

## Functional Interplay between Type I Collagen and Cell Surface Matrix Metalloproteinase Activity\*

Received for publication, June 27, 2000, and in revised form, April 4, 2001  
Published, JBC Papers in Press, April 30, 2001, DOI 10.1074/jbc.M005631200

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**Type I collagen stimulation of pro-matrix metalloproteinase (pro-MMP)-2 activation by ovarian cancer cells involves  $\beta_1$  integrin receptor clustering; however, the specific cellular and biochemical events that accompany MMP processing are not well characterized. Collagenolysis is not required for stimulation of pro-MMP-2 activation, and denatured collagen does not elicit an MMP-2 activation response. Similarly, DOV13 cells bind to intact collagen utilizing both  $\alpha_2\beta_1$  and  $\alpha_3\beta_1$  integrins but interact poorly with collagenase-treated or thermally denatured collagen. Antibody-induced clustering of  $\alpha_3\beta_1$  strongly promotes activation of pro-MMP-2, whereas  $\alpha_2\beta_1$  integrin clustering has only marginal effects. Membrane-type 1 (MT1)-MMP is present on the DOV13 cell surface as both an active 55-kDa TIMP-2-binding species and a stable catalytically inactive 43-kDa form. Integrin clustering stimulates cell surface expression of MT1-MMP and co-localization of the proteinase to aggregated integrin complexes. Furthermore, cell surface proteolysis of the 55-kDa MT1-MMP species occurs in the absence of active MMP-2, suggesting MT1-MMP autolysis. Cellular invasion of type I collagen matrices requires collagenase activity, is blocked by tissue inhibitor of metalloproteinases-2 (TIMP-2) and collagenase-resistant collagen, is unaffected by TIMP-1, and is accompanied by pro-MMP-2 activation. Together, these data indicate that integrin stimulation of MT1-MMP activity is a rate-limiting step for type I collagen invasion and provide a mechanism by which this activity can be down-regulated following collagen clearance.**

The MMP family is composed of at least 25 zinc-dependent extracellular endopeptidases whose activities are regulated predominantly by expression as inactive precursors, or zymogens (1–3). Although precise physiological activators of MMPs<sup>1</sup> are unknown, a variety of serine proteinases and other

MMPs in the extracellular milieu execute the initial propeptide cleavage events *in vitro* (2). An exception to serine protease activation is pro-MMP-2 (72-kDa gelatinase A), which lacks the necessary basic amino acid cleavage sites in its pro-domain (4). A primary mechanism of pro-MMP-2 activation involves zymogen association with the cell surface via formation of a ternary complex containing tissue inhibitor of metalloproteinase (TIMP)-2 and membrane type 1-MMP (MT1-MMP, MMP-14) (3–6). Following trimeric complex formation, it is hypothesized that a neighboring MT1-MMP molecule that is not associated with TIMP-2 cleaves pro-MMP-2 at the Asn<sup>37</sup>–Leu<sup>38</sup> peptide bond within the pro-domain (7). Intermediately processed MMP-2 (Leu<sup>38</sup>–MMP-2) undergoes further concentration-dependent autolytic cleavage(s) to generate mature enzymes that can be released into the soluble phase or remain surface-associated (8). Although biological mechanisms of active MMP-2 release from the cell surface are not well characterized and dissociation kinetics provide little insight, cellular binding affinities may shift following pro-MMP-2 cleavage.

Culturing a variety of cell types within a three-dimensional gel of type I collagen stimulates cellular activation of pro-MMP-2 (9–12). Although MT1-MMP is implicated in MMP-2 processing, regulation of cellular events that promote MMP processing are poorly understood. As cellular interaction with type I collagen is mediated largely through integrin receptors, it has been postulated that collagen stimulation occurs either directly or indirectly through integrin signaling (12–16). In support, we have previously demonstrated that culturing DOV13 ovarian cancer cells in a three-dimensional collagen gel elicits a strong pro-MMP-2 activation response that can be mimicked by clustering of  $\beta_1$  integrin receptors (12). Furthermore, pro-MMP-2 activation coincides with the processing of MT1-MMP into truncated 55- and 43-kDa forms on the cell surface. In this study, we utilize a variety of approaches to elucidate the biochemical requirements of type I collagen stimulation of MMP zymogen activation, characterize processed forms of MT1-MMP that are generated in this response, and examine the proteinase requirements for cellular invasion of type I collagen gels. Our findings illustrate a general mechanism by which cells may regulate cell surface-associated MMP activity via interactions with pericellular collagen matrix.

### EXPERIMENTAL PROCEDURES

**Materials**—Bovine serum albumin, gelatin, cell culture reagents, human placental type I collagen, aminophenylmercuric acetate, anti-(rabbit IgG)-peroxidase conjugates, purified mouse immunoglobulins, 2.97- $\mu$ m diameter latex beads, concanavalin A (ConA), and *ortho*-phenanthroline were all purchased from Sigma. Anti-human  $\beta_1$  integrin

\* This work was supported by National Institutes of Health Training Grant 5T32 GM08061 (to S. M. E.), United States Army MRMC Training Grant DAMD170010386 (to Y. I. W.), and NCI Research Grant R01 CA86984 (to M. S. S.) from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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<sup>1</sup> The abbreviations used are: MMP, matrix metalloproteinase; MT1-MMP, membrane type-1 matrix metalloproteinase; TIMP, tissue inhibitor of matrix metalloproteinase; ConA, concanavalin A; rCBD123, recombinant collagen binding domain; rCD, recombinant carboxyl hemopexin domain; PBS, phosphate-buffered saline; TBS, Tris-buffered

saline; BSA, bovine serum albumin; mAb, monoclonal antibody; CR, collagenase-resistant; MES, 4-morpholineethanesulfonic acid; MMPI, MMP inhibitor; CHO, Chinese hamster ovary.

mAb clones 21C8 and P5D2, anti-human  $\alpha_2$  mAb clones P1E6 and AK7, anti-human  $\alpha_3$  integrin mAb clones P1B5 and ASC-6, anti-human  $\alpha_3\beta_1$  heterodimer integrin mAb clone M-KD102, anti-human (carboxyl domain) TIMP-2 mAb clone 67-4H11, and anti-human MT1-MMP polyclonal antibody (AB815, hinge domain) were all obtained from Chemicon (Temecula, CA). Hydrobond-P:polyvinylidene difluoride membrane was obtained from Amersham Pharmacia Biotech. Super-Signal-enhanced chemiluminescence reagents were purchased from Pierce. The general hydroxamic acid MMP inhibitor INH-3850-PI (MMPI) was purchased from Peptides International (Louisville, KY). Purified TIMP-2 and TIMP-1 and anti-MT1-MMP (raised against amino acids 160–173/catalytic domain) polyclonal antibody MTK3 were generous gifts of Dr. Hideaki Nagase (Kennedy Institute of Rheumatology, Imperial College School of Medicine, UK). Recombinant MMP-2 type II fibronectin domain repeats (rCBD123) and hemopexin carboxyl domain (Gly<sup>417</sup>–Cys<sup>631</sup>) were generated as described previously (17, 18). Collagenase-resistant (CR) murine type I collagen ( $\alpha_1(I)$  chain mutations, Gln<sup>774</sup> → Pro<sup>774</sup>, Ala<sup>777</sup> → Pro<sup>777</sup>, and Ile<sup>776</sup> → Met<sup>776</sup>) and wild type murine type I collagen were the generous gifts of Dr. Stephen Crane (Harvard University) (19).

**Cell Culture**—The ovarian carcinoma cell line DOV13 was provided by Dr. Robert Bast, Jr. (M.D. Anderson Cancer Center, Houston, TX). Cell culture was maintained under standard conditions in 75-cm<sup>2</sup> cell culture flasks (20).

**Quantification of Cell Adhesion**—96-well cluster plate chambers were coated with 50  $\mu$ l of 10  $\mu$ g/ml type I collagen,  $\frac{3}{4}$ ,  $\frac{1}{4}$  collagen fragments (prepared as described below), or type I gelatin (1.6  $\mu$ g/cm<sup>2</sup>) in sterile phosphate-buffered saline (PBS) for 4 h at 25 or 37 °C, blocked with 3% BSA in minimal essential medium for 1 h at 37 °C, washed with PBS, and air-dried. Cells ( $1 \times 10^5$  cells/ml in serum-free medium) were incubated for 20 min at 37 °C with specific integrin function-blocking antibodies or nonspecific control antibodies, plated at a density of  $2 \times 10^4$  cells/well, and allowed to adhere for 75 min. Unbound cells were removed by washing with PBS, and adherent cells were fixed in ethanol (10 min), stained with 0.5% crystal violet (20 min), washed extensively with water, and solubilized with 100  $\mu$ l 1% SDS. Relative adhesion was quantified by monitoring the absorbance of released dye at 540 nm ( $n = 5$ ).

**Isolation of Collagen Fragments and Preparation of Collagen-containing Surfaces**—Human placental collagen (7 mg) was cleaved into  $\frac{3}{4}$  and  $\frac{1}{4}$  fragments by incubating with 2  $\mu$ g of MMP-1 for 16 h at 25 °C in Tris-buffered saline (TBS) (pH 7.4) containing 60 mM CaCl<sub>2</sub>. Cleaved collagen was isolated using a modified ammonium sulfate precipitation protocol (21). Ammonium sulfate (12%) was added to collagen suspension on ice at 4 °C under constant stirring for 1 h and then centrifuged (15,000  $\times g$ , 1 h, 4 °C). The 12% pellet containing intact collagen was washed with ice-cold TBS containing 12% ammonium sulfate, re-centrifuged (15,000  $\times g$ , 1 h, 4 °C), solubilized with 0.2 M acetic acid, and dialyzed against PBS. To isolate the  $\frac{3}{4}$  and  $\frac{1}{4}$  fragments, ammonium sulfate was added to the 12% supernatant to a final concentration of 25%, incubated for 1 h, and centrifuged (15,000  $\times g$ , 1 h, 4 °C). The 25% pellet containing  $\frac{3}{4}$  and  $\frac{1}{4}$  collagen fragments was washed with ice-cold TBS containing 25% ammonium sulfate, re-centrifuged (15,000  $\times g$ , 1 h, 4 °C), solubilized, and dialyzed as described above. Type I gelatin was produced by thermal denaturation of type I collagen for 20 min at 60 °C. Protein concentrations were determined using the Bio-Rad D<sub>C</sub> kit and bovine albumin as a standard.

Assays using thin deposits of type I collagen were performed by dialyzing acid-solubilized collagen against PBS, diluting to 10  $\mu$ g/ml in 100 mM sodium carbonate (pH 9.6), and coating 24-well cluster plate chambers with 200  $\mu$ l (10  $\mu$ g/ml, 1.1  $\mu$ g/cm<sup>2</sup>) of collagen. Chambers were incubated for 1 h at 37 °C, washed with sterile PBS, and air-dried. For three-dimensional collagen gel experiments, dialyzed type I collagen was diluted to 1.5 mg/ml with cold minimum essential medium containing 20 mM Hepes (pH 7.4). Diluted collagen (200  $\mu$ l, 158  $\mu$ g/cm<sup>2</sup>) was added to 24-well plates and allowed to gel at 37 °C for 30 min before the addition of cells ( $2.5 \times 10^5$ ) to wells. Cells were incubated for 48–72 h in serum-free medium at 37 °C before collection of conditioned media.

**Integrin Clustering**—Anti-integrin subunit-specific antibodies or control IgG were passively adsorbed onto 2.97- $\mu$ m diameter latex beads as described previously using the following modifications (12, 24). Latex beads were incubated at a final suspension of 0.1% in 100 mM MES buffer pH 6.1, with 25  $\mu$ g/ml appropriate antibody in 1-ml volumes overnight at 4 °C under agitation, and then blocked with 10 mg/ml BSA for 2 h at room temperature. Blocked beads were pelleted (3,000  $\times g$ , 3 min, 25 °C), washed twice with 1 ml of serum-free media, and resuspended to 1% by volume. Protein concentration assays using a BCA detection kit (Sigma) indicated 60–70% adsorption of immunoglobulins.

Cells were plated at a density of  $2.5 \times 10^5$  cells/well in 24-well cluster plates (Becton Dickinson) overnight in serum-containing medium, incubated for 2 h in serum-free medium prior to the addition of fresh medium containing soluble antibodies (10  $\mu$ g/ml), concanavalin A (20  $\mu$ g/ml), (25) or antibody-adsorbed latex beads (3–4  $\mu$ g/ml; 0.02% beads by final volume) for 18–20 h. All final volumes were 500  $\mu$ l/well.

**Gelatin Zymography**—Gelatinase activities in conditioned media were determined using SDS-polyacrylamide gel electrophoresis zymography. Conditioned media (20  $\mu$ l) from an equivalent number of cells were electrophoresed without reduction on SDS-polyacrylamide gel electrophoresis gels prepared with 9% acrylamide containing 0.1% gelatin (23). SDS was removed through a 1-h incubation in 2.5% Triton X-100, and gels were incubated in 20 mM glycine, 10 mM CaCl<sub>2</sub>, 1  $\mu$ M ZnCl<sub>2</sub> (pH 8.3), at 37 °C for 24 h prior to staining for gelatin with Coomassie Blue. Enzyme activity was visualized as zones of gelatin clearance within the gels.

**MMP-2 Competition Experiments**—Cells were grown to confluency in 24-well chamber plates and incubated for 2 h in serum-free medium. Fresh medium containing various concentrations of rCBD123 (17) or recombinant hemopexin carboxyl domain (18) were added to cells in a 500- $\mu$ l volume. After 2 h, ConA was added to a final concentration of 20  $\mu$ g/ml, and cells were incubated for an additional 18 h. Conditioned media and cell lysates were collected and processed as described above.

**MT1-MMP Immunoblots**—Cells were incubated under various conditions, collected with lysis buffer, and protein concentration of lysates was analyzed using the Bio-Rad D<sub>C</sub> detection kit and bovine albumin standards. Cell lysates (5–15  $\mu$ g) were electrophoresed on 9% SDS-polyacrylamide gels, transferred to polyvinylidene difluoride membrane, and blocked with 3% BSA in 50 mM Trizma (Tris base) (pH 7.5), 300 mM NaCl, 0.2% Tween 20 (TBST). Membranes were incubated for 1 h at room temperature with a 1:4000 dilution of anti-human MT1-MMP polyclonal antibody in 3% BSA/TBST. Immunoreactive bands were visualized with a peroxidase-conjugated anti-rabbit-IgG (1:5000 in 3% BSA/TBST) and enhanced chemiluminescence.

**Isolation of Biotin-labeled Cell Surface Proteins**—Cells were grown to confluency in 6-well cluster dishes, washed with PBS, and incubated for 2 h in serum-free medium. Fresh serum-free medium (1 ml) containing a 0.06% antibody-coated bead suspension or 20  $\mu$ g/ml concanavalin A was added, and cells were incubated for 20 h. Conditioned medium was removed; cells were washed with  $2 \times 2$  ml of PBS, and surface proteins were labeled with a non-cell-permeable sulfo-NHS biotin analog (500  $\mu$ l at 500  $\mu$ g/ml PBS, Pierce) under gentle shaking at 4 °C for 30 min. After washing, cells were incubated with 1 ml of 100 mM glycine/PBS for an additional 20 min under gentle shaking at 4 °C. Washed cells were lysed with 500  $\mu$ l of lysis buffer (50 mM sodium phosphate buffer (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 0.5% Triton X-100, 1  $\mu$ g/ml aprotinin, 1  $\mu$ M pepstatin, 10  $\mu$ M leupeptin, and 10  $\mu$ M E64), collected with a cell scraper, and clarified by centrifugation (10,000  $\times g$ , 10 min, 4 °C). Protein concentrations were calculated as described. To precipitate biotin-labeled cell surface proteins, lysate (1 ml, 750  $\mu$ g/ml) was added to either ImmunoPure immobilized monomeric avidin or multimeric streptavidin gel (40  $\mu$ l) (Pierce) and incubated overnight at 4 °C on a rotator. Gels were washed 5 $\times$  with lysis buffer. In some experiments, 10 mM free D-Biotin/PBS (Pierce) was added to aliquots of monomeric avidin gel-immobilized protein to compete off bound protein at 4 °C overnight. Eluates (30  $\mu$ l) were analyzed by gelatin substrate zymography or immunoblotting. Control conditions utilized non-biotinylated cell lysates.

**Isolation of Plasma Membranes**—DOV13 cells were grown to confluency in 15-cm culture dishes, washed with PBS, switched to serum-free medium for 2 h, and incubated in 5 ml of fresh serum-free medium containing 20  $\mu$ g/ml ConA for 20 h. Cells were washed twice with 5 ml of PBS, collected with a cell scraper, and pelleted by centrifugation (1500  $\times g$ , 10 min, 4 °C). Cells were resuspended in ice-cold 5 mM Tris/HCl (pH 7.8) and incubated for 10 min prior to homogenization by 30 passages through a 26 $\frac{1}{4}$ -gauge needle (8). Crude membrane preparations were isolated by centrifuging whole cell lysates (10,000  $\times g$ , 10 min, 4 °C), retaining the supernatant, and centrifuging the supernatant (100,000  $\times g$ , 1 h, 4 °C). The recovered pellet was washed in 20 mM Tris/HCl (pH 7.8), 10 mM CaCl<sub>2</sub>, 0.05% Brij 35 and recentrifuged (100,000  $\times g$ , 45 min, 4 °C). The plasma membrane-containing pellet was resuspended in washing buffer, and protein concentration was assessed as described above.

**Cross-linking and Immunoprecipitation**—To isolate TIMP-2-binding MT1-MMP species, cell surface proteins were cross-linked, and TIMP-2 complexes were isolated through immunoprecipitation. Confluent cells were incubated for 16 h in 5 ml of serum-free medium containing 20  $\mu$ g/ml ConA and 125  $\mu$ g/ml TIMP-2. After washing ( $2 \times 5$  ml of PBS),

cell surface proteins were cross-linked with 2 mM 3,3'-dithiobis(sulfosuccinimidylpropionate) (Pierce) in PBS at 4 °C for 25 min under gentle shaking. Cells were washed (2 × 10 ml of PBS), and lysates were collected in cold lysis buffer. Clarified cell lysates (700 µg/ml) were incubated with 5 µg of anti-TIMP-2 (carboxyl-terminal) mAb clone 67-4H11 or murine IgG (κ) for 2.5 h at 4 °C on a rotator prior to the addition of a 50% slurry of protein A-agarose (20 µl) for 90 min. Immunoprecipitates were centrifuged (10,000 × g, 3 min, 4 °C), washed with lysis buffer (5 × 1 ml), and protein solubilized with 50 µl of 5× Laemmli buffer and processed for MT1-MMP immunoblotting as described above. To evaluate MT1-MMP activity, immunoprecipitations using anti-MT1-MMP hinge antibodies were performed using lysates from cells that were not exposed to cross-linking agent. Lysates were evaluated by gelatin zymography as described above.

**Stable Transfection of MT1-MMP**—The eukaryotic plasmid vector pCR3.1-Uni (Invitrogen, Carlsbad, CA) containing the MT1-MMP gene under a cytomegalovirus promoter was the generous gift of Dr. Duanqing Pei (University of Minnesota). A 6 histidine repeat was incorporated 3' of the carboxyl terminus using a polymerase chain reaction based approach with a 5' primer of ATgggCAGCgATgAAgTC and 3' primer of CgTCTAgATCAGTgATgATggTggTgATggACCTTgTCCAgCaggA. The amplified polymerase chain reaction product was cloned back into the vector using a unique *FseI* restriction site and the *XbaI* restriction site in the 3' polylinker region of the vector. Plasmid DNA was sequenced and then isolated for transfection experiments using a Qiafilter maxi-kit (Qiagen, Valencia, CA). The vector control used in experiments is the pCR3.1-Uni vector driving the chloramphenicol transferase gene (pCR3.1/CAT, Invitrogen). pCR3.1/MT1-His<sub>6</sub> and control pCR3.1/CAT were transfected with LipofectAMINE (Pierce) as a delivery vehicle and isolated transformants screened for neomycin resistance selection (450 µg/ml G418). Soluble recombinant MT1-MMP lacking the linker from the carboxyl-terminal hemopexin domain through the cytoplasmic tail was expressed in CHO cells using the pW1HG vector. Soluble MT1-MMP purified from CHO serum-free conditioned medium was examined by electrophoresis on SDS-polyacrylamide gels and silver staining to visualize proteins.

**Immunocytochemistry**—Cells were cultured on glass coverslips overnight prior to addition of a 1-ml volume of serum-free medium containing 0.005% β<sub>1</sub> integrin mAb-coated beads. Cells were fixed with 3.7% formaldehyde without permeabilization and incubated with anti-MT1-MMP polyclonal antibody (MTK3) or normal rabbit serum in PBS. Immunoreactivity was visualized with a fluorescein-conjugated anti-rabbit secondary antibody previously purified against cross-reactivity to mouse immunoglobulins (Chemicon). Phase contrast and indirect fluorescent images were collected using a Zeiss fluorescence microscope (model II) and Adobe PhotoShop Software (version 4.0).

**Cellular Migration and Invasion**—Type I collagen was dissolved in 0.5 M acetic acid at a concentration of 2 mg/ml. For invasion experiments, the collagen stock was neutralized with 100 mM Na<sub>2</sub>CO<sub>3</sub> (pH 9.6) to a final concentration of 0.4 mg/ml. Transwell inserts (0.8 µm, Becton Dickinson, Bedford, MA) were coated on the underside with 500 µl of collagen diluted to a concentration of 100 µg/ml at 37 °C for 1 h. Collagen gels were prepared in the inner well by adding 50 µl of collagen (20 µg) at room temperature and allowing gels to air-dry overnight. Collagen-coated inserts were then washed with minimum essential medium three times to remove salts and used immediately. In some experiments, transwell inserts were coated with collagenase-resistant (19), rather than wild type, collagen. Cells were trypsinized, washed with serum-free medium, and 1 × 10<sup>5</sup> cells were added to the inner invasion chamber in a volume of 200 µl. The outer wells contained 400 µl of culture medium (serum-free, except in experiments using murine collagen). To evaluate the MMP dependence of invasion, after a 2-h incubation, the MMP inhibitors TIMP-1 (10 nM), TIMP-2 (10 nM), or MMPI (10 µM) were added to the inner and outer chambers as indicated. Control wells for MMPI contained the solvent Me<sub>2</sub>SO. Cells were allowed to invade for 24–48 h as indicated; non-invading cells were removed from inner wells using a cotton swab, and invading cells adherent to the bottom of membrane were fixed and stained using a Diff-Quick staining kit (DADE AG, Miami, FL). Invading cells were enumerated by dividing membranes into 4 quadrants and counting the number of cells in 3 distinct areas for each quadrant under a 10× objective using an ocular micrometer. Assays were performed in triplicate. To evaluate the integrin dependence of invasion, after a 2-h incubation anti-integrin antibodies (15 µg/ml) or control IgG (15 µg/ml) were added to the inner and outer chambers, as indicated. To evaluate the collagen structural requirements of invasion, wells were coated with thermally denatured collagen (gelatin, 50 µg/well in a 50-µl volume) and allowed to invade for 24 h in the presence of TIMP-1 (10 nM),

TABLE I

Rapid attachment of DOV13 cells requires α<sub>2</sub>β<sub>1</sub> and α<sub>3</sub>β<sub>1</sub> integrins

DOV13 cells (2 × 10<sup>4</sup>/well) were preincubated with the indicated concentration of antibodies for 20 min under standard cell culture conditions prior to addition to collagen-coated wells. Cell attachment was measured as described under "Experimental Procedures." DOV13 cells utilize both α<sub>2</sub>β<sub>1</sub> and α<sub>3</sub>β<sub>1</sub> integrins to bind native type I collagen. Values were corrected for control binding to BSA-coated wells.

Matrix + condition	Absorbance at 540 nm
Type I collagen	226 ± 41
+ 15 µg/ml mouse IgG	196 ± 51
+ 15 µg/ml anti-β <sub>1</sub> integrin (P5D2)	48 ± 4 <sup>a</sup>
+ 5 µg/ml anti-α <sub>2</sub> integrin (P1E6)	167 ± 40
+ 15 µg/ml anti-α <sub>2</sub> integrin	126 ± 24 <sup>b</sup>
+ 5 µg/ml anti-α <sub>3</sub> integrin (P1B5)	149 ± 10 <sup>b</sup>
+ 15 µg/ml anti-α <sub>3</sub> integrin	117 ± 23 <sup>b</sup>
+ 7.5 µg/ml anti-α <sub>2</sub> integrin/µg/ml anti-α <sub>3</sub> integrin	83 ± 12 <sup>a</sup>

<sup>a</sup> *p* < 0.05.

<sup>b</sup> *p* < 0.005 relative to IgG controls.

TIMP-2 (10 nM), or aprotinin (20 µg/ml), as indicated. Invasion of native and denatured collagen was also evaluated in the presence of the MMP-2 carboxyl hemopexin-like domain (rCD, 100 nM) (18) or the fibronectin type II-like domain (rCBD, 100 nM) (17) under the conditions described above.

To assess cell motility, migration through transwell membranes coated with a thin layer of collagen (100 µg/ml, 37 °C, 1 h) on both the upper and lower surfaces was evaluated as described above using an incubation time of 5.0 h. Haptotactic motility was assessed as described previously by plating cells on coverslips coated with colloidal gold overlaid with type I collagen (100 µg/ml) (22). Cells were allowed to migrate for 18 h, and phagokinetic tracks were monitored by visual examination using a Zeiss microscope with dark-field illumination. Semi-quantitative analysis of phagokinetic tracks was performed by measuring track area using computer-assisted image analysis and NIH Image.

## RESULTS

**Collagen Structure Regulates Cell Adhesion**—To evaluate the relative contribution of α<sub>2</sub>β<sub>1</sub> and α<sub>3</sub>β<sub>1</sub> integrins to collagen binding, attachment of DOV13 cells to type I collagen in the presence of integrin function-blocking antibodies was evaluated. DOV13 cells adhere rapidly to type I collagen-coated microtiter wells in an integrin-dependent fashion (Table I). Addition of anti-β<sub>1</sub> integrin function-blocking monoclonal antibodies (15 µg/ml, clone P5D2) significantly reduced binding to collagen relative to a nonspecific IgG control (75%), whereas equivalent amounts of either α<sub>2</sub> (P1E6) or α<sub>3</sub> (P1B5) integrin-blocking antibodies inhibited adhesion by 35–40% (Table I). Combination of α integrin blocking antibodies (7.5 µg/ml each) together reduced adhesion nearly 60%, supporting the conclusion that both α<sub>2</sub>β<sub>1</sub> and α<sub>3</sub>β<sub>1</sub> integrin heterodimers contribute to DOV13 cell attachment to type I collagen.

To assess the collagen structural requirements for integrin-mediated binding, adhesion to both collagenase-cleaved and thermally denatured collagen was analyzed. Native type-I collagen was incubated with collagenase-1 (MMP-1) and the ¾ and ¼ digestion products isolated through ammonium sulfate precipitation (21). Intact collagen heterofibrils, also isolated through this approach, were gelatinized by thermal denaturation. Intact collagen, ¾ and ¼ collagen fragments, or gelatin were coated onto microtiter wells at either 25 or 37 °C, and adhesion was evaluated as described above. Both intact collagen and ¾ and ¼ fragments support adhesion of DOV13 cells when matrices are coated at 25 °C; however, adhesion to ¾ and ¼ fragments was significantly reduced when matrices were coated at physiologic temperature (Table II). Although cells will slowly adhere to thermally denatured collagen (3–4 h), little adhesion to this matrix was observed under any coating condition over the course of the assay (75 min) (Table II). These results indicate that ¾ and ¼ collagen fragments coated at

TABLE II  
Intact collagen is required for DOV13 attachment

Type I collagen,  $\frac{3}{4}$  and  $\frac{1}{4}$  collagen fragments, and type I gelatin were coated at the indicated temperature ( $1.6 \mu\text{g}/\text{cm}^2$ ), blocked with BSA, and cell attachment ( $2 \times 10^4/\text{well}$ ) measured as described under "Experimental Procedures." Values are reported as the percent absorbance at 540 nm in comparison to intact type I collagen, coated at the specified temperature.

10 $\mu\text{g}/\text{ml}$ matrix	% Bound relative to collagen
	%
Coating at 25 °C	
Type I collagen	100
Type I gelatin	$28.1 \pm 10.7^a$
$\frac{3}{4}$ and $\frac{1}{4}$ type I collagen	$94.3 \pm 11.7$
Coating at 37 °C	
Type I collagen	100
Type I gelatin	$9.7 \pm 9.2^a$
$\frac{3}{4}$ and $\frac{1}{4}$ type I collagen	$9 \pm 6.3^a$

<sup>a</sup>  $p < 0.005$  relative to intact type I collagen coated at the specified temperature.

25 °C retain a triple helical conformation that is required for recognition by  $\alpha_2\beta_1$  and  $\alpha_3\beta_1$  integrins, whereas coating at 37 °C results in destabilization of fragment helices (21). Together, these data suggest that collagenase activity at the cell/matrix interface can reduce the efficiency of integrin-mediated adhesion on the surface of DOV13 cells, thereby potentially affecting matrix-induced signaling events that are involved in cellular invasion.

**Integrin Clustering Promotes Cell Surface MMP-2 Processing**—To evaluate the matrix structural requirements for collagen induction of pro-MMP-2 activation, cells were cultured in the presence of native or thermally denatured collagen. Processing of pro-MMP-2 was not observed by cells cultured on type I gelatin (Fig. 1, lanes 4 and 5). However, culturing cells with collagenase-resistant collagen (19) stimulated pro-MMP-2 processing as efficiently as wild type collagen (Fig. 1, lanes 6 and 7), indicating that collagen processing is not necessary to promote a cellular gelatinase activation response. Similar to the wild type protein, gelation of collagenase-resistant collagen abrogated the ability to elicit pro-MMP-2 activation (Fig. 1, lane 5). In conjunction with cell adhesion studies, these data indicate that integrin interaction with intact triple-helical collagen is necessary to stimulate pro-MMP-2 activation and that destabilization of the collagen matrix following collagenase activity will reduce the ability of pericellular collagen to elicit an MMP response.

We have demonstrated previously that  $\beta_1$  integrin clustering stimulates a pro-MMP-2 processing event that correlates with enhanced gelatinolytic activity in conditioned media, indicating that integrin signaling is sufficient to elicit pro-MMP-2 activation (12). As adhesion of DOV13 cells to type I collagen is supported by both  $\alpha_2\beta_1$  and  $\alpha_3\beta_1$  integrins (Table I), integrin clustering was induced through the  $\alpha$ -subunit to evaluate the effects of clustering each specific integrin heterodimer on pro-MMP-2 activation. Consistent with adhesion blocking assays,  $\alpha_2$ ,  $\alpha_3$ , and  $\beta_1$  integrin antibody-coated beads attached with similar efficacy (data not shown); however, analysis of conditioned media by zymography demonstrates that clustering  $\alpha_3$  or  $\beta_1$  integrins elicits a stronger pro-MMP-2 activation response than  $\alpha_2$  integrins (Fig. 2A, lower panel). The observed difference in  $\alpha$  integrin subunit specificity was independent of the antibody clone used to promote clustering, and utilization of antibodies recognizing an  $\alpha_3\beta_1$  heterodimer-specific epitope also resulted in a pro-MMP-2 activation response (Fig. 2A, lower panel, lane 8). Soluble integrin antibodies did not influence pro-MMP-2 expression or activation (Fig. 2A, upper panel), supporting the hypothesis that multivalent integrin

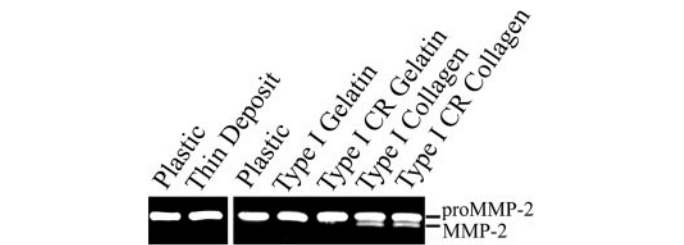


FIG. 1. **Collagen stimulation of pro-MMP-2 activation.** DOV13 cells ( $2.5 \times 10^5$  cells/well) were incubated on plastic or a thin deposit of type I collagen ( $1.1 \mu\text{g}/\text{cm}^2$ ), in the presence of thermally denatured wild type or collagenase-resistant (CR) collagen (Gelatin or CR Gelatin, respectively,  $158 \mu\text{g}/\text{cm}^2$ ), or within wild type or CR type I collagen gels (Collagen or CR Collagen, respectively,  $158 \mu\text{g}/\text{cm}^2$ ), as indicated, in serum-free medium for 48 h. Conditioned media were analyzed by gelatin zymography. The relative migration positions of pro- and active MMP-2 are indicated.

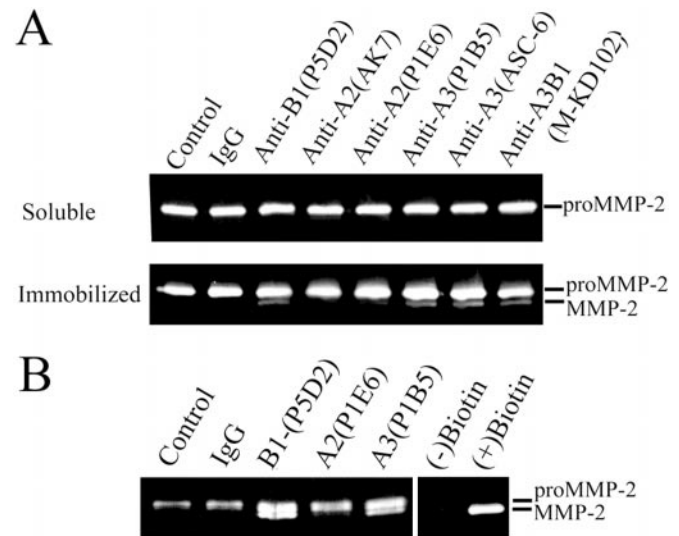


FIG. 2.  **$\alpha_3\beta_1$  integrin clustering promotes MMP-2 processing and surface association.** A, DOV13 cells were treated with the indicated soluble ( $10 \mu\text{g}/\text{ml}$ ) (upper panel) or bead-immobilized ( $4 \mu\text{g}/\text{ml}$ ) (lower panel) antibodies for 20 h, and conditioned media were analyzed by gelatin zymography. Antibody clone numbers are given in parentheses. The relative migration positions of pro- and active MMP-2 are indicated. B, cells were untreated (Control) or treated with the indicated bead-immobilized antibodies ( $12 \mu\text{g}/\text{ml}$ ) for 20 h prior to biotinylation of surface proteins using a non-cell-permeable biotin and cell lysis. Labeled protein ( $750 \mu\text{g}$ ) was captured with monomeric avidin gels, eluted with D-biotin, and eluates analyzed by gelatin zymography. The lack of activity eluted from non-biotinylated ConA-treated ( $20 \mu\text{g}/\text{ml}$ ) cell lysates (–Biotin) demonstrates the specificity of the system for cell surface proteins, as shown in the biotinylated, ConA-treated ( $20 \mu\text{g}/\text{ml}$ ) control (+Biotin).

aggregation is necessary for proteinase induction.

As the transmembrane proteinase MT1-MMP is predicted to catalyze cell surface integrin-mediated pro-MMP-2 activation, MMP-2 association with the plasma membrane was evaluated by surface labeling with cell-impermeable s-NHS-biotin and isolating biotinylated proteins with monomeric avidin-agarose gels. Proteins were eluted from avidin-agarose gels using 10 mM free D-biotin and analyzed by zymography for MMP activity. Clustering of both  $\alpha_3$  and  $\beta_1$  integrins promoted a significant association of MMP-2 to the cell surface (Fig. 2B, lanes 3 and 5) relative to cells subjected to  $\alpha_2$  integrin clustering (Fig. 2B, lane 4) or controls (Fig. 2B, lanes 1 and 2), confirming that  $\alpha_3\beta_1$  integrins elicit a more robust pro-MMP-2 processing response.

**Release of MMP-2 from the Cell Surface**—Secreted pro-MMP-2 binds to the cell surface through the carboxyl-terminal hemopexin domain, and an intermediately processed form is

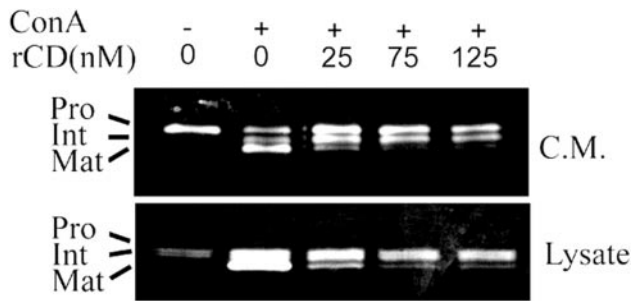


FIG. 3. **Competition of MMP-2 from the cell surface.** Cells were cultured for 20 h in the presence or absence of ConA (20  $\mu$ g/ml) and recombinant MMP-2 carboxyl hemopexin domain (rCD, 0–125 nM), as indicated. Conditioned media (C.M.) and cell lysates (5  $\mu$ g of total protein) were analyzed by gelatin zymography for processed forms of MMP-2. *Pro*, pro-MMP-2 (66 kDa); *Int*, intermediately processed MMP-2 (64 kDa); *Mat*, mature MMP-2 (62 kDa).

generated through MT1-MMP-mediated cleavage of the MMP-2 pro-domain at the Asn<sup>37</sup>–Leu<sup>38</sup> peptide bond (7). The MMP-2 intermediate is then predicted to undergo maturation to an active enzyme through concentration-dependent autolysis at the cell surface (8). Although formation of the trimeric MT1-MMP·TIMP-2·MMP-2 activation complex at the cell surface is well established (reviewed in Ref. 3), the mechanism by which active MMP-2 is released from the plasma membrane is presently unclear. Saturation binding studies are not technically feasible due to the instability of the intermediate and mature forms of the activated enzyme in concentrated solution. Thus, the ability of the MMP-2 hemopexin domain to dissociate surface-bound MMP-2 was evaluated. In the presence of increasing concentrations of recombinant hemopexin domain, active MMP-2 is not associated with the cell surface (Fig. 3A). In control experiments, competition of MMP-2 from the cell surface was not observed with rCBD123 at the same molar concentrations (data not shown).

**Integrin Clustering Promotes Cell Surface MT1-MMP Processing**—The appearance of active MMP-2 on the cell surface implicates the involvement of MT1-MMP in integrin stimulation of pro-MMP-2 processing. To evaluate the effect of  $\alpha$  integrin clustering on events further upstream in the zymogen activation pathway, cells were treated with integrin antibody-coated beads, and cell lysates were immunoblotted with an antibody reactive against the hinge domain of MT1-MMP. Consistent with previous observations (12), stimulation of DOV13 cells with ConA promotes accumulation of 55- and 43-kDa forms of MT1-MMP in cell lysates (Fig. 4A, lane 6). Clustering of  $\beta_1$  integrins, and to a lesser extent  $\alpha_3$  integrins, promoted processing of MT1-MMP to a 43-kDa form (Fig. 4A, lanes 3 and 5, respectively), whereas little or no change in MT1-MMP expression was observed following clustering of  $\alpha_2$  integrins (lane 4) or under control conditions with nonspecific IgG (lane 2). Furthermore,  $\beta_1$  integrin clustering results in a small, but reproducible, accumulation of 55-kDa MT1-MMP in cell lysates (lane 3).

To address whether integrin clustering stimulates MT1-MMP expression on the cell surface, biotin-labeled cell surface proteins were isolated and immunoblotted for MT1-MMP species. ConA treatment promoted a strong accumulation of 55- and 43-kDa MT1-MMP species on the cell surface (Fig. 4B, lane 1). Higher molecular weight species that were detected in immunoblots of cell lysates (brackets, Fig. 4A) were absent in cell surface preparations (brackets, Fig. 4B), indicating that these proteins are likely intracellular. Consistent with cell lysate results, clustering of  $\beta_1$  integrins promoted cell surface expression of 55- and 43-kDa forms of MT1-MMP (Fig. 4B, lane 5), whereas clustering of either  $\alpha$  integrin subunit was not suffi-

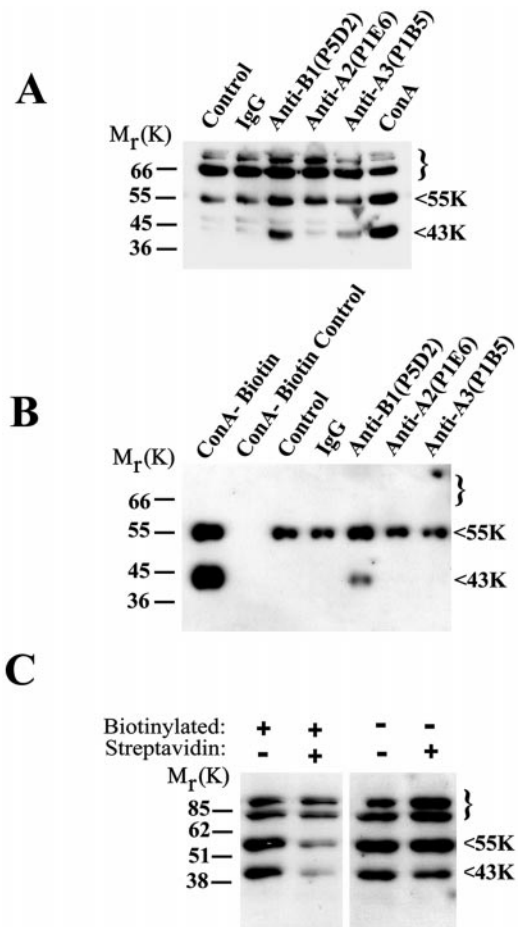
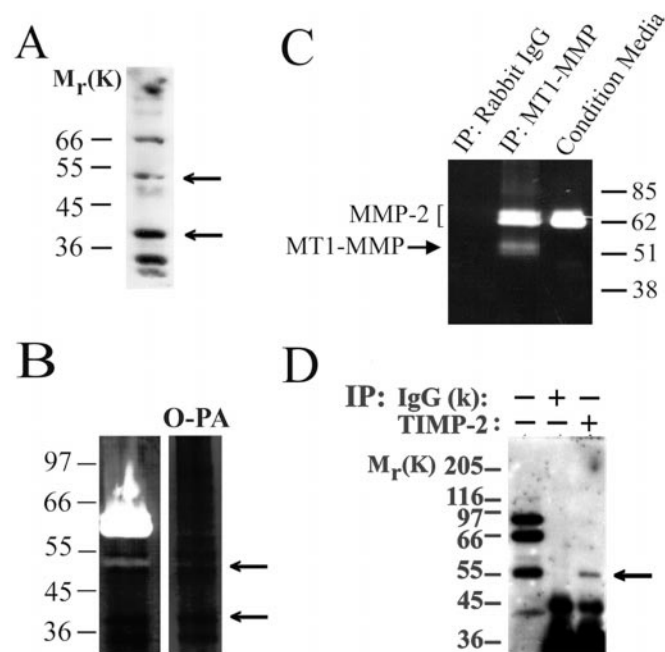


FIG. 4. **Integrin clustering promotes MT1-MMP cell surface expression and processing.** A, integrin-induced MT1-MMP processing. Cells were treated for 20 h with bead-immobilized antibodies (4  $\mu$ g/ml) as indicated, and lysates (10  $\mu$ g for all samples except ConA, 7  $\mu$ g) were electrophoresed on 8% polyacrylamide gels and Western-blotted using an antibody to the MT1-MMP hinge region. Blots were developed using enhanced chemiluminescence. The migration position of molecular weight standards is indicated in the left margin, and the migration positions of the 55- and 43-kDa species of MT1-MMP are indicated in the right margin. B, cells were treated with ConA (20  $\mu$ g/ml) or the indicated bead-immobilized antibodies (12  $\mu$ g/ml) for 20 h prior to biotinylation of surface proteins using a non-cell-permeable biotin and cell lysis. Labeled protein (750  $\mu$ g) was captured with monomeric avidin gels, eluted with D-biotin, and eluates analyzed by electrophoresis and Western blotting for MT1-MMP. The lack of protein eluted from non-biotinylated ConA-treated cell lysates (lane designated *ConA-Biotin Control*) demonstrates the specificity of the system. C, to evaluate what fraction of the total pool of 55- and 43-kDa MT1-MMP species resides on the cell surface, cells were stimulated with ConA (20  $\mu$ g/ml) for 20 h, surface-biotinylated, lysed, and the lysate (750  $\mu$ g, + Biotin) depleted of biotin-labeled proteins through precipitation using multimeric streptavidin-conjugated agarose gels (+ *Streptavidin*). Streptavidin-depleted or control lysates (7.5  $\mu$ g) were analyzed by electrophoresis and Western blotting for MT1-MMP.

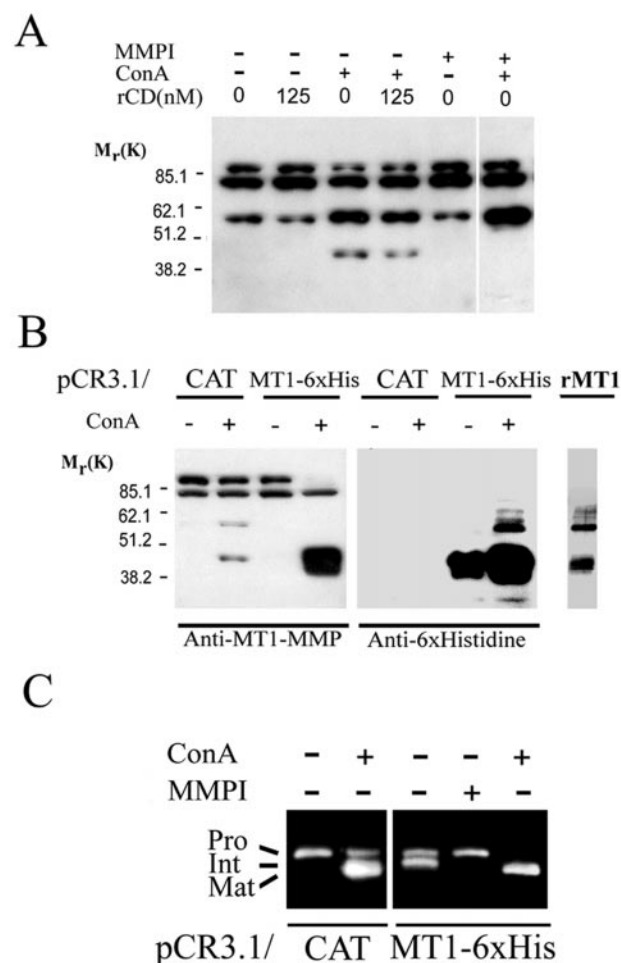
cient to promote detectable changes in the cell surface expression profile of MT1-MMP (Fig. 4B, lanes 6 and 7). Together, these data suggest that clustering of  $\beta_1$  integrins promotes pro-MMP-2 cell surface binding and activation through increased expression of cell surface-localized MT1-MMP. Furthermore, cell surface gelatinolytic profiles indicate that  $\alpha_3\beta_1$  integrins potentiate a stronger MMP response than  $\alpha_2\beta_1$  integrins (Fig. 2B). The ability to detect enhanced levels of cellular, but not cell surface, MT1-MMP following  $\alpha_3$  integrin clustering likely reflects the technical limitations of the assay, as  $\beta_1$  integrin clustering induces a more robust overall response. To evaluate further the pool of MT1-MMP species that reside on the cell surface, cells were stimulated with ConA, surface



**FIG. 5. Identification of active MT1-MMP species.** DOV13 cells were cultured in the presence of ConA (20  $\mu\text{g}/\text{ml}$ ) for 20 h, lysed, and homogenized. Plasma membranes were isolated by centrifugation, and aliquots (20  $\mu\text{g}$ ) were analyzed for MT1-MMP protein (A) and activity (B) by immunoblotting and gelatin zymography, respectively. A, Western blot of plasma membranes showing proteins cross-reactive with MT1-MMP antibody. The arrows indicate the migration positions of the 55- and 43-kDa MT1-MMP species. B, zymogram depicting MT1-MMP gelatinolytic activity associated with the 55-kDa species (upper arrow). (Note that the prevalent gelatinase activity in this sample is due to plasma membrane-bound MMP-2.) All gelatinase activity is inhibited by the zinc chelating agent *ortho*-phenanthroline (O-PA). C, immunoprecipitation (IP) of DOV13 cell lysates. Lysates (non-denatured) were immunoprecipitated with either IgG control or anti-MT1-MMP antibodies as indicated and analyzed by gelatin zymography. Although the immunoprecipitating antibody recognizes both the 43- and 55-kDa forms of MT1-MMP by immunoblotting (A), gelatinase activity corresponding to only the 55-kDa species is observed (arrow). MMP-2 coprecipitated as a component of the ternary complex, as evidenced by the co-migration with an MMP-2 standard (bracket). Lane designated *conditioned media*, DOV13 conditioned medium to designate immunoprecipitation position of MMP-2 (not subjected to immunoprecipitation). D, cross-linking and immunoprecipitation. Cells were treated with ConA (20  $\mu\text{g}/\text{ml}$ ) and an excess of free TIMP-2 (125 ng/ml) for 18 h. After washing, cell surface proteins were cross-linked with a reducible cross-linking agent (2 mM 3,3'-dithiobis(sulfosuccinimidylpropionate), 25 min, 4  $^{\circ}\text{C}$ ). Clarified cell lysates (700  $\mu\text{g}$ ) were incubated with 5  $\mu\text{g}$  of anti-TIMP-2 (carboxyl-terminal) mAb clone 67-4H11 or murine IgG ( $\kappa$ ) as indicated and precipitated using protein A-agarose. Immunoprecipitates were solubilized with Laemmli sample buffer, electrophoresed under reducing conditions on 8% polyacrylamide gels, and immunoblotted with the MT1-MMP hinge polyclonal antibody. The arrow designates the migration position of 55-kDa MT1-MMP, and the left margin indicates the migration position of molecular weight standards.

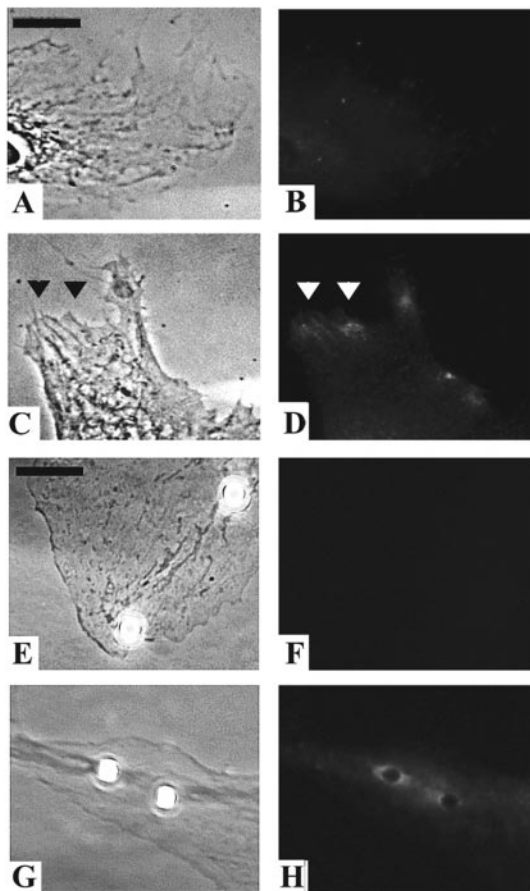
biotin-labeled, and lysates were depleted of biotinylated cell surface proteins using multimeric streptavidin-conjugated agarose. A selective depletion of only the 55- and 43-kDa forms of MT1-MMP was observed in streptavidin-treated samples compared with non-treated controls (Fig. 4C, lane 2), supporting the conclusion that these species, but not the higher molecular weight immunoreactive material present in Fig. 4A, are stably expressed on the cell surface.

**Characterization of Cell Surface MT1-MMP**—As MT1-MMP has been reported to have weak gelatinase activity (30, 31), plasma membrane preparations of ConA-treated DOV13 cells were analyzed by gelatin zymography to determine whether either of the surface-associated MT1-MMP species are proteolytically active. In addition to surface-bound MMP-2, an *ortho*-



**FIG. 6. MT1-MMP processing in the absence of cell surface MMP-2.** A, cells were cultured in the presence or absence of MMPI (10  $\mu\text{M}$ ), ConA (20  $\mu\text{g}/\text{ml}$ ), or rCD (125 nM) as indicated. Lysates (7.5  $\mu\text{g}$ ) were electrophoresed on 8% polyacrylamide gels and immunoblotted for MT1-MMP. B, stable DOV13 transfectants expressing polyhistidine-tagged MT1-MMP (MT1-6xHis) or control vector (CAT) were isolated, cultured in the presence or absence of ConA (20  $\mu\text{g}/\text{ml}$ ) as indicated, and 7.5  $\mu\text{g}$  of cell lysates analyzed by electrophoresis and immunoblotting for MT1-MMP (lanes 1–4) or polyhistidine (lanes 4–8). Lane 9 contains soluble recombinant MT1-MMP (rMT1) lacking the transmembrane and cytoplasmic domain purified from CHO cell-conditioned medium and visualized by silver staining. C, DOV13 stable transfectants expressing polyhistidine-tagged MT1-MMP (MT1-6xHis) or vector controls (CAT) were isolated, cultured in the presence or absence of ConA (20  $\mu\text{g}/\text{ml}$ ) or MMPI (10  $\mu\text{M}$ ) as indicated, and conditioned media analyzed for pro-MMP-2 activation by gelatin zymography. The relative migration positions of the pro-, intermediate (Int), and activated mature (Mat) forms of MT1-MMP are indicated in the left margin.

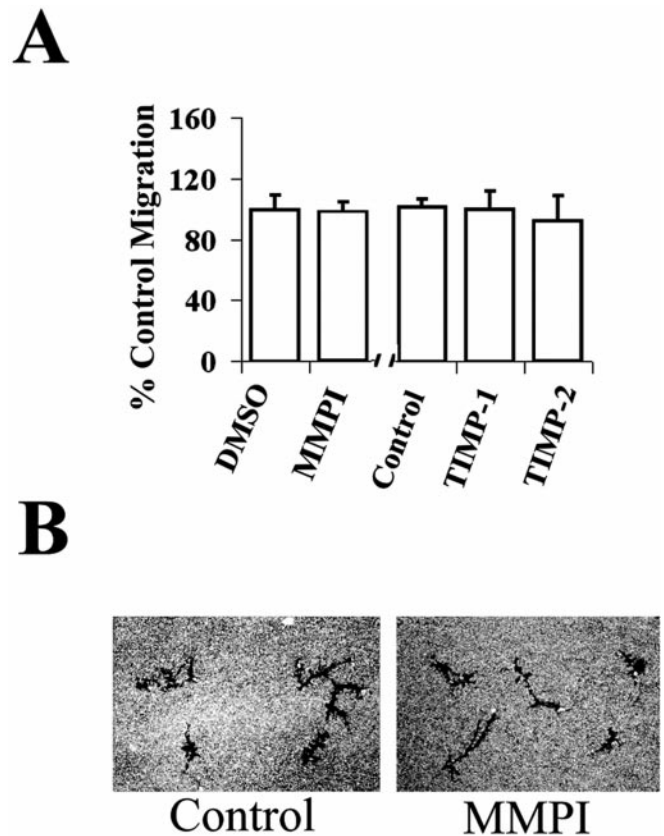
phenanthroline-sensitive gelatinolytic band that co-migrated with the non-reduced 55-kDa form of MT1-MMP was observed (Fig. 5B), whereas no gelatinase activity was attributable to the 43-kDa species. Control immunoblots demonstrate that both the 55- and 43-kDa species were prevalent in the experimental sample (Fig. 5A). To confirm that the observed 55-kDa gelatinolytic activity is a property of MT1-MMP, cell lysates were immunoprecipitated with an anti-MT1-MMP-specific antibody and the immunoprecipitates were analyzed by gelatin zymography. A 55-kDa gelatinolytic activity was recovered together with MMP-2, suggesting that both proteinases co-precipitate as components of the ternary complex (Fig. 5C). The relative ratio of MT1-MMP to MMP-2 gelatinase activity is enhanced by the immunoprecipitation approach (Fig. 5, C versus B). Similar to results obtained in whole plasma membrane preparations, there was no observable gelatinolytic activity attributable to



**FIG. 7. MT1-MMP localizes to cellular processes and redistributes to clustered  $\beta_1$  integrins.** Cells were cultured on glass coverslips in the absence (A–D) or presence (E–H) of anti- $\beta_1$  integrin (clone 21C8)-coated beads for 8 h and then processed for immunofluorescence as described under “Experimental Procedures.” Cells were incubated with normal rabbit serum (B and F) or anti-MT1-MMP antibody (D and H). Immunoreactivity was visualized with fluorescein-conjugated anti-rabbit secondary antibody previously purified against cross-reactivity to mouse immunoglobulins. Phase contrast images A, C, E, and G correspond with B, D, F, and H, respectively, and identify cellular processes and 2.97  $\mu\text{m}$  diameter latex beads. Arrowheads denote MT1-MMP immunoreactivity in cell surface projections. Magnification bar is 5  $\mu\text{m}$  (A–D) or 10  $\mu\text{m}$  (E–H), and images were collected with a 63 $\times$  objective using a Zeiss fluorescence microscope.

the 43-kDa form of MT1-MMP. As these results indicated that the 55-kDa form of MT1-MMP is an active species on the cell surface, the ability to bind TIMP-2 was assessed. Exogenous TIMP-2 was added directly to ConA-treated cells, followed by cross-linking with a reducible, cell-impermeable cross-linker. Cell lysates were then immunoprecipitated with an antibody specific to the carboxyl-terminal domain of TIMP-2, reduced, and analyzed by electrophoresis and immunoblotting for MT1-MMP. The 55-kDa species of MT1-MMP was specifically precipitated through TIMP-2 (Fig. 5D, lane 3), providing additional evidence that it is an active, TIMP-2-binding protein.

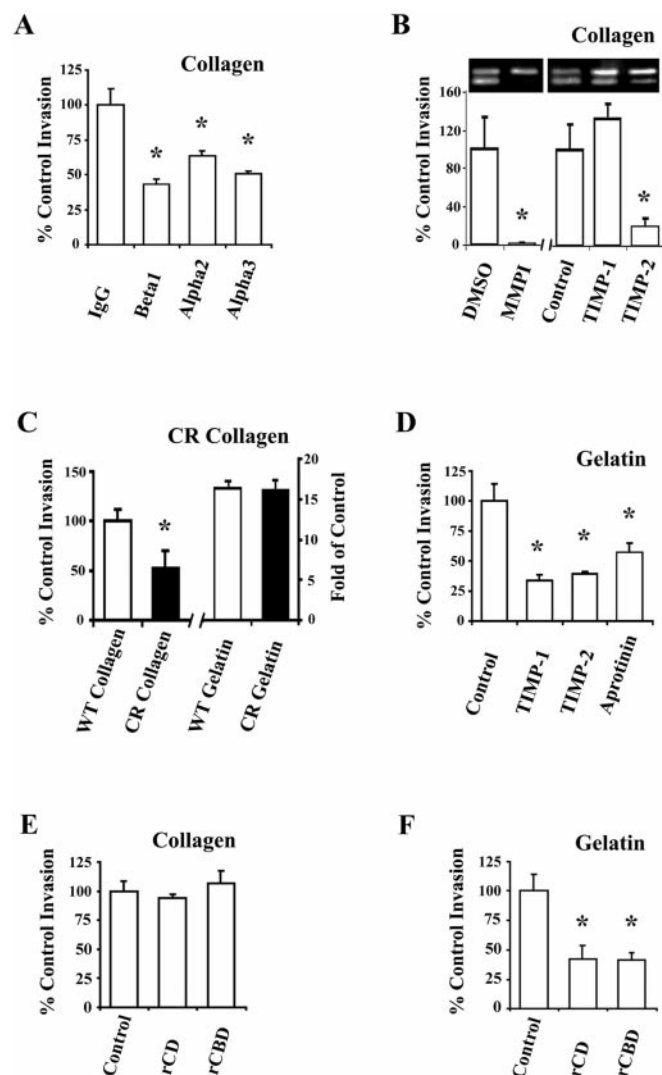
**MT1-MMP Processing in the Absence of Cell Surface MMP-2**—As reported previously (12), active 55-kDa MT1-MMP is converted to an inactive 43-kDa form through MMP-dependent proteolysis in DOV13 cells (Fig. 6A, compare lanes 3 and 6) (12). In a regulated cellular system generating an endogenous MMP-2/MT1-MMP activation response, it is unclear whether this cleavage is mediated by activated MMP-2 or through concentration-dependent autolysis of MT1-MMP. As active MMP-2 is effectively removed from the cell system with 125 nM heparin (Fig. 3), cellular MT1-MMP processing in the absence of MMP-2 was assessed by immunoblotting. Con-



**FIG. 8. Migration of DOV13 cells is MMP-independent.** A, cells ( $2.5 \times 10^5$ ) were seeded onto Transwell filters (8  $\mu\text{m}$  pore) coated with a thin layer of collagen (as described under “Experimental Procedures”) in the presence or absence of MMPI (10  $\mu\text{M}$ ), Me<sub>2</sub>SO (DMSO, vehicle for MMPI), TIMP-1 (10 nM), or TIMP-2 (10 nM) as indicated and incubated for 5 h to permit migration. Non-migrating cells were removed from the upper chamber; filters were stained, and migrating cells adherent to the underside of the filter were enumerated using an ocular micrometer. Data are expressed as % of control migration (Me<sub>2</sub>SO for MMPI and PBS for TIMP-1 and -2). There is no significant difference in migration of inhibitor-treated samples relative to respective controls ( $p > 0.05$ ). B, haptotactic motility of DOV13 cells. Cells (1000) were plated onto coverslips coated with colloidal gold overlaid with type I collagen (100  $\mu\text{g}/\text{ml}$ ) in the presence or absence of MMPI (10  $\mu\text{M}$ ) as indicated, allowed to migrate for 24 h, and phagokinetic tracks visualized using dark field illumination.

sion of 55-kDa MT1-MMP to the 43-kDa form was not affected by the loss of active MMP-2 from the cell surface (Fig. 6A, lanes 3 and 4), indicating that MMP-2 is not required for proteolytic processing of endogenous 55-kDa MT1-MMP in DOV13 cells.

To determine whether MT1-MMP degradation proceeds through autolysis or through activity of an unidentified metalloprotease, DOV13 cells that stably overexpress full-length histidine-tagged MT1-MMP were generated. Overexpression of MT1-MMP resulted in accumulation of 40–45-kDa species of recombinant enzyme as detected by Western blotting with anti-MT1-MMP (Fig. 6B, lane 4) and confirmed using an antibody against the polyhistidine label (Fig. 6B, lanes 7 and 8). Similar results were obtained following expression of soluble recombinant MT1-MMP lacking the transmembrane and cytoplasmic domain, in which autolytic processing to 40–45-kDa species was also observed (Fig. 6B, lane 9, labeled *rMT1*), supporting the conclusion that the 55-kDa MT1-MMP-processing activity is directly attributable to the level of cell surface MT1-MMP activity (32). Together, these data indicate that active 55-kDa MT1-MMP undergoes concentration-dependent autolysis to an inactive 43-kDa form on the surface of DOV13 cells. Control experiments demonstrated MMP-dependent processing of pro-



**FIG. 9. Characterization of the proteinase requirements for invasion.** A–C and E, invasion of collagen. Cells ( $1.0 \times 10^5$ ) were added to collagen-coated Transwell chambers ( $20 \mu\text{g}$ ) in serum-free medium for 2 h prior to addition of reagents indicated below and allowed to invade for 48 h under the indicated conditions. Following invasion, cells were removed from the top chamber with a cotton swab; membranes were stained, and invading cells were enumerated using an ocular micrometer. C, D, and F, invasion of gelatin. Cells ( $1.0 \times 10^5$ ) were added to gelatin-coated Transwell chambers ( $50 \mu\text{g}$ ) in serum-free medium for 2 h prior to addition of reagents indicated and allowed to invade for 24 h under the indicated conditions. Invading cells were quantified as described. A, integrin subunit-specific antibodies block invasion of collagen. Integrin subunit-specific antibodies or control IgG ( $15 \mu\text{g}/\text{ml}$ ) were added as indicated. Results are expressed as % of control invasion (IgG), normalized to 100%. (\*,  $p < 0.05$  relative to control.) B, effect of MMP inhibitors on collagen invasion. Invasion was quantified in the presence or absence of MMPI ( $10 \mu\text{M}$ ), TIMP-1 ( $10 \text{ nM}$ ), or TIMP-2 ( $10 \text{ nM}$ ), as indicated. Results are expressed as % of control invasion. MMPI data are normalized to Me<sub>2</sub>SO controls (designated 100%), and TIMP-1 and -2 data are normalized to PBS controls (designated 100%). (\*,  $p < 0.05$  relative to control.) *Inset*, analysis of conditioned media from invasion chambers by gelatin zymography. C, invasion of wild type and CR collagen gels. Cells were allowed to invade gels composed of wild type or CR collagen or wild type and CR gelatin, as indicated. Invasion of CR collagen is expressed as % of control invasion (with wild type collagen designated 100%). Invasion of gelatin is designated as fold increase relative to wild type collagen control. (\*,  $p < 0.05$  relative to control.) D, effect of proteinase inhibitors on invasion of gelatin. Invasion was evaluated in the presence or absence of TIMP-1 ( $10 \text{ nM}$ ), TIMP-2 ( $10 \text{ nM}$ ), or aprotinin ( $20 \mu\text{g}/\text{well}$ ), as indicated. Invasion of gelatin is expressed as % of control invasion (designated 100%). (\*,  $p < 0.05$  relative to control.) E, effect of MMP-2 domains on collagen invasion. Invasion was quantified in the presence or absence of rCD ( $100 \text{ nM}$ ) or rCBD ( $100 \text{ nM}$ ), as indicated. Results are expressed as

MMP-2, confirming surface expression of the recombinant enzyme in DOV13 cells (Fig. 6C).

**Localization of Cell Surface MT1-MMP**—In previous studies, MT1-MMP has been localized to membrane protrusions, termed invadopodia, which are rich in matrix proteinases and integrins, including  $\alpha_3\beta_1$  integrins (27–29). Immunocytochemical analysis of MT1-MMP surface staining in non-permeabilized DOV13 cells demonstrates MT1-MMP localization to distinct cell surface projections (Fig. 7D), characteristic of integrin-rich sites of cell-matrix contact. Following  $\beta_1$  integrin clustering using antibody-coated beads, MT1-MMP immunoreactivity is substantially redistributed to the periphery of the aggregated integrins (Fig. 7H). These data indicate that MT1-MMP can be actively recruited to membrane sites containing clustered  $\beta_1$  integrins on the surface of DOV13 cells.

**MMP Dependence of Migration and Collagen Gel Invasion**—Three-dimensional collagen culture and clustering of collagen binding integrins up-regulate surface MMP activity in DOV13 cells. To assess the functional consequences of matrix-enhanced proteolytic potential, the ability of cells to migrate and to invade a three-dimensional collagen matrix was evaluated. MMP activity was not required for general cell motility, as cells migrated through uncoated transwell filters with equal efficiency in the presence or absence of a broad spectrum MMP inhibitor (MMPI), TIMP-1, or TIMP-2 (Fig. 8A). Haptotactic motility over collagen-coated colloidal gold surfaces is also unaffected by a broad spectrum MMP inhibitor (Fig. 8B). Semi-quantitative analysis of phagokinetic tracks from 30 cells in the absence and presence of MMPI using computer-assisted image analysis gave relative migration areas of  $1.4 \pm 0.1$  and  $1.5 \pm 0.3$ , respectively, indicating that MMP activity does not contribute to collagen-driven migration of DOV13 cells.

DOV13 cells efficiently penetrate three-dimensional collagen gels via  $\alpha_2\beta_1$  and  $\alpha_3\beta_1$  integrin receptors, and obstructing collagen-induced integrin clustering using integrin function blocking antibodies inhibits invasion (Fig. 9A). Invasion correlates with collagen-induced pro-MMP-2 processing (Fig. 9B, *inset*), and in contrast to cellular migration, invasion of collagen is MMP-dependent, as both a broad spectrum MMPI and exogenous TIMP-2 abrogate invasion and reduce or eliminate pro-MMP-2 processing (Fig. 9B, *lanes 2 and 5*). TIMP-1, a poor inhibitor of MT1-MMP (26), failed to reduce either collagen invasion or MT1-MMP-mediated pro-MMP-2 processing (Fig. 9B), suggesting that MT1-MMP collagenolytic activity may potentiate invasion. The serine proteinase inhibitor aprotinin had no effect on invasion of intact collagen gels (not shown). Furthermore, although collagenase-resistant collagen is sufficient to stimulate pro-MMP-2 activation (Fig. 1), cellular penetration of this matrix is significantly inhibited relative to wild type collagen (Fig. 9C), providing further support for the hypothesis that collagenolysis is required for invasive activity. Disrupting the triple helical structure of collagen by thermal denaturation removes the requirement for collagenase activity, as no difference in invasion of cells through gelatin derived from either wild type or collagenase-resistant collagen is observed (Fig. 9C). This is supported by data using wild type gelatin, in which invasion is effectively blocked by both TIMP-1 and -2 as well as by the serine proteinase inhibitor aprotinin (Fig. 9D), indicating that additional (non-MMP) gelatinolytic proteinases potentiate invasion following destabilization of collagen triple helical structure. Collagen invasion is unaltered in

% of control invasion (designated 100%). F, effect of MMP-2 domains on gelatin invasion. Invasion was quantified in the presence or absence of rCD ( $100 \text{ nM}$ ) or rCBD ( $100 \text{ nM}$ ), as indicated. Results are expressed as % of control invasion (designated 100%). (\*,  $p < 0.05$  relative to control.)

the presence of either the rCD or rCBD of MMP-2 (Fig. 9E). However, both the rCD, which inhibits MMP-2 cell surface activation (Fig. 3) (6, 8), and rCBD, which prevents MMP-2 binding to native and denatured collagen (17), effectively inhibit the MMP-2-dependent component of gelatin invasion (Fig. 9F). Together these data suggest that the stimulation of MT1-MMP collagenolytic activity (31) through collagen-binding integrins is a rate-limiting step for invasion of native type I collagen-rich matrices.

#### DISCUSSION

MT1-MMP is a cell surface activator of pro-MMP-2 and has been implicated in collagen invasion and turnover (33–36). In this study, DOV13 ovarian cancer cells activate MT1-MMP as a consequence of culture in type I collagen gels and display MMP-dependent invasion of type I collagen, indicating that MMP activity is required for removal of collagen matrix constraints during invasion. However, migration over two-dimensional collagen is not impeded by MMP inhibitors. Although TIMP-1 does not interact with MT1-MMP, TIMP-2 specifically binds the proteinase, functioning in both inhibition and stabilization of the enzyme on the cell surface (26, 32). The ability of exogenous TIMP-2, in contrast to TIMP-1, to inhibit DOV13 collagen gel invasion implicates a cell surface proteolytic cascade initiated by MT1-MMP. This is further supported by data demonstrating that inhibition of MMP-2 cellular activation or collagen binding using rCD and rCBD (17, 18), respectively, has no effect on collagen invasion. As the cellular events that govern the collagen-induced MMP-2/MT1-MMP response are unclear and technically difficult to assess in three-dimensional collagen gel systems, a variety of biochemical approaches were employed in this study to dissect the interplay between collagen-cell interactions and regulation of cell surface MMP activity.

DOV13 cells bind type I collagen via  $\alpha_2\beta_1$  and  $\alpha_3\beta_1$  integrins. Recognition of collagen by these integrins depends on retention of the triple helical conformation, as thermal gelation of collagen abrogates cellular adhesion. Collagenase-cleaved type I collagen produces  $\frac{3}{4}$  and  $\frac{1}{4}$  fragments that display a lower  $T_m$  than intact fibrils (37). Adhesion data in the current study demonstrate that the triple helical conformation of the collagen fragments is stabilized at low coating temperatures but is lost under physiological coating conditions (21). Together, these data suggest that pericellular type I collagenolysis will reduce  $\alpha_2\beta_1$  and  $\alpha_3\beta_1$  integrin-mediated cell-matrix contacts. By using a similar approach, the appearance of cryptic  $\alpha_V\beta_3$  integrin-binding sites (RGD) in collagenase-generated collagen fragments was reported (21). However, DOV13 cells adhere weakly to type I gelatin or  $\frac{3}{4}$  and  $\frac{1}{4}$  fragments, and aggregation of  $\alpha_V\beta_3$  integrins on the surface of DOV13 cells does not elicit a cellular MMP processing response (12, 20). Nevertheless, the exposure of cryptic  $\alpha_V\beta_3$ - or  $\alpha_V\beta_5$ -binding sites in collagenase-cleaved collagen may further influence MMP expression in a cell type-specific manner. In support of this observation, we have previously demonstrated that vitronectin-induced aggregation of melanoma cell  $\alpha_V\beta_3$  integrins up-regulates MMP-2 expression (38). Relative to collagen gel penetration, DOV13 cells rapidly invade a gelatin matrix. Although pro-MMP-2 activation is not up-regulated over basal levels under these conditions, additional proteinases of other mechanistic classes can provide gelatinase activity (1, 39, 40). Together these data indicate that collagenase activity provided by MT1-MMP is critical to invasion of an intact collagen matrix. Subsequent clearance of resultant fragments can then proceed by activation of cell surface MMP-2 along with contributions from other cell surface proteinases including seprase (39) and the components of the plasminogen activator/plasmin system (40), which have

been implicated in DOV13 cellular invasion of Matrigel (22).

Stimulation of pro-MMP-2 activation does not require collagenolysis, as collagenase-resistant collagen is as efficacious as wild type type I collagen at inducing pro-MMP-2 processing. However, thermal denaturation of either collagen abolishes the ability to enhance MMP activation, suggesting that  $\alpha_2\beta_1$  and/or  $\alpha_3\beta_1$  integrin binding to intact triple-helical collagen mediates the MMP activation response in DOV13 cells. By using subunit-specific antibodies to dissect integrin requirements for MMP processing, our data demonstrate that clustering of  $\alpha_3$  integrins promotes a stronger cellular MMP processing response than  $\alpha_2$  integrin aggregation. A potential role for  $\alpha$  integrin-specific regulation has been demonstrated previously for type I collagen-induced cellular responses, including those involving MMP-1 expression (41, 42). Administration of function blocking antibodies against either  $\alpha$  integrin subunit reveals a role for both receptors during invasion. Although this study implicates the  $\alpha_3\beta_1$  heterodimer in mediating the MT1-MMP response, it is likely that both  $\alpha_2\beta_1$  and  $\alpha_3\beta_1$  integrins provide a mechanical advantage to the migration component of invasion. Furthermore, it is unclear at this level of investigation whether  $\alpha_2\beta_1$  integrins are required for effective dispersal of  $\alpha_3\beta_1$  into focal adhesions (43, 44). Interestingly,  $\alpha_3\beta_1$  integrins have recently been hypothesized to play a major organizational role in the formation of invadopodia in response to cellular engagement of type I collagen (45). As MT1-MMP also localizes to invadopodia (27–29), it is possible that  $\alpha_3\beta_1$  clustering in DOV13 cells selectively initiates cellular events that mimic formation of invadopodial projections, which in turn regulate MT1-MMP activity. Moreover, our data demonstrate localization of MT1-MMP immunoreactivity to the periphery of clustered  $\beta_1$  integrins, indicating that MT1-MMP redistribution occurs during integrin clustering events. This observation, together with previous reports of MT1-MMP localization to integrin-rich cellular protrusions, suggests a cellular regulatory mechanism for MT1-MMP aggregation, thereby promoting effective pro-MMP-2 processing and efficient matrix degradation. As MT1-MMP can function as a collagenase (31) and MT1-MMP null mice exhibit severe deficiencies in collagen remodeling (33), localization of the enzyme to cellular collagen receptors could clearly influence physiologic events such as collagen gel contraction, adhesion, and invasion. In addition, pro-MMP-2 bound to intact peri-cellular collagen may readily infiltrate the MT1-MMP activation pathway, resulting in a switch from a collagenase to a gelatinase environment as pro-MMP-2 activation and collagen triple helix denaturation ensues (17).

It has recently been demonstrated that exogenous MT1-MMP overexpressed in a MMP-2 null background undergoes autolysis to a 43-kDa form, the rate of which is regulated by TIMP-2 (32). Similarly, endogenously expressed MT1-MMP in DOV13 cells exists in two major forms of 55 and 43 kDa (12). The current data indicate that the 55-kDa form of MT1-MMP is the active TIMP-2-binding species, whereas the 43-kDa form is an inactive autolysis product. This result is consistent with the amino-terminal sequences of similar MT1-MMP species obtained from overexpression systems, which demonstrate a loss of essential amino acids in the zinc-binding consensus sequence (30, 32). Although catalytically inactive, the 43-kDa form of MT1-MMP is nevertheless retained on the cell surface. As this 43-kDa species contains the carboxyl-terminal domains necessary for invadopodial localization and enzyme aggregation, it is interesting to speculate that MT1-MMP-mediated proteolysis may be down-regulated through the dilution of active enzyme with truncated proteinase.

In summary, our data support the hypothesis that as DOV13

cells interact with type I collagen, integrin receptors cluster on the cell surface, resulting in up-regulation of MT1-MMP and pro-MMP-2 processing, recruitment of MT1-MMP to sites of cell-matrix contact, MMP-2 surface association, and MT1-MMP-dependent collagen gel invasion. As a consequence of MT1-MMP collagenolysis, the resulting collagen cleavage products thermally denature, providing a substrate for a number of proteinases. In addition, MMP-2 is released from the cell surface to further advance matrix clearance through directed gelatinase activity on denatured collagen fragments. As  $\alpha_2\beta_1$  or  $\alpha_3\beta_1$  integrin occupancy is reduced, collagen matrix stimulation of proteolysis is attenuated. Furthermore, MT1-MMP activity can be down-regulated by autolytic processing to a stable, inactive 43-kDa form that may functionally dilute productive enzyme-substrate interactions. Together, these data support an hypothesis wherein matrix status influences cell surface matrix-degrading potential to facilitate cellular functions including migration, invasion, and matrix remodeling.

**Acknowledgments**—We thank Dr. Jonathan Jones (Northwestern University) for the use of the Zeiss fluorescence photomicroscope and Yueying Liu for valuable help with computer-assisted image analysis.

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