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Research Article

Binding of matrilysin-1 to human epithelial cells promotes its activity

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Abstract. Matrix metalloproteinase-7 (MMP-7, matrilysin-1) modulates crucial biological events by processing many epithelial cell surface-associated effectors. We addressed MMP-7 interaction with human epithelial cells and its resulting activity. In human endometrium, a model of controlled tissue remodeling, proMMP-7 was diffusely immunolocalized inside epithelial cells, whereas MMP-7 delineated their entire plasma membrane. Endometrial explants preferentially retained active MMP-7, but not proMMP-7. Endometrial epithelial cells and carcinoma cells from various tissues bound active MMP-7. Endometrial carcinoma-derived Ishikawa cells showed high affinity (K_D of ~2.5 nM) and capacity (~260 000 sites per cell) for MMP-7. MMP-7 binding decreased by extracting membrane sterols or interfering with heparan sulfate proteoglycans, and was abrogated by tissue inhibitors of metalloproteinase-2 (TIMP-2) or synthetic MMP inhibitors. Bound MMP-7 not only remained fully active towards a macromolecular substrate but also became resistant to TIMP-2. We conclude that MMP-7-selective targeting to the plasma membrane of epithelial cells promotes its activity by conferring resistance to TIMP-2.

Keywords. MMP-7, epithelium, cell surface, proteolytic activity, resistance to inhibition.

Introduction

Matrix metalloproteinases (MMPs) are zinc-dependent endoproteases that not only degrade structural constituents of the extracellular matrix (ECM) at neutral pH [1], but also process a variety of key pericellular regulatory molecules, including other proteinases, proteinase inhibitors, chemotactic molecules, latent growth factors, growth-factor binding proteins, cell surface receptors, and cell-cell adhesion molecules [2]. MMPs activity is finely tuned by transcriptional and post-transcriptional regulation. First, the expression of several MMPs is controlled by soluble local regulators such as growth factors, cytokines, and ECM-derived peptides termed matrikines [3]. Second, all MMPs being produced as inactive zymogens must be proteolytically processed to generate active forms. Third, active MMPs are specifically inhibited by tissue inhib-

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itors of metalloproteinases (TIMPs) [4]. Fourth, some MMPs (MMP-2, -9 and -13) are specifically cleared by receptor-mediated endocytosis [5]. Membrane-type (MT)-MMPs are structurally anchored to plasma membrane by a C-terminal transmembrane domain or by a glycosylphosphatidylinositol anchor [6]. In contrast, focused activity of secreted MMPs to the immediate pericellular space requires their recruitment to specific plasma membrane binding sites [7]. For example, MMP-1 association to $\alpha_2\beta_1$ integrin facilitates migration of smooth muscle cells on type I collagen [8]. MMP-7 (matrilysin-1, EC 3.4.24.23), the smallest MMP, is predominantly expressed by epithelial cells [9]. Studies with MMP-7-null mice have highlighted the key role of MMP-7 in re-epithelialization [10] and apoptosis [11]. In human endometrium, whereas several MMPs such as MMP-1 are significantly expressed only during the menstrual phase, MMP-7 mRNA content remains abundant during the proliferative and secretory phases [12, 13]. This suggests that MMP-7 participates not only to the ECM breakdown during menstrual phase but also in cellular processes for ongoing remodeling of nonmenstrual endometrium, such as epithelial cell migration, proliferation and apoptosis, which are essential for re-epithelialization and angiogenesis. MMP-7 indeed processes numerous cell-associated molecules that modulate epithelial cell behavior. These include E-cadherin [14], β4 integrin [15], tumor necrosis factor-α [16], Fas (CD95) [17], Fas ligand [11], heparin-binding epidermal growth factor [18], insulin-like growth factor binding proteins [19], and plasminogen. The latter cleavage generates angiostatin, which controls the formation of new blood vessels [20]. Furthermore, since most non-ECM substrates of MMP-7 are associated to the epithelial cell plasma membrane, MMP-7 has to be recruited at this surface to hydrolyze biologically relevant molecules. CD151, a member of the tetraspanin superfamily anchors latent proMMP-7 onto the epithelial cell surface, allowing for its local activation [21]. In addition, a spliced form of CD44, the CD44v3 heparan sulfate proteoglycan (HSPG) [22], as well as cholesterol sulfate [23] have both been proposed as plasma membrane binding sites for active MMP-7. While active MMP-7 has been localized at the apical surface of the endometrial epithelium of rat uterus [24], its distribution in humans is unknown. Furthermore, to what extent membrane-bound MMP-7 is catalytically active remains to be investigated. We have established that culture of non-menstrual human endometrial explants in the absence of ovarian steroid hormones closely mimics the menstrual breakdown, by triggering MMPs secretion and activation [25]. Remarkably, whereas most active MMPs were released as soluble enzymes in the conditioned medium [26], we found that active MMP-7, but not its latent form, was selectively retained in the endometrial explants. This observation triggered the present investigation to view the potential role of MMP-7 at the epithelial cell surface in the human endometrium. By immunohistochemistry, we found that MMP-7 is not restricted to the apical surface but associates with the entire cell surface of human endometrial epithelial cells, which expands the scope of its activity. Partial characterization of ^{125}I-labeled MMP-7 binding sites to the human endometrium-derived Ishikawa cells indicates interaction with both CD44v3 and cholesterol sulfate, which allows to target the enzyme to different microdomains of the plasma membrane. Our data further demonstrate that membrane-bound MMP-7 remains fully active towards a macromolecular substrate and that cell surface binding confers resistance to inhibition by TIMP-2.

Materials and methods

Materials. Cell culture medium, fetal calf serum (FCS), and other cell culture reagents were from Gibco (Invitrogen, Carlsbad, CA, USA). Recombinant proMMP-7, MMP-7 and proMMP-3, as well as anti-human proMMP-7 (clone 141-B2, mouse IgG_1) and anti-human active MMP-7 (clone 176-5F12, mouse IgG_1) antibodies were from Oncogene (Calbiochem, San Diego, CA, USA). Anti-human CD44v3 (clone 3G5, mouse IgG_{2b}) antibody was from Zymed Laboratories (South San Francisco, CA, USA). Non-immune mouse IgG_1 or IgG_{2b} (Ancell, Bayport, MN, USA) were included in all experiments for negative controls. The synthetic MMP inhibitors were either generously provided by Dr. P. Mitchell (SC-44463; Pfizer, Groton, CT, USA) or purchased from Calbiochem (MMP inhibitor-II, MMPI-II). TIMP-2 was a kind gift of Dr. H. Nagase (Kennedy Institute of Rheumatology Division, London, UK). Quenched casein was from Molecular Probes (BODIPY FL casein; Eugene, OR, USA). Nitrocellulose membranes and protein G-Sepharose were from Amersham (Roosendaal, The Netherlands). Tween 20 and Triton X-100 were from Merck (Darmstadt, Germany). Bovine serum albumin (BSA), 4-aminophenylmercuric acetate (APMA), methyl-β-cyclodextrin (MCD), protamine, heparin and *o*-phenanthroline were from Sigma-Aldrich (Bornem, Belgium). SDS-PAGE molecular weights standards were from Fermentas (St Leon-Rot, Germany).

Tissue sampling and immunohistochemical staining. The study was approved by the Ethical Committee of the University of Louvain, in accordance with the Declaration of Helsinki of the World Medical Association. Normal endometrial tissue was obtained from either hysterectomy specimens or biopsies sampled at various phases of the menstrual cycle. Tissue was embedded in tissue-freezing medium (Tissue-Tek, Sakura, Zoeterwoude, The Netherlands) and 5-µm-thick cryosections were fixed in acetone for 5 min. Sections were blocked with 10 % normal goat serum in phosphate-buffered saline (PBS) for 1 h at room temperature. Immunostaining was performed overnight at 4°C with 10 µg/ml monoclonal antibodies. Bound antibodies were revealed with Envision system (Dako Cytomation, Glostrup, Denmark) according to the manufacturer's instructions, and 3,3'-diaminobenzidine (Sigma-Aldrich) as chromogen.

Alternatively, cryosections were incubated overnight at 4°C with anti-MMP-7 or anti-CD44v3 antibodies. After washing with PBS, sections were incubated with Alexa 488 (green signal)-conjugated goat anti-mouse IgG_1 or Alexa 568 (red signal)-conjugated goat anti-mouse IgG_{2b} (Molecular Probes, Invitrogen) at room temper-

ature for 45 min, washed, and analyzed in a Axiovert M135 microscope (Zeiss, Zaventem, Belgium) coupled to an MRC 1024 confocal microscopy equipment (Bio-Rad Laboratories, Nazareth, Belgium) using a plan-apochromatic 40x1.40 oil immersion objective. Images were acquired with Bio-Rad LaserSharp 2000 v.4.3 software with global adjustment of contrast and brightness using Bio-Rad LaserPix v.4.0.

Explant and cell cultures. Endometrial explants were cultured as described [25]. Briefly, tissue samples were cut in pieces of about 1-mm wide with a sterile surgical blade. Six explants were placed on a Biopore™ membrane per 12-mm tissue culture insert (Millipore, Cork, Ireland), and 300 µl Dulbecco's modified Eagle's medium (DMEM) devoid of serum, phenol red and ovarian steroids were added in the lower chamber. When indicated, explants were incubated with or without 10 mM MCD dissolved in serum-free medium at 37°C for 8 h. Explant viability upon MCD treatment was verified, based on negligible LDH release in conditioned media (data not shown). At the end of culture, media were collected and kept frozen at –20°C until biochemical analysis; explants were frozen at –20°C until homogenization in 200 µl PBS with a Dounce homogenizer for biochemical analysis. Each culture condition was tested in triplicate with explants from the same endometrium.

Ishikawa cells, derived from an endometrial adenocarcinoma (kind gift from Dr. Masato Nishida, Kasumigaura National Hospital, Japan) [27], and MCF-7 cells, derived from a breast carcinoma (kind gift of Dr. M. Berlière, our institution) were cultured in DMEM/F12 medium devoid of phenol red, supplemented with 10 % charcoal-stripped FCS. CaCo-2 and Calu-3 cells, respectively derived from a colon adenocarcinoma and a lung carcinoma (kind gifts of Drs. P. Van der Bruggen and P. Tulkens, both from our institution) were maintained in DMEM containing phenol red, supplemented with 10 % FCS.

Purified epithelial cells were isolated by incubating PBS-washed endometrial tissue with 2 mg/ml collagenase A (Sigma-Aldrich) at 37°C for 45 min. Epithelial glands were then separated by sequential filtration through nylon meshes with pore sizes of 300 and 35 µm, respectively. The resulting suspension of epithelial glands was plated onto 35-mm dishes coated with 1 mg/ml Matrigel™ (BD Biosciences San Jose, CA, USA) and cultured in OPTIMEM I medium supplemented with 2 µM insulin, 130 nM transferrin, 58 µM sodium selenite, 15 µM BSA and 600 µM Na_2SO_4 (all from Sigma-Aldrich).

Zymography. Conditioned media and cultured endometrial explants were analyzed by casein zymography (12 % polyacrylamide gel) to estimate the distribution of latent and active MMP-7 [28]. Zymograms were quantified using KODAK 1D Image Analysis Software (Eastman Kodak Company, Rochester, NY, USA). Data were normalized according to the protein concentration, using the Bradford method for conditioned media [29] (Bio-Rad, Hercules, CA, USA) or by the bicinchoninic acid method for explant homogenates [30] (Sigma-Aldrich).

ProMMPs activation. Activation of proMMPs was performed in 50 mM Tris-HCl, pH 7.5, containing 5 mM $CaCl_2$, 1 µM $ZnCl_2$ and APMA. ProMMP-7 was fully activated by 1 mM APMA after 2 h at 37°C.

ProMMP-3 was treated with 2 mM APMA at 37°C for 4 h to generate the 28-kDa catalytic domain, which presents high sequence homology with active MMP-7 [31].

MMPs binding onto the cell surface. For a qualitative analysis, Ishikawa cells were plated at high density (4×10^5 cells per 35-mm dishes) and cultured for 24 h as described above. After washing with ice-cold DMEM/F12 medium, cell monolayers were incubated at 4°C for 2 h in DMEM/F12 medium containing 0.1 % BSA (assay medium) supplemented with 50 nM (pro)MMP-7 or MMP-3 (*i.e.*, without or after activation by APMA). After extensive washing on ice (3× PBS, 3× PBS with 0.1 % BSA, 3× PBS) for 5 min each, cells were collected by scraping with a rubber policeman, and sonicated for 10 s on ice. Conditioned media and cell lysates were analyzed by casein zymography as above.

For a quantitative analysis, 10 µg MMP-7 was radiolabeled with 1 mCi of $Na^{125}I$ (Amersham) in iodogen-precoated tubes (Pierce, Rockford, IL, USA) for 10 min and ^{125}I-labeled MMP-7 (^{125}I-MMP-7) was separated from free iodine by gel filtration. The specific radioactivity ranged from 10 to 15 µCi/µg MMP-7. Activity of ^{125}I-MMP-7 was assayed by zymography as above, followed by a PhosphorImager analysis (Molecular Dynamics, Los Alamos, CA, USA). Binding of ^{125}I-MMP-7 on epithelial cells from different tissue origins was performed as above. Nonspecific binding was determined in the presence of a 100-fold excess of unlabeled MMP-7. After incubation, supernatants were removed and cells were rinsed as described above. Cells were scraped, sonicated and counted with a gamma counter (Minaxi γ 5000 series, Perkin-Elmer, MA, USA). Scatchard analysis was performed using the Grafit computer software (R. J. Leatherbarrow, Erithacus Software, Staines, UK).

To characterize binding interactions, Ishikawa and MCF-7 cells were treated with 5 mM MCD, 500 µM protamine, 500 µM heparin, or a combination of MCD and protamine or heparin at 37°C for 30 min before adding 1 nM ^{125}I-MMP-7, in the continuous presence of agents. Cell viability upon MCD treatment was verified as above (data not shown). Alternatively, ^{125}I-MMP-7 was first mixed at 37°C for 30 min with natural (50 nM TIMP-2) or synthetic MMP inhibitors (10 nM SC44463, 100 nM MMPI-II), or 1 mM *o*-phenanthroline before addition to Ishikawa cells.

Catalytic activity of membrane-bound MMP-7. To measure enzymatic activity, Ishikawa cells were cultured to confluency in 24-well plates (9×10^4 cells seeded/well). After washing with ice-cold DMEM/F12 medium, cells were incubated in assay medium with 10 nM MMP-7 at 4°C for 2 h as above, then fixed with 4 % formaldehyde at 4°C for 90 s to prevent release of cell-bound MMP-7 during enzymatic assays and washed (3× PBS, 3× PBS with 0.1 % BSA, 3× PBS). Activity of the membrane-bound MMP-7 was quantified by incubating cells in 300 µl assay medium containing 500 ng BODIPY FL-casein/well at 37°C for 3 h. Fluorescence generated by release of self-quenching upon casein degradation was measured at $\lambda_{exc}=485$ nm and $\lambda_{em}=530$ nm (Fluorocount, Packard, Boston, MA, USA). To test the effect of inhibitors on plasma membrane-bound MMP-7, soluble MMP-7 and MMP-7 bound to Ishikawa cells (both 0.5 nM) were incubated at 37°C for 30 min with or without 0.5–500 nM MMPI-II or 1–500 nM TIMP-2 before the fluorescence assay. Additionally, TIMP-2 efficiency towards bound MMP-7 has been evaluated using Ishikawa cells first treated with MCD, protamine or heparin, as described above. IC_{50} values were determined by nonlinear regression analysis using the Grafit computer software.

To localize caseinolytic activity, Ishikawa cells were detached from regular 35-mm dishes by 2 mM EDTA in PBS, pH 7.5, then washed three times in PBS. This cell suspension was incubated at 4°C for 2 h in the absence or presence of 10 nM MMP-7, washed three times in PBS, fixed with formaldehyde (as above) and washed again three times in PBS. Cells were then plated onto quenched casein-coated LabTek chambers (previously coated with quenched substrate in sterile PBS, 100 µg/cm^2, at 37°C for 2 h, followed by three rinses in PBS and drying for 8 h under laminar flow; 5×10^4 cells per chamber), cultured at 37°C for 3 h in assay medium, extensively washed with PBS (6× for 5 min) and mounted in Moviol supplemented with 2.5 % 1,4-diazabicyclo[2.2.2]octane to reduce photobleaching. Fluorescence generated by casein degradation was localized by confocal microscopy.

Statistical analysis. Statistical significance was tested using the Student's *t*-test; $p<0.05$ was considered significant.

Results

Distinct localization of latent and active forms of MMP-7. Two approaches were used to analyze MMP-7 localization in the human endometrium. First, as a static assay, proMMP-7 and MMP-7 were immunolocalized in fixed tissue and cultured explants using

monoclonal antibodies specific for each form. Both in noncultured and cultured samples, proMMP-7 was diffusely localized in the cytoplasm of essentially all epithelial cells, while MMP-7 immunolabeling finely delineated the contour of a large fraction of, but not all, epithelial cells (Fig. 1a). Second, as a dynamic assay, we measured the release of MMP-7 by endometrial explants cultured in the absence of steroid hormones, under which condition MMP-7 expression is the highest [13] and essentially all MMP-2 is released in the medium, as shown by gelatin zymography [26]. Zymographic analysis of conditioned medium and endometrial tissue explants at various intervals of culture time revealed a distinct profile between latent and active MMP-7 (Fig. 1b). While conditioned medium was rapidly enriched in proMMP-7 (maximum level at 12–24 h), active MMP-7 was preferentially and progressively retained by the explants (maximum level after 48 h). Moreover, an intermediate active MMP-7 form, referred to as MMP-7i, can be fully activated upon APMA treatment (data not shown), and was only detected in explants of some cultures. Combined data suggest that MMP-7 is released as a proenzyme, and that fully active MMP-7 and MMP-7i are secondarily recruited to epithelial cells. In the latter case, membrane-bound MMP-7i could undergo a conformational molecular change, leading to its full activation by limited autoproteolysis, as previously described for proMMP-2 [32].

Cell-surface docking candidates for active MMP-7. We next attempted to better define the nature of MMP-7 binding sites on epithelial cell plasma membrane. Since the HSPG CD44v3, which is expressed by epithelial cells in the human endometrium [33], had been reported as a putative binding site for MMP-7 in mice [22], we first looked for their colocalization in noncultured human endometrial tissue by immunofluorescence using confocal microscopy (Fig. 2a). As already observed by immunoperoxidase (Fig. 1a), MMP-7 delineated the entire plasma membrane of labeled glandular epithelial cells, whereas CD44v3 showed a restricted apical distribution in this polarized glandular epithelium (Fig. 2a). Merged image suggested overlap of MMP-7 with CD44v3 signals at the apical membrane, with a dotted pattern compatible with a co-localization in this compartment (Fig. 2a).

Alternatively, cholesterol sulfate has been recently proposed as a major cell-surface binding site for active MMP-7, but not for proMMP-7, in various cancer cell lines [23]. Accordingly, the role of this lipid in the plasma membrane retention of MMP-7 in endometrial glands was evaluated by treating endometrial explants with MCD, a cyclic heptasaccharide deriva-

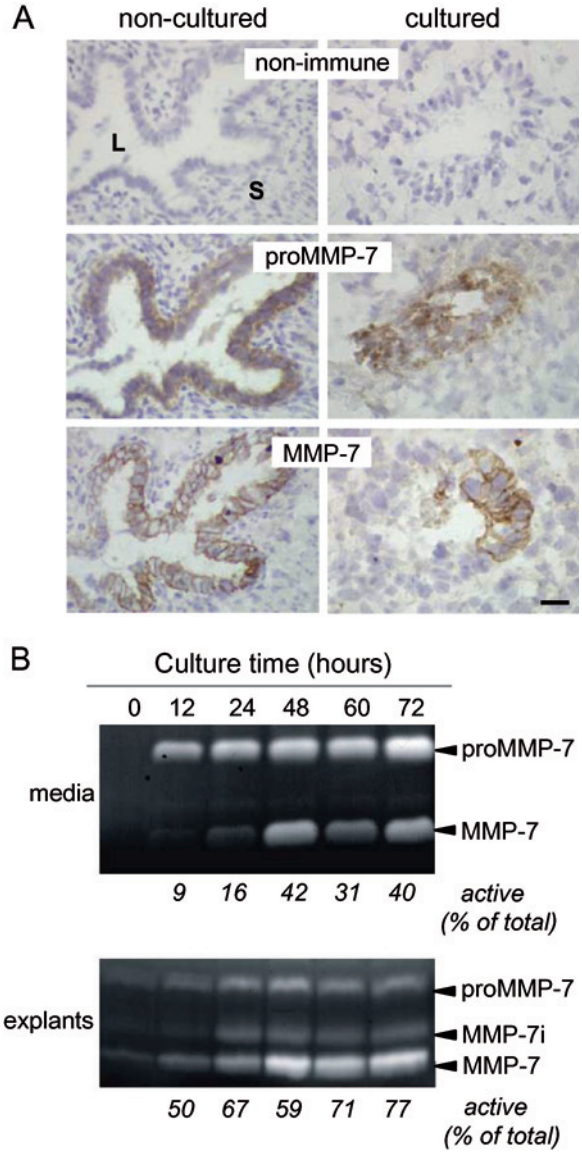

Figure 1. Distinct distribution of latent and active forms of matrix metalloproteinase (MMP)-7 in the human endometrium. (*a*) Immunolocalization of proMMP-7 and MMP-7. Serial cryosections of non-cultured proliferative endometrium (left) or explants cultured for 24 h (right) were processed for immunoperoxidase with irrelevant mouse IgG_1 (non-immune), proMMP-7-specific monoclonal antibody 141-7B2 (proMMP-7), or MMP-7-specific monoclonal antibody 176-5F12 (MMP-7). Immunolabeling appears in brown, cell nuclei were counterstained with hematoxylin in blue. L: lumen of glands; S: stroma. Bar, 25 µm. (*b*) Casein zymography. A series of explants from a menstrual endometrium were cultured in 300 µl medium for 72 h. Media and explants were collected immediately at the beginning (time 0) or after 12, 24, 48, 60 and 72 h of culture and explants were homogenized in 300 µl PBS. The same volumes of conditioned media or of explants homogenate were analyzed by casein zymography. Protein content of media was similar in all lanes, except at time 0 when no protein was detected. The (pro)MMPs bands are identified at right. Percentage of active form of MMP-7 is shown in italics below each lane (total = active + latent), with the intermediate form of MMP-7 (MMP-7i) being not taken in account. Results are representative of experiments from nine distinct endometria, sampled at various phases of the menstrual cycle.

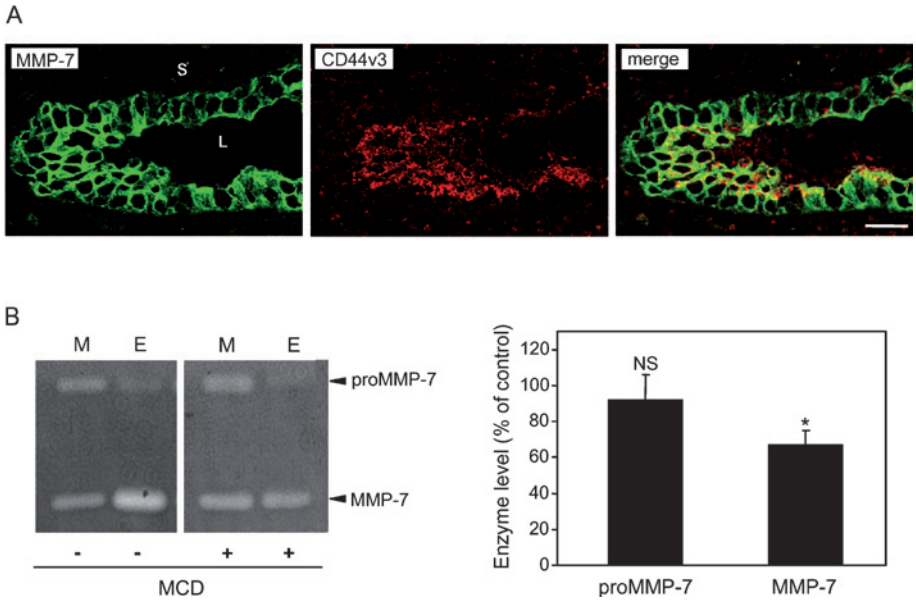

Figure 2. Analysis of MMP-7 binding sites in human endometrial tissue. (*a*) Comparative distribution of active MMP-7 and CD44v3 in the epithelium. Frozen sections were incubated with anti-MMP-7 IgG_1 and anti-CD44v3 IgG_{2b} monoclonal antibodies, followed by Alexa 488-anti-IgG_1 (green fluorescence) and Alexa 568-anti-IgG_{2b} (red fluorescence) antibodies, then observed with a confocal microscope. Merge signals appear in yellow. L: lumen of glands; S: stroma. Bar, 25 μm. (*b*) Elution of active MMP-7 from endometrial explants by methyl-β-cyclodextrin (MCD) treatment. After culture in 300 μl serum-free medium for 48 h, in the absence of estradiol and progesterone (-H), explants were carefully rinsed with PBS and further cultured for 8 h, in the presence or absence of 10 mM MCD. Media (M) and explants (E) were then collected to be analyzed by casein zymography (left). Active and latent forms of MMP-7 were quantified as described in "Materials and methods". They were expressed as ratios of (pro)MMP-7 in explants/total (pro)MMP-7 (explants + media) (right). Changes in these ratios in response to MCD addition are presented after normalization to the corresponding samples without MCD treatment. Data are means ± SD of n=3 separate cultures. NS, non significant; * $p<0.05$.

tive able to cage various sterols and resulting in rafts disruption [34], followed by casein zymography. After 8 h, the tissue content of proMMP-7 was unaffected, but that of MMP-7 was clearly decreased (Fig. 2b). Combined data are compatible with both CD44v3 and cholesterol sulfate acting as binding sites for active MMP-7.

Selective binding of active MMP-7 to Ishikawa cells. To further analyze MMP-7 plasma membrane interaction in a convenient single cell system, we selected the Ishikawa cell line, derived from a human endometrial carcinoma. Ishikawa cells contained no detectable (pro)MMP-7 nor released any such enzyme detectable by the highly sensitive zymographic assay after 2 h of culture at 4°C (Fig. 3, control). Incubation with a mixture of recombinant human proMMP-7 and MMP-7 for 2 h at 4°C, a temperature that arrests endocytosis, resulted in the exclusive cell-association of MMP-7, but not of proMMP-7 [Fig. 3, (pro)MMP-7, -APMA]. As expected, full activation of recombinant proMMP-7 by APMA before incubation increased the amount of cell-associated MMP-7 [Fig. 3, (pro)MMP-7, +APMA], showing that MMP-7 can directly interact with the cells, without proMMP-7 having to bind first to the cell surface for local activation. Under similar experimental conditions, the MMP-3 catalytic domain failed to bind to the cells (Fig. 3, MMP-3_{cat}), compatible with specific docking sites for MMP-7. Remarkably, as much as 10% of added MMP-7 became cell-associated after this incubation, indicating high affinity and/or binding capacity.

Quantitation of MMP-7 binding to Ishikawa cells. Binding parameters of MMP-7 on Ishikawa cells were further determined using ^{125}I-MMP-7. Radio-iodination preserved the caseinolytic activity of MMP-7 (Fig. 4a, *Inset*). A time course indicated that binding approached equilibrium after 120 min (Fig. 4a); this time was then selected to measure the binding isotherm (Fig. 4b). Scatchard plot analysis of specific binding suggested the occurrence of abundant binding sites (~260 000 per Ishikawa cell), with a high affinity (K_D value of ~2.5 nM) (Fig. 4b, *Inset*). These two high values combined explain the efficient cell recruitment noticed above. The linearity of Scatchard plot was compatible with a single class of binding sites, or with two classes of comparable affinities.

We then tested whether active MMP-7 binding was a general characteristic of human epithelial cells. Purified endometrial epithelial cells and Caco-2 colon-derived cells both showed the highest levels of ^{125}I-MMP-7 binding (137±3% and 112±15% of Ishikawa

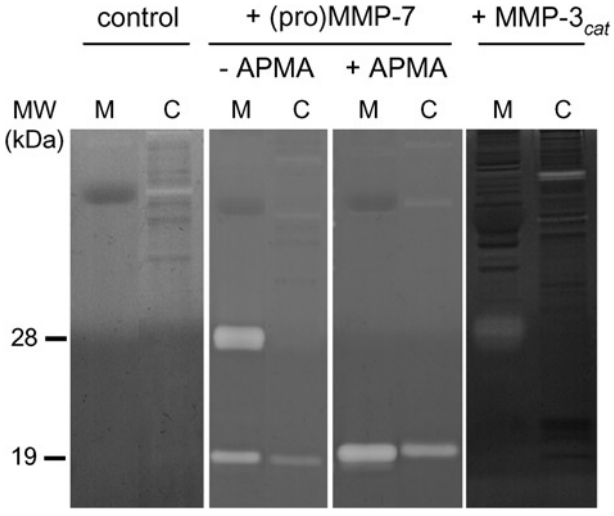

Figure 3. Selective binding of active MMP-7 to human endometrial Ishikawa cells. Confluent Ishikawa cells were incubated at 4°C for 2 h with DMEM/F12 medium containing 0.1% BSA alone [(control), no endogenous (pro)MMP-7 detected]; supplemented with 50 nM recombinant proMMP-7 (28 kDa) without organomercurial activation [-APMA (4-aminophenylmercuric acetate); notice that a minor fraction was found to be spontaneously activated, at 19 kDa]; the same amount of proMMP-7 fully activated by 1 mM APMA at 37°C for 2 h prior to binding (+APMA); or with 50 nM recombinant proMMP-3 processed into its catalytic domain upon treatment with 2 mM APMA at 37°C for 4 h (MMP-3_{cat}, 28 kDa). After incubation, the medium was collected and cells were extensively washed, scraped and sonicated. Enzymes remaining in the conditioned media (M) or cell-associated (C) were analyzed by casein zymography (20 µg cell protein or corresponding amount of medium). The molecular mass of the respective forms of recombinant (pro)MMPs are indicated in kDa on the left. These results are representative of five separate experiments.

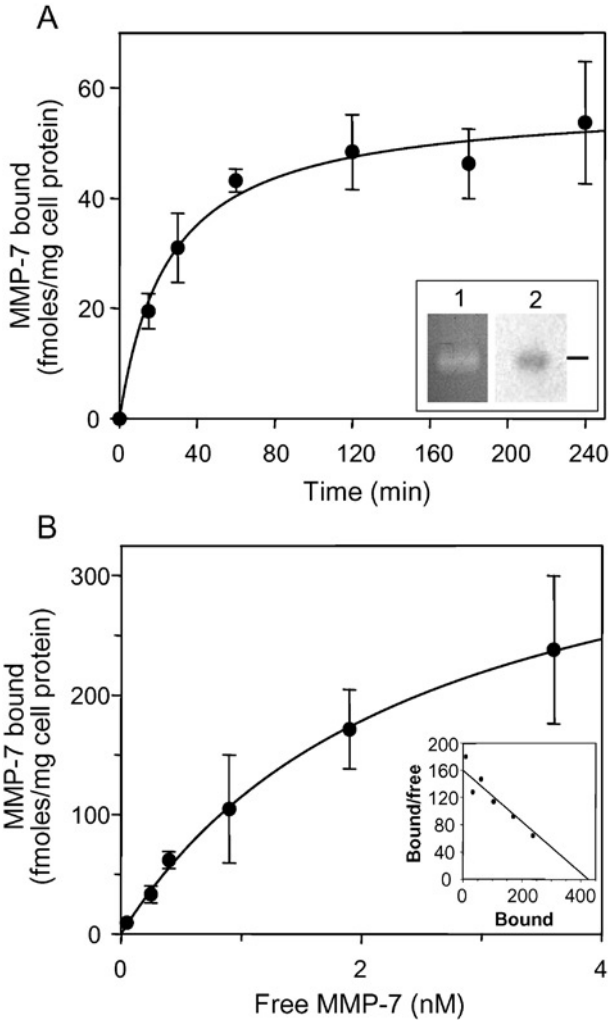

Figure 4. Quantitative analysis of ^{125}I-MMP-7 binding onto Ishikawa cells. (*a*) Time course: Confluent cells were incubated with 1 nM ^{125}I-MMP-7 at 4°C for the indicated times. After washing, cells were scraped and cell-associated radioactivity was normalized to protein content (means ± SD of triplicate dishes). Inset, preservation of ^{125}I-MMP-7 activity : comparison of casein zymography (1) and autoradiography of the same gel (2). (*b*) Binding isotherm. The indicated concentrations of ^{125}I-MMP-7 were allowed to bind to Ishikawa cells at 4°C for 2 h, then supernatants were removed and cells were washed. Supernatant and washes were pooled for each concentration to yield "free" values. After scraping, cell-associated ^{125}I-MMP-7 was measured; nonspecific binding measured in the presence of a 100-fold molar excess of unlabeled MMP-7 was subtracted from total binding to yield specifically "bound" values (means ± SD of triplicate, representative of three separate experiments). Inset, Scatchard plot analysis. Binding parameters were derived after curvilinear fitting, using Grafit computer software.

cells, respectively), whereas Calu-3 lung-derived and MCF-7 breast-derived cells bound MMP-7 at lower levels (41±28% and 34±2% of Ishikawa cells, respectively).

Partial characterization of MMP-7 cell membrane binding mechanism. The nature of these binding sites was explored using a screening based on controlled perturbations, highly charged agents and MMPs inhibitors. Since MCD preferentially extracted MMP-7 from endometrial explants, we first investigated whether a similar treatment of Ishikawa cells would likewise impair ^{125}I-MMP-7 binding. This treatment indeed strongly decreased binding of MMP-7 to cells (to levels close to 40% of untreated control; Fig. 5a), confirming the results on explants. Thus, part of MMP-7 could indeed either involve direct interaction to cholesterol sulfate, or this constituent could be necessary to stabilize docking proteins clustered in rafts. Second, in view of the preferential interaction of MMP-7 with HSPGs, we next looked at the effect of highly charged molecules such as protamine or heparin. Incubation of Ishikawa cells with either of these agents also significantly diminished ^{125}I-MMP-7 binding (to levels close to 60% of untreated control; Fig. 5a). Combination of MCD and protamine or heparin almost completely suppressed the binding (residual binding lower than 10% of untreated control; Fig. 5a). On MCF-7 cells, a similar almost complete inhibition of binding (2% residual binding)

was measured upon treatment with combined MCD and heparin. However, unlike Ishikawa cells, MCD treatment only slightly decreased MMP-7 binding, while heparin largely prevented it (to levels close to about 85 % and 15 % of untreated control, respectively). These results suggest that cholesterol sulfate and HSPGs represent the major MMP-7 binding sites in both cell lines and that relative levels of each receptor class may depend on tissue origin.

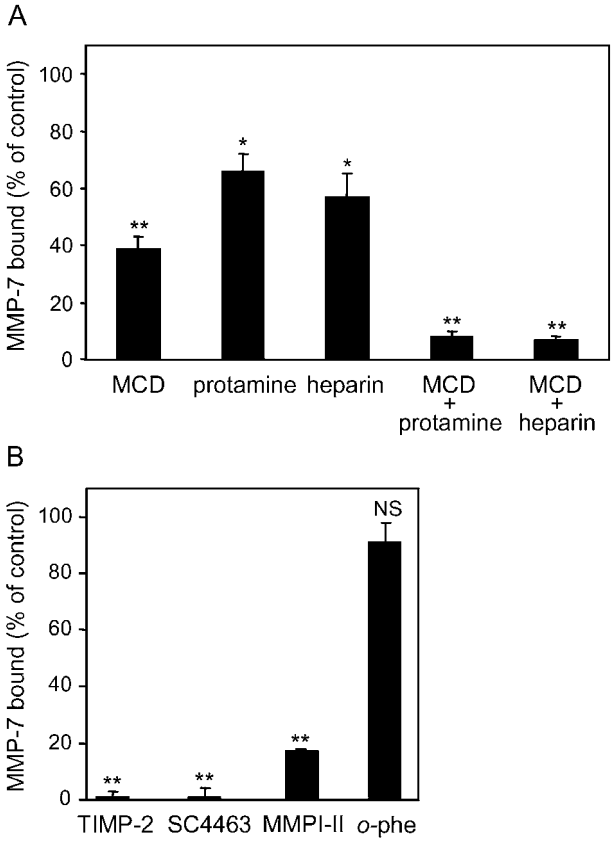

Figure 5. Nature of ^{125}I-MMP-7 interaction with Ishikawa cells. (*a*) Nature of cell-surface binding sites. Confluent Ishikawa cells were preincubated at 37°C for 30 min with 5 mM MCD, 500 µM protamine, 500 µM heparin or a combination of MCD with protamine or heparin in serum-free DMEM/F12 medium before adding 1 nM ^{125}I-MMP-7 in serum-free medium at 4°C for 2 h. (*b*) Role of MMP active site. Before exposure to the cells, 1 nM ^{125}I-MMP-7 was preincubated at 37°C for 30 min with 50 nM tissue inhibitor of MMPs (TIMP)-2, 10 nM SC44463, 100 nM MMP inhibitor II (MMPI-II), or 1 mM *o*-phenanthroline (*o*-phe). After washing, cells were scraped and cell-associated radioactivity was measured, normalized to protein content and expressed as a percentage of the control. Values are means ± SD of n=12 from 4 separate cultures, except for the combined treatments, which are triplicates from a single culture, for (*a*) and of n=9 from 3 separate cultures for (*b*). NS, non significant; * $p<0.05$; ** $p<0.01$.

In addition, the selective binding to the cell surface of active MMP-7, the smallest MMP devoid of the hemopexin domain found in other members of this family, suggested that the catalytic site itself could be involved in the interaction. We thus investigated the effect of preincubating ^{125}I-MMP-7 with natural (TIMP-2) or synthetic MMP inhibitors (SC44463 and MMPI-II) on its binding to Ishikawa cells (Fig. 5b). All MMP inhibitors strongly (MMPI-II) or completely (TIMP-2, SC4463) inhibited ^{125}I-MMP-7 cell surface binding. In contrast, prior incubation of active MMP-7 with 1 mM *o*-phenanthroline had no effect. We conclude that MMP-7 binds to the plasma membrane by a cluster of positively charged amino acid residues localized at the vicinity of the active site, independently of the catalytic zinc atom.

Functionality of membrane-bound MMP-7. Since the above experiments indicated that MMP-7 interaction with the cell-surface binding sites involves its active domain, one would expect that, once bound, MMP-7 would exhibit altered catalytic properties, due to impaired accessibility to substrates and/or to inhibitors. To test these hypotheses, we first cross-linked bound enzyme to the cell surface by a brief exposure to formaldehyde, compatible with partial preservation of activity, then investigated its ability to degrade soluble BODIPY dye-labeled casein, as assayed by the release of self-quenched fluorescence of BODIPY. Formaldehyde treatment partially prevented MMP-7 elution from cell surface upon TIMP-2 and substrate addition (data not shown). Membrane-bound, formaldehyde-fixed MMP-7 exhibited 79±11 % of the caseinolytic activity generated by the same amount of untreated soluble MMP-7 (mean ± SD, n=6). This slight decrease could be due to formaldehyde. We conclude that membrane binding remains compatible with essentially intact MMP-7 activity, at least towards soluble substrates.

We next tested for a topological relationship between membrane-bound MMP-7 and substrate degradation. Ishikawa cells were incubated in suspension in the absence or presence of 10 nM MMP-7, washed, fixed with formaldehyde as above, then plated onto LabTek chambers coated with BODIPY dye-labeled casein. After 3 h of incubation, cells were observed by confocal microscopy (Fig. 6). Non-treated Ishikawa cells generated only few fluorescence spots, preferentially associated with membrane ruffles known to be enriched in MT1-MMP [35], and no fluorescence was detected between cells (Fig. 6a). When Ishikawa cells were first incubated with MMP-7, a strong fluorescence signal was generated all around the surface (Fig. 6b), with a minor signal between cells that could result from release of incompletely fixed enzyme. In contrast, equimolar amount of soluble MMP-7 incubated on quenched substrate only generated a weak diffuse fluorescence (Fig. 6c). Altogether, we conclude that membrane-bound MMP-7 is catalytically active and shows no preferential binding topology on cells in suspension, as already found by immunohistochemistry.

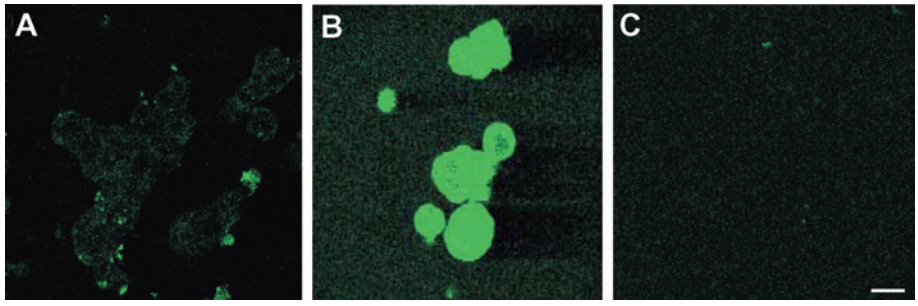

Figure 6. Membrane-bound MMP-7 on Ishikawa cells is catalytically active. Ishikawa cells were detached by EDTA treatment, washed, then allowed to bind MMP-7 in suspension. After brief cross-linking by formaldehyde, cells were plated onto LabTek chambers coated with BODIPY-casein (a quenched substrate). Fluorescence signals generated by casein degradation after 3 h at 37°C were observed by confocal microscopy. (*a*) Untreated Ishikawa cells show discrete focalized fluorescent spots at ruffling zones. (*b*) MMP-7-treated Ishikawa cells exhibit a strong fluorescence all around the cell surface. (*c*) Soluble MMP-7 incubated on quenched casein reveals a weak diffuse fluorescence. Bar, 20 μm.

We evaluated the effectiveness of increasing concentrations of MMP inhibitors against equal amounts of soluble and membrane-bound MMP-7, by comparing a small synthetic inhibitor, MMPI-II (514 Da) with a large natural inhibitor, TIMP-2 (21 000 Da) (Fig. 7). As shown in Figure 7a, the IC_{50} values for inhibition by MMPI-II were comparable for soluble (27 nM, as previously reported) [36], and membrane-bound MMP-7 (53 nM), indicating that the low molecular weight inhibitor had still adequate access when MMP-7 was bound to plasma membrane. In contrast, the IC_{50} value for inhibition by TIMP-2 of membrane-bound MMP-7 was much higher (64 nM) than that for inhibition of soluble MMP-7 ($IC_{50}=9$ nM; Fig. 7b) and even a large excess of TIMP-2 failed to totally inhibit membrane-bound MMP-7. To exclude that TIMP-2 resistance may depend on fixative agent, we compared TIMP-2 effect on cell-bound MMP-7, treated or not by formaldehyde, and found that fixation did not alter the inhibitory capacity of TIMP-2 (data not shown). These data suggest that binding of MMP-7 to plasma membrane strengthens its catalytic efficiency by (i) concentrating its proteolytic activity to the pericellular space, and (ii) conferring resistance to its natural inhibitor TIMP-2. We then evaluated the influence of binding sites on TIMP-2 resistance, by first treating Ishikawa cells with MCD, protamine or heparin. MMP-7 bound to CD44v3 was less resistant to TIMP-2 inhibition than MMP-7 bound to cholesterol sulfate ($IC_{50}=43$ nM for MCD condition *versus* 63 and 61 nM for protamine and heparin conditions).

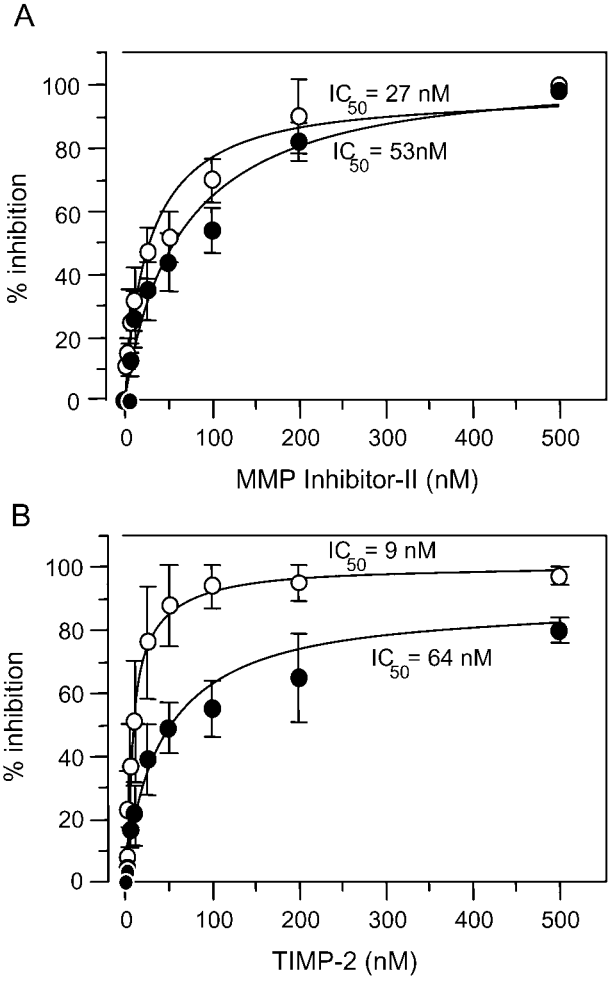

Figure 7. Membrane-binding of MMP-7 on Ishikawa cells confers resistance to inhibition by TIMP-2. Equal amounts (0.5 nM) of soluble MMP-7 (open symbols) or membrane-bound MMP-7 (filled symbols) was incubated at 37°C for 30 min with the indicated concentrations of (*a*) MMP inhibitor II or (*b*) TIMP-2. Residual MMP-7 activity was quantified using the soluble BODIPY-casein assay. IC_{50} values were calculated by nonlinear regression analysis. Data are mean values ± SD (n=6).

Discussion

This study addressed the selective binding of active MMP-7 onto epithelial cells, which promotes its pericellular activity. Our investigations were initiated

by the observation that active MMP-7, but not its latent proform, was preferentially retained in cultured endometrial explants. Analysis of the binding mechanism of MMP-7 in an endometrium-derived epithelial cell line revealed high-affinity and high-capacity binding sites and suggests new directions for their identification. The key observations that membrane-bound MMP-7 remains fully active to a macromolecular substrate and becomes refractory to TIMP-2 support the hypothesis that MMP-7 is strategically located and potentiated at the plasma membrane. Localization of active MMP-7 at the plasma membrane of epithelial cells from various tissue origins suggest that this protease processes multiple signaling molecules involved in cell proliferation and motility that occur in carcinomas and in the cyclic remodeling human endometrium.

By immunolabeling of human endometrial tissues, MMP-7 was restricted to the cell surface of glandular epithelia, contrasting with the diffuse intracellular pattern of proMMP-7, but showed no polarized distribution. It has been previously reported that rat MMP-7 is associated with HSPGs at the surface of rat uterus cells [24]. Moreover, the HSPG CD44v3 and MMP-7 were found to colocalize on the apical surface of murine endometrial epithelial cells and invalidation of CD44v3 caused redistribution from the apical to the basal surface [22], probably on other cell surface HSPG(s), such as syndecans [37], consistent with MMP-7 elution by heparin treatment [24]. Our observations in the human endometrium are compatible with a partial colocalization of CD44v3 and MMP-7 at the apical surface of epithelial cells, suggesting that, as in murine system, human CD44v3 can dock MMP-7 and represents one type of binding site. Indeed, protamine and heparin interfered with MMP-7 binding to Ishikawa cells. However, confocal microscopy clearly distinguished the non-polarized MMP-7 distribution from the apical pattern of CD44v3 in human endometrial cells, and the effect of protamine and heparin was partial. Altogether, these data indicate that additional binding sites, different from HSPG partners, are present on human epithelial cell surface, in particular at the basolateral domain, which is most relevant for interaction with structural ECM constituents and signaling molecules.

Binding of active MMP-7 to the human colon carcinoma Colo 201 cell line was not impaired by heparin treatment [38], but was reported to depend on cell surface sulfatides including cholesterol sulfate [23]. In agreement with this suggestion, we found that MCD treatment, which extracts various sterols from the plasma membrane [34], efficiently extracted active MMP-7, but not proMMP-7, from endometrial explants and inhibited binding of active MMP-7 to Ishikawa cells. These data suggest that lipids present in rafts, such as cholesterol sulfate, could represent additional MMP-7-docking sites in human endometrial epithelial cells. Although a single class was evidenced by Scatchard plot, this type of analysis cannot resolve two populations of binding sites with similar affinities, such as CD44v3 and cholesterol sulfate for MMP-7, both reported in the nanomolar range [22, 23]. Moreover, MMP-7 binds to cholesterol sulfate but not to cholesterol [23], and cholesterol sulfotransferase type 2B isoform 1b, which catalyzes the sulfate conjugation of cholesterol [39], is expressed by human endometrial epithelial cells and not by stromal cells (Héloïse Gaide Chevronnay, personal communication). These data pinpoint the role of sulfate group(s) present in sulfated MMP-7-docking compounds localized on the epithelial cell membranes and could explain the strict epithelial localization of active MMP-7 observed in human endometrium.

As previously reported for human colon carcinoma cells [38], we did not observe binding of proMMP-7 to Ishikawa cells, although proMMP-7 was shown to be activated through interaction with the plasma membrane CD151 protein in human rectal and lung carcinoma cells [21]. The selective detection of an intermediate form of active MMP-7 inside endometrial explants in some cultures suggests that proMMP-7 is preferentially activated in close vicinity to cells. The lack of proMMP-7 binding to Ishikawa cells is probably due to the propeptide domain that prevents interaction between the catalytic domain and its docking sites at the plasma membrane, for instance by masking a critical protein stretch. Furthermore, the strong inhibition of MMP-7 surface binding by TIMP-2 and by synthetic MMP inhibitors that occupy its catalytic site suggests that interaction involves amino acid residues located at, or near the active site of MMP-7. Alternatively, engagement of the synthetic inhibitor into the catalytic site of MMP-7 could modify the secondary structure of the protein, not only close to the S_1' pocket, but also of the entire catalytic domain, as already described for MMP-3 [40], and thus could mask positively charged residues involved in MMP-7 interaction with sulfated cell membrane compounds.

Proteolytic activity of MMP-7 is not required for its binding because the E215A MMP-7 inactive mutant can still bind to cell membranes [23]. Furthermore, we found that *o*-phenanthroline, that chelates catalytic zinc ion without occupying substrate-binding S_1' pocket of the metalloenzyme [41] had no effect on MMP-7 binding. Although catalytic domains of MMPs possess high sequence homology [42], the catalytic domain of MMP-3 did not bind to epithelial

cells, confirming the specificity of interaction between MMP-7 and sulfated compounds, as recently reported for cholesterol sulfate [23].

Active MMP-7 binding induces colon carcinoma cell aggregation [38]. Our data clearly show that membrane-bound MMP-7 remained able to degrade macromolecular substrates, such as casein, and was still efficiently inhibited by a low-molecular weight MMP inhibitor but not by the high-molecular weight TIMP-2, similarly to MMP-8 and MMP-9 bound to polymorphonuclear cell membrane [43, 44]. Most synthetic inhibitors of MMPs are derived from the peptide structure of the α-chain of type I collagen, at the point at which collagenase first cleaves the molecule [41], *i.e.*, P' residues of inhibitors match S' subsites of enzymes. This strongly suggests that MMP-7 S' subsites interacting with the low-molecular weight MMP inhibitor or macromolecular substrate and those involved in cell-surface binding are different. The indirect relationship between inhibitors size and their efficiency against membrane-bound MMPs suggest that steric hindrance may explain the resistance of membrane-bound MMP-7 to TIMP-2. Such steric hindrance could be stronger in close contact to membrane lipid rafts than at the heparan sulfate chains that extend from the large extracellular domain of CD44v3.

In conclusion, this study shows that specific binding of active MMP-7 to human endometrial epithelial cell surface may involve two different classes of sulfated binding sites and shows that bound MMP-7 not only remains catalytically active but exhibits resistance against a main natural inhibitor. Overall, these data may contribute to explain the important role exerted by MMP-7 in various physiopathological conditions, such as tumor invasion and metastasis [45], and antibacterial defense [46]. However, this by no means contradicts the role of secreted proteases such as secreted MMP-9, which appears to be more efficient in promoting angiogenesis than its tumor cell membrane-bound counterpart [47].

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