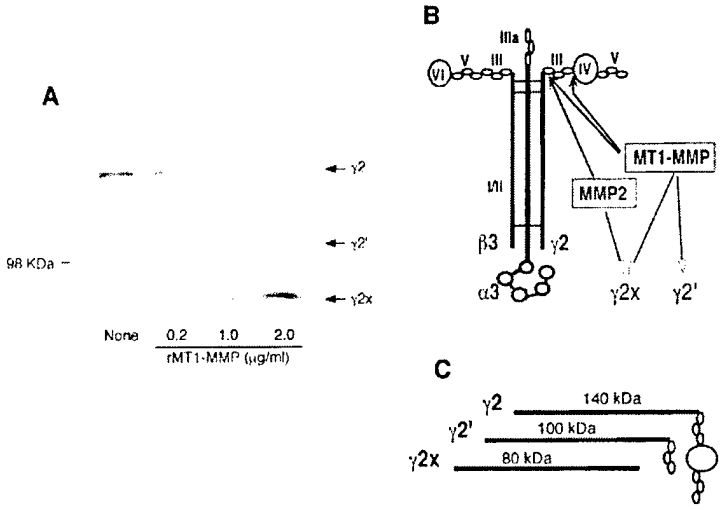

Figure 5. Cleavage of Ln-5 by MT1-MMP. (A) Ln-5 (1 μg) was treated with recombinant soluble MT1-MMP at the indicated concentrations, electrophoresed by 6% SDS-PAGE under reducing conditions, and then analyzed by Western blotting with a polyclonal antibody (1963) against rat Ln-5 γ2 chain. The positions of Ln-5 γ2 (140 kD), γ2′ (100 kD), and γ2x (80 kD) chains are indicated. In the presence of increasing concentrations of MT1-MMP, the γ2 appears to chase into the γ2′ and the γ2x chain. (B) Schematic depiction of the position of the MMP2 cleavage site (Giannelli et al., 1997) and the predicted position of the MT1-MMP sites. The γ2′ has been described (Vailly et al., 1994). (C) Scheme of the predicted size of MMP2 and MT1-MMP cleavage products of the Ln-5 γ2 chain.

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.

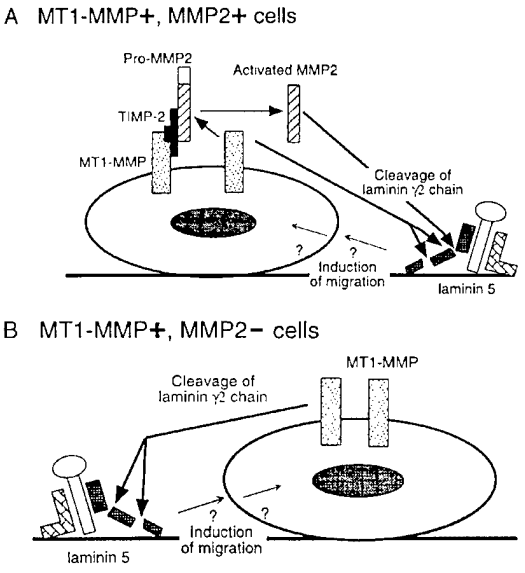

Figure 6. Schematic model for mechanisms of MMP-dependent Ln-5 cell migration.

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

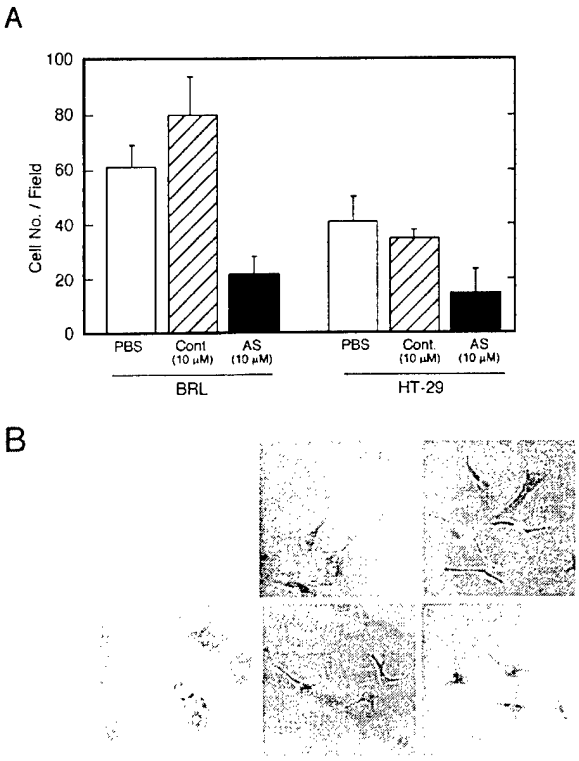

Figure 7. Effect of MT1-MMP antisense oligonucleotides on Ln-5 migration by BRL or HT-29 cells. (A) Cells were pretreated for 2 d in culture with MT1-MMP antisense (AS) or control, scrambled (Cont.) oligonucleotides, and tested in migration assays. Ln-5 and oligonucleotides, at the same concentrations as treatment, or PBS were present throughout the assay in the lower Transwell chambers. Each bar represents the mean + SD for cell migration of two (BRL) or four (HT-29) wells. (B) BRL cells were pretreated with MT1-MMP AS or control oligonucleotides, and tested in the scattering assay as in Fig. 2, in the presence of either intact or MMP2-cleaved Ln-5. Addition of either intact or MMP2-cleaved Ln-5 causes a morphological change in BRL cells, which loosen cell-cell contacts, elongate, and scatter. MT1-MMP AS treatment inhibits these morphological changes when they are induced by intact Ln-5. In contrast, cleaved Ln-5 still causes scattering of AS-treated cells, supporting a link between reduction of MT1-MMP expression, Ln-5 cleavage and scattering response.

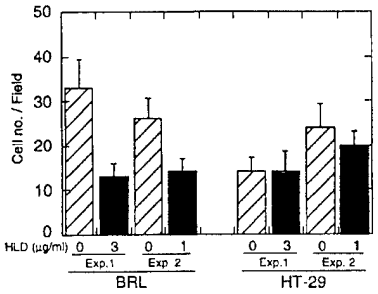

Figure 8. Effect of MMP2 HLD on Ln-5 migration by BRL or HT-29 cell. Purified HLD was added to the lower Transwell chambers at 0, 1, or 3 μg/ml. Results shown are from two separate, representative experiments (1 and 2) performed on different occasions. Each bar represents the mean + SD for cell migration of four wells.

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+, MMP2+ cells, and nondetectable in MT1-MMP+, MMP2− 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 NH_2-terminal amino acids (γ2′). 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).

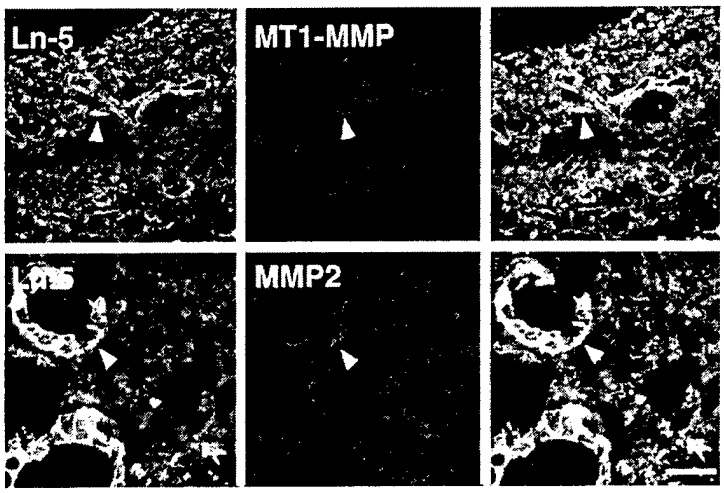

Figure 9. Detection of Ln-5, MT1-MMP, and MMP2 in human colon carcinoma tissue specimens by double immunofluorescence and confocal microscopy. Cryostat sections were double immunostained for either Ln-5 and MT1-MMP or Ln-5 and MMP2. Expression of Ln-5 is discrete, in some cases limited to the outside layer of cell nests. Colocalization with MT1-MMP and MMP2 is shown in the panels on the right (yellow) by combining the specific fluorophore spectra recorded for Ln-5 (green) and MT1-MMP or MMP2 (red). Examples of colocalization are indicated by arrowheads. A slight difference in the appearance of Ln-5–specific immunoreactivity in top and middle panels is due to the use of either mouse mAb D4B5 (combination with rabbit anti–MT1-MMP) or rabbit polyclonal 1963 (combination with mouse monoclonal to MMP2). Control sections incubated with normal mouse IgGs and rabbit IgGs were completely negative (not shown). Bar size, 43 μm.

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 ($\alpha3\beta1$ or $\alpha6\beta4$). In contrast, epithelial cells do not express receptors for collagen IV (integrin $\alpha1\beta1$) 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).

All 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 $\alpha3$ 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 $\alpha v\beta3$ modulate glioma cell migration on ECM. MMP2 also modulates melanoma cell attachment and facilitates migration and invasion (Ray and Stetler-Stevenson, 1995). Ln-5 also has been associated with carcinoma cell invasion. In particular, the $\gamma2$ 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. An 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 (Yu et al., 1997; Kadono et al., 1998). Alternatively, 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. Another 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. Additional studies are now necessary to further our understanding of these molecular mechanisms of invasion, both in normal and neoplastic tissues.

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Comment

Cell Migration through Extracellular Matrix: Membrane-Type Metalloproteinases Make the Way

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Every so often, a paper comes along that brings clarity to an issue. Clarity is not necessarily a final resolution, but rather a conceptual framework for productively addressing that issue. Such a paper could be based on a breakthrough discovery, an intriguing observation, a flash of intuition, or a systematic analysis. An excellent example of the latter is in this issue of *The Journal of Cell Biology* (Hotary et al., 2000). With a deceptively simple experimental layout, Hotary et al. (2000) explore the relative role of soluble versus membrane-anchored matrix metalloproteinases (MMP) in tissue morphogenesis. In their in vitro morphogenesis and matrix invasion models, the conclusion is clear cut: membrane-anchored (MT-MMPs), not soluble MMPs, effectively regulate cell migration through extracellular matrix and affect self-organization of cells into tubular structures (Fig. 1).

Cell migration through the matrix is a key component of morphogenesis, i.e., how tissue or organs attain their shape. It is truly an invasive process, whereby cells move into and possibly colonize new territory, and that is why one can speak of cell invasion and morphogenesis in the same breath, and test them with the same assay (Hotary et al., 2000). According to their differentiated type, the migratory cells may give rise to new structures within the matrix they invaded, e.g., tubules, alveoli, or acini, shaping tissues and organs. It is evident, then, why we would like to know the molecular details of migration through the matrix: what motivates cells to migrate, how they do it, how is the process controlled.

There is general agreement that MMPs are important in the execution of migration through the matrix and of invasion, based on abundant data correlating invasive phenomena with the presence of MMPs (Stetler-Stevenson et al., 1993; Werb, 1997). In recent years, mainly via isolation of gelatinolytic activities and homology cloning, the burgeoning protein family of MMPs has come to include, in man, close to 20 members (Table I). Several of these degrade collagens (Table I), the most abundant components of the extracellular matrix, though fine substrate specificity is still at issue (Koshikawa et al., 2000). The majority of MMPs are secreted proteins generally requiring activation for enzymatic activity. A few are true transmembrane proteins, the membrane-type metalloproteinases or MT-MMPs, which are expressed at the cell surface in activated form.

Does the MMP structural diversity reflect functional redundancy or specialization? With their systematic expression of proteinases in select cell types, followed by challenge of specified complex extracellular matrices, Hotary et al. (2000) addressed this fundamental question and came away with an unexpected mechanistic insight (Fig. 1). Key to this accomplishment was their effort to merge modern trends from the fields of proteinase biochemistry, cell migration, tumor invasion, and morphogenesis. Such multidisciplinary approaches often electrify fields and, as in most cell biology problems today, are badly needed in MMP research at this junction. For historical reasons, and because MMPs have been for the most part characterized in biochemistry laboratories, the emphasis of the field has been on enzymatic activities, mechanisms of activation, kinetics and substrate specificity (Nagase and Woessner, 1999). The combined output of several outstanding groups has produced an extraordinary in depth understanding of MMP structure–function relationships, of intricate activation mechanisms and proteinase interactions with natural or man made inhibitors, and provided a solid foundation for MMP enzyme biochemistry (Docherty et al., 1992; Strongin et al., 1993; Morgunova et al., 1999). In contrast, the cell biology of MMPs has lagged behind. Targeted gene disruption by homologous recombination has produced MMP knockout mouse strains with phenotypes ranging from the mild to the dramatic (Itoh et al., 1998; Vu et al., 1998; Holmbeck et al., 1999). Attempts to explain these phenotypes in molecular terms have further raised our discomfort for the currently poor understanding of MMPs in terms of their cell biology. The Hotary et al. (2000) paper is an important step towards bridging the gap between enzyme biochemistry and whole organism analyses of MMP phenotypes. Their findings already begin to make some sense of the fact that soluble MMP knockout mice present themselves with mild developmental phenotypes, whereas MT1-MMP knockout mice display severe abnormalities in bone formation, angiogenesis and collagen turnover, leading to dwarfism, dysmorphic skull, and precocious death (Holmbeck et al., 1999; Zhou et al., 2000). Thus, the next wave in this arena should be analyzing the effects of MMPs on cell behavior in complex model systems, allowing us to dissect the in vivo functions of MMPs in greater detail.

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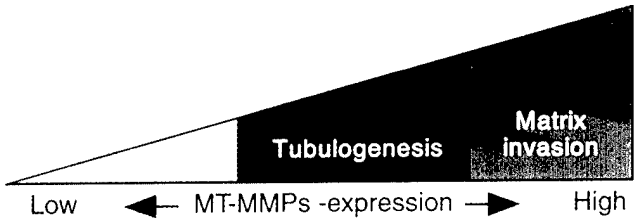

Figure 1. Schematic representation of a proposed correlation between expression of membrane-anchored MMPs (MT-MMPs), invasion, and tubulogenesis (drawing by N. Koshikawa, based on results published in this issue by Hotary et al., 2000).

Migration through the matrix may be a property also of neoplastic cells, even though they may have originated from nonmigratory cells. Contrary to morphogenesis, the results of neoplastic cell migration are often disastrous: tumor invasion and metastasis set in. Important questions then arise: do neoplastic cells in fact migrate through the matrix and ultimately invade tissues by the same mechanisms as normal cells? And, how do they acquire ability to migrate? A result of Hotary et al. (2000) offers an opportunity for reflection on possible answers. In their system, the genesis of tubular structures requires invasion of the matrix, and MT-MMPs appear critical for this. However, express too much of them, and the morphogenetic program is lost. Rather, nondescript matrix invasion takes place (Fig. 1). This result suggests that intriguingly simple rules may determine whether matrix invasion will give rise to organized structure. Admittedly, this hypothetical conclusion may stretch data interpretation a little too far, but it could nonetheless stimulate appropriate experimentation for testing its validity. The attractiveness of this hypothesis is that it may offer some mechanistic underpinning to the process of cancer invasion.

The stakes in MMP research are high because of their involvement in human pathology, e.g., cancer invasion, metastasis, or tissue degenerative diseases, (Matrisian, 1992; Stetler-Stevenson et al., 1993). There have been substantial investments in identifying drug targets based on our knowledge of soluble MMPs (Nelson et al., 2000). The payoff, though, is still below expectations. Inhibitors of MMPs are being taken all the way to Phase III clinical trials, e.g., for cancer treatment. Not all results are in yet, but thus far outcomes are less than spectacular (Yip et al., 1999). In hindsight, was too much being asked of the soluble MMPs? Perhaps. In fairness, though, soluble MMPs used to be the only game in town and, being secreted, their expression, handling, and characterization is easier than membrane-bound proteins. The membrane-anchored forms of MMPs, of which MT1-MMP is the best known, are late arrivals. Furthermore, the fact that MT1-MMP physiologically activates MMP2 (a soluble collagenase), might have distracted investigators from looking at it as an MMP in its own right (Sato et al., 1994). For instance, substrates for MT1-MMP are not well understood. There had been signs in the field that some fresh looks were necessary, and the Hotary et al. (2000) paper may crystallize this mood, signaling a shift in focus towards membrane-anchored MMPs and providing impetus for new experimental frameworks.

A major challenge facing us remains: How do MMPs operate? The matrix surrounding the cells, of which collagens tend to be a dominant component, is often thought of as a physical barrier constraining movement. MMPs, several of which show collagenolytic activity, can degrade the matrix, creating openings. A long-standing view maintains that matrix degradation should be enough to form such openings, but not too much so as to reduce traction. Such a view is easily accepted because it is rooted in human experience in the macroscopic world: ever got your car stuck in mud (SUV owners need not reply)? On the other hand, traction may take on a whole different meaning on the scale at which cells operate. Efforts to quantify the mechanical properties of extracellular matrices should help define whether or not a substrate is permissive for migration, and one should be prepared for surprises. After all, overexpression of MT1-MMP disrupts tubulogenesis,

Table I. The Matrix Metalloproteinase Family

	MMP number	Common name	Substrate
Secreted	1	Interstitial collagenase	Collagens
	2	Gelatinase A	Gelatin, collagens, laminin-5
	3	Stromelysin 1	Collagens, laminin-1, fibronectin
	7	Matrilysin	Gelatin, fibronectin, laminin-1
	8	Neutrophil collagenase	Collagens
	9	Gelatinase B	Gelatin, collagens
	10	Stromelysin 2	Collagens, laminin-1, fibronectin
	11	Stromelysin 3	Alpha-1–antiproteinase
	12	Macrophage elastase	Elastin
	13	Collagenase 3	Collagens
	18	Collagenase 4	?
	19	None	?
	20	Enamelysin	?
Membrane anchored	14	MT1-MMP	Pro-MMP2, gelatin, collagens, laminin-5
	15	MT2-MMP	Pro-MMP2, gelatin
	16	MT3-MMP	Pro-MMP2, collagens
	17	MT4-MMP	TNF-α

but enhances invasion (Fig. 1). Thus, proteinases may affect cell detachment, cell–cell adhesion, receptor-matrix interactions, or, perhaps, the way cells perceive surrounding matrix (Giannelli et al., 1997; Koshikawa et al., 2000; Pozzi et al., 2000), in addition to removing mechanical barriers.

An intriguing result of Hotary et al. (2000) is that altering a topogenic signal had no effect on the ability of MT1-MMP to disrupt tubulogenesis, suggesting that its delivery by intracellular transport mechanisms to precise locations on the cell surface does not matter much. One cannot discount the possibility that MMPs wandering across the plasma membrane are recruited to hot spots of activity by polarized receptors (Brooks et al., 1996). More radically, though, we might have to revisit the well-rooted concept that, for effectiveness, proteinases must be concentrated at the leading edge of invading cells. Again, this concept derives its popularity more from an anthropomorphic view of how a moving cell should be engineered, than from hard data. An alternative view could be that proteolysis of the close pericellular matrix, whether or not focused at a hot spot, sets in motion morphogenetic programs. Maintaining an open mind (Werb, 1997) and looking for informative model systems should be a high priority.

Having made all of these considerations, it is still surprising that, in the Hotary experiments, none of the seven soluble MMPs had any effect on tubulogenesis in collagen gels, particularly since most of these MMPs are well characterized collagenases (Table I). Could it be that soluble MMPs, stimulated by SF/HGF, were already at a maximum in that system, so that no further disruptive effects were detectable upon overexpression? This is possible but unlikely, because the disruptive effects on tubulogenesis of MT1-MMP overexpression required membrane anchoring, seemingly regardless of expression levels. Could it be that soluble MMPs are generally not included in morphogenetic programs because of their lack of spatial specificity? Time will tell, though the available knockouts of two major collagenases, MMP2 and MMP9, would already suggest that their participation in morphogenesis is not essential. Thus, MMP2 deficient mice display minor growth retardation, but appear to develop normally otherwise (Itoh et al., 1998). In MMP9 deficient mice, vascularization of growth plates causes skeletal defects eventually overcome after birth by compensation (Vu et al., 1998).

In summary, to identify which MMPs are important in matrix invasion, Hotary et al. (2000) took the direct route: express them one at a time in informative, albeit complex, cell model system, and see what happens. The difficulty of this approach lays not in its conception, but rather in committing to its elaborate execution. Hence, the considerable lag from the time reagents first became available to the time one laboratory produced the experimental data. In retrospect, what was required was the blending of several distinct skills in one place, and an effort thorough enough to allow for meaningful side by side comparisons. The study reported in this issue (Hotary et al., 2000) did just that, and the reward is an insight that, though glimpsed at by others, remained unproven: membrane-anchored MMPs, the MT-MMPs, play a primary role in cellular invasion of collagenous matrices and, at the right levels of expression, may promote tubulogenesis. Final answer? Yes, in this system, based on the thoroughness of the Hotary study and the permutations they tested. For generalization or textbooks status, this conclusion is definitely one to be reckoned with and to be falsified, in a popperian sense, using other in vitro, and more importantly in vivo systems. Along this road, no doubt, the cell biology of MMPs holds in reserve many surprises for us.

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