The Propeptide Domain of Membrane Type 1 Matrix Metalloproteinase Is Required for Binding of Tissue Inhibitor of Metalloproteinases and for Activation of Pro-gelatinase A*

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Jian Cao‡, Michelle Drews§, Hsi M. Lee¶, Cathleen Conner§, Wadie F. Bahou‡, and Stanley Zucker‡§

From the Departments of ‡Medicine and §Oral Biology, Schools of Medicine and Dentistry, State University of New York, Stony Brook, New York 11794 and ¶Department of Veterans Affairs Medical Center, Northport, New York 11768

Activation of secreted latent matrix metalloproteinases (MMPs) is accompanied by cleavage of the N-terminal propeptide, thereby liberating the active zinc from binding to the conserved cysteine in the pro-domain. It has been assumed that an analogous mechanism is responsible for the activation of membrane type 1 MMP (MT1-MMP). Using recombinant wild-type MT1-MMP cDNA and mutant cDNAs transfected into COS-1 cells lacking endogenous MT1-MMP, we have examined the function of the propeptide domain of MT1-MMP. MT1-MMP was characterized by immunoblotting, surface biotinylation, gelatin substrate zymography, and 125I-tissue inhibitor of metalloproteinases 2 (TIMP-2) binding. In contrast to wild-type MT1-MMP-transfected COS-1 cells, transfected COS-1 cells containing a deletion of the N-terminal propeptide domain of MT1-MMP or a chimeric construction (substitution of the pro-domain of MT1-MMP with that of collagenase 3) were functionally inactive in terms of binding of 125I-labeled TIMP-2 to the cell surface and initiating the activation of pro-gelatinase A. These results support the concept that in its native plasma membrane-inserted form, the pro-domain of MT1-MMP plays an essential role in TIMP-2 binding and subsequent activation of pro-gelatinase A.

Matrix metalloproteinases (MMPs, 1 matrixins) are a large family of neutral zinc endopeptidases, which display homologous structural features consisting of a N-terminal propeptide domain, a zinc-coordinated catalytic domain, and a C-terminal hemopexin- and vitronectin-like domain (1–3). During the process of activation of secreted latent MMPs in the pericellular and extracellular environment, conformational perturbation or limited proteolysis within the N-terminal propeptide domain causes a change in the molecule that disrupts the unpaired Cys-Zn2+ interaction and frees the Zn2+ to participate in proteolytic cleavage. The modified MMP then attacks the peptide sequence downstream of the PRCGVPD sequence in an autolytic manner and cleaves the propeptide, thus producing a lower molecular weight activated enzyme (popularly described as a cysteine switch or velcro mechanism (4)). Activation of MMPs is inhibited by a family of proteins collectively known as tissue inhibitors of metalloproteinases (TIMPs).

Membrane-type matrix metalloproteinases (MT-MMPs) represent a newly described group of matrixins (5) that are widely but selectively distributed in tissues. As the name implies, MT-MMPs are localized to the plasma membranes of cells by a stretch of hydrophobic amino acids (transmembrane domain; Ref. 6) followed by a short cytoplasmic sequence. MT-MMPs have been the subject of great interest because of their role in activating a secreted MMP, pro-gelatinase A, at the cell surface (5). Expression of MT1-MMP in embryonic tissue (7) and in malignant tumors has been correlated with the degree of activated gelatinase A in these tissues.

The mechanism of pericellular activation of pro-gelatinase A appears to involve the formation of a unique bimolecular complex between TIMP-2 and MT1-MMP (5, 8, 9). Our recent study (10) documented that the N-terminal domain of TIMP-2 binds avidly to the catalytic domain of MT1-MMP, thereby producing a complex on the cell surface. The C-terminal domain of progelatinase A then binds to the available C-terminal domain of TIMP-2 (stabilization site), forming a trimolecular complex (9). According to this theory, a second MT1-MMP molecule (not complexed to TIMP-2) then attacks a single bond in complexed pro-gelatinase A, which is followed by an autolytic cleavage (11, 12), thus resulting in pro-gelatinase A activation. Excess TIMP-2 interferes with this activation mechanism by binding and inhibiting all available MT1-MMP molecules.

A special feature of MT-MMPs, as well as stromelysin-3 (13), is a 10-residue insert immediately preceding the final processing site between the propeptide and catalytic domain, which harbors a paired basic amino acid cleaving enzyme recognition motif (RXKK) (5, 6). Unlike stromelysin-3, which is a secreted MMP, latent membrane-bound MT1-MMP does not appear to be attacked at the RXKK sequence by the propeptide convertase-dependent pathway (furin) in MT1-MMP transformed COS cells (14), but is attacked and converted to the activated form when secreted as a C-terminal truncated proenzyme (13). Secreted MT-MMPs are of unknown biological significance.

In the current study, we have examined structural-functional relationships within the propeptide domain of MT1-MMP. Whereas the peptide sequence of the N-terminal domain...
main of MT-MMPs shares more than 70% homology with naturally secreted matrixins between amino acids Met68 and Asp97, the peptide sequence (Ser34-Ala67), following the signal peptide of MT-MMPs shares <10% homology with secreted matrixins. Considerable sequence homology (>60%) between Ser34-Ala67 of MT1-MMP, MT2-MMP, MT3-MMP, and chicken MT-MMP (5, 15, 16) suggests that this peptide domain may be required for function of MT-MMPs. To examine this possibility, we have transfected COS-1 cells with human MT1-MMP cDNAs containing mutations within the propeptide domain. Whereas non-membrane-bound matrixins with critical mutations in the conserved PRCGVPD sequence of the N-terminal propeptide have been reported to be secreted as activated enzymes (stromelysin-1, collagenase, and matrilysin) (17–20), we demonstrated that transfected COS-1 cells expressing a deletion or substitution of the N-terminal propeptide domain of MT1-MMP are functionally inactive in terms of activating progelatinase A and binding TIMP 2. Thus, contrary to observations with secreted matrixins, retention of the propeptide domain of MT1-MMP on the cell surface is required to maintain certain biological functions of the enzyme. Other studies have likewise identified only the 63-kDa latent form of MT1-MMP in cells demonstrating MT1-MMP-induced pro-gelatinase A activation (21).

MATERIALS AND METHODS

Reagents—Bovine serum albumin, IGEPAL CA-630, EDTA, dithiothreitol, leupeptin, aprotinin, polyoxyethylene ether (W1), and chloroamne T were purchased from Sigma. Sulfo-NHS-LC-biotin was purchased from Pierce. Horseradish peroxidase-conjugated streptavidin and protein A-agarose beads were purchased from Life Technologies, Inc. Restriction enzymes were purchased from Stratagene (La Jolla, CA). NuSearum was purchased from Collaborative Biomedical Products (Bedford, MA). The pcDNA3 expression vector was purchased from Invitrogen (San Diego, CA). Rabbit polyclonal antibodies to a synthetic peptide CDGNFDTVAMLRGEM (catalytic domain) (22) and to the prodomain of MT1-MMP (Chemicon International, Temecula, CA) were used. Recombinant progelatinase A was produced by COS-1 cells transfected with gelatinase A cDNA as described previously (6, 14). Collagenase-3 cDNA was generously provided by Dr. Lopez-Otin (Universidad de Oviedo, Oviedo, Spain) (23).

Cell Culture and Transfection—COS-1 cells were maintained in Dulbecco’s modified Eagle’s medium (Life Technologies) containing 10% fetal bovine serum (Atlanta Biologicals) and 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin under 5% CO2 atmosphere. Plasmids were transfected into cells using calcium phosphate as described previously (6). Construction of Plasmids—MT1-MMP cDNA encoding an open reading frame from amino acid residues Met1-Val592 and mutant MT1-MMP were cloned in a pcDNA3 expression vector using a cytomegalovirus promoter as we have previously described (14). A mutant of MT1-MMP with Arg183, Lys196, and Arg197 substituted with alanine was described as previously (14). Another mutagenesis strategy used overlap extension mutagenesis using two-step PCR (24). To generate a deletion mutant lacking the entire N-terminal propeptide of MT1-MMP (MTΔpro), a PCR fragment coding for the signal domain of MT1-MMP (Met1–Phe25) was amplified using the MT1-MMP cDNA template with the MT1-MMP forward primer (5′ to 3′, CACGAAATCCCGACCATGTCCTCCCGCCCCAAGA) and the reverse primer (5′ to 3′, ACCTGGATGTCGCTGCTTGTG) (bold nucleotides indicate the complementary region), which complemented with the C-terminal MT1-MMP fragment starting from the putative catalytic domain as described below. Another fragment extending from the beginning of the catalytic domain to the intracellular end (Tyrs127–Val592) was generated by amplifying the MT1-MMP cDNA template with the forward primer (5′ to 3′, TACGCCATCCAGGGTCTCAAATGG), and the reverse primer (5′ to 3′, CACGAAATCCGACCTTGTCGACAGGAAAC). Both products were used as templates to generate full-length mutant MT1-MMP lacking the entire propeptide domain by PCR amplification with MT1-MMP forward and reverse primers. The resulting PCR fragment was cloned into the pcDNA3 expression vector. Using the same strategy, a deletion mutation of MT1-MMP lacking part of the N-terminal pro-domain (Ser21–Arg57) was generated (MT343–51) using the reverse primer (5′ to 3′, CAAAGCGAAGCTTCCACCAAGACACACG- GCCA) and forward primer (5′ to 3′, CAGAAGGCGACTTCCACCAAGACACACG-GCTCA).

A chimera between collagenase 3 and MT1-MMP (Col3/MT) was constructed by a two-step PCR using the introduced collagenase-3 fragment extending from the N-terminal signal and propeptide domain (from Met1 to Arg97) and on MT1-MMP cDNA. This chimera by PCR with collagenase-3 cDNA as a template. A forward primer (5′ to 3′, TGGTGGTTCAGCAAGAC) was paired with a reverse primer of MT1-MMP as described above to generate an MT1-MMP fragment lacking the signal and most of the propeptide domains. The full-length chimeric fragment was amplified by PCR using T7 and reverse primers using collagenase-3 and MT1-MMP fragments as templates. The PCR fragment was then cloned into pcDNA3 vector. All of the mutants were confirmed by sequencing as described previously (14).

RNA Isolation and Northern blot Hybridization—Total RNA was extracted from COS-1 cells transfected with desired plasmids by guanidine solubilization, phenol-chloroform extraction, and serial precipitation as described previously (14). Approximately 15 μg of total RNA was subjected to denaturing gel electrophoresis followed by Northern transfer to nylon membranes (Schleicher & Schuell, Keene, NH). Blots were hybridized to a 32P-labeled MT1-MMP insert at 42 °C as described (22) and analyzed after 6-h exposure with an intensity screen at −80 °C. The amount of the samples applied to the lanes was normalized by β-actin RNA.

Cell Surface Binding of TIMP 2—Recombinant TIMP 2 was iodinated to a specific activity of 5 × 1010 dpm/mg as recently described (26). Binding of 125I-labeled TIMP 2 to COS-1 cells propagated in 24-well dishes was performed in duplicate (10% variation between duplicates). For equilibrium binding experiments, dilutions of 125I-labeled TIMP 2 (0.25–8.0 nM) in bovine serum albumin/PBS were added to cells in 200 μl of serum-free media in the presence or absence of excess unlabeled recombinant TIMP 2. After 3 h of incubation at 4 °C, supernatant fluid and washes were collected as the unbound 125I TIMP 2 fraction. Cell monolayers were then lysed in 0.1% SDS in 0.5 M NaOH and collected as the bound fraction. Bound and unbound 125I were measured by gamma counting. The residual radioactivity associated with cells in the nonspecific binding experiment (50-fold excess TIMP 2) was subtracted from the total bound fraction (no unlabeled TIMP 2) to give specific binding. Scatchard plot analysis of binding data used best fit curves (26).

Cell Surface Biotinylation and Immunoprecipitation—COS-1 cells transfected with vector pcDNA3, MT1-MMP, or MTΔpro cDNA were washed twice with PBS. Sulfo-NHS-LC-biotin (Pierce) at a concentration of 1 mg/ml in PBS was added, and cells were incubated at 4 °C with gentle shaking. After 30 min, the cells were washed three times with PBS and further resuspended in PBS containing 100 μg/ml proteinase K for 30 min to remove unincorporated biotin. After washing three times with PBS, surface-biotinylated cells were solubilized at 4 °C for 30 min in 50 mM Tris-HCl (pH 7.5), 10 mM EDTA, 150 mM NaCl, 1% IGEPA CA-630 (a nonionic detergent), 0.25 mM dithiothreitol, and protease inhibitors (1 μg/ml leupeptin, 1 μg/ml aprotonin). Nuclei and intact cells were removed by centrifugation at 10,000 rpm for 5 min. Aliquots of biotin labeled cell extracts were incubated overnight at 4 °C with polyclonal anti-MT1-MMP antibodies. Immune complexes were precipitated with protein A-agarose followed by brief centrifugation and washing. Surface-biotinylated immunoprecipitates were resolved by SDS-PAGE (10%) and transferred to nitrocellulose filters. The filters were blocked for 60 min using 5% (w/v) bovine serum albumin/PBS, and then incubated for 60 min in the same buffer containing streptavidin conjugated to horseradish peroxidase. After extensive washes in 20 mM Tris-HCl, pH 7.6, 137 mM NaCl, and 0.1% Tween 20, protein was detected by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech).

Digestion of 3H-Labeled Type I Gelatin by Membrane Fractions—Crude plasma membrane fractions of COS-1 cells transfected with vector pcDNA3, MT1-MMP, or MTΔpro were prepared following nitrogen cavitation and differential centrifugation as recently described (22). Two μg of each membrane fraction was incubated with 10 μg of [3H]methionyl type I gelatin at 37 °C for 4–18 h in the presence or absence of 0.5 mM TIMP 2. The reaction was terminated by adding 10× SDS-sample buffer under reducing conditions. The degradation of type I gelatin was quantified by scanning the fluorogram with a laser densitometer.
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The gelatinolytic activity was calculated as the percentage of degraded fragments versus total gelatin in the incubation mixture.

Binding of 125I-Labeled TIMP-2 to Membrane-extracted MT1-MMP—Plasma membrane-enriched fractions (100,000 g) from COS-1 transfected cells were prepared following nitrogen cavitation and differential centrifugation as described previously (10). Membrane proteins were extracted using 0.25% polyoxyethylene ether (W1) in HEPES buffer. 125I-TIMP-2 (40 nM final concentration) was incubated with membrane-extracted proteins for 1 h at 4 °C to permit binding. To assess the specificity of binding, a similar 125I-TIMP-2 incubation with membrane-extracted proteins was performed in the presence of 10-fold excess unlabeled TIMP-2. Affinity-purified rabbit polyclonal antibodies to MT1-MMP (catalytic domain) were added to each reaction mixture to immunoprecipitate MT1-MMP and complexes. The mixtures were then incubated with protein A-coated Sepharose beads and then thoroughly washed in buffer. Beads were then added to SDS-PAGE sample buffer containing -mercaptoethanol. After boiling, samples were subjected to SDS-PAGE followed by autoradiography to identify 125I-TIMP-2 bound to MT1-MMP as described previously (10).

Gelatin Substrate Zymography and Western Blotting—Basic protocols for these techniques have been described in our recent papers (14, 22).

RESULTS

N-Terminal Pro-domain-deleted MT1-MMP Loses Pro-gelatinase A Activation but Not Gelatinolytic Function—The expression plasmid encoding cDNA for a deletion mutant of MT1-MMP (MT1pro) lacking the entire propeptide domain from Ser34 to Arg111 (5, 25) was constructed by a PCR-based overlap extension method as described under “Materials and Methods” (Fig. 1). COS-1 cells, which do not produce endogenous MT1-MMP proteins, as demonstrated by Northern blotting (Fig. 2), were used as the recipient for the transfection assay. Substituted mutant MT1-MMP Ala108-Ala110-Ala111 (MT1 Ara AA), which is not attacked by furin (14), was examined in selected experiments. , MT1-MMP, MT1pro, and MT1 Ara AA proteins were expressed in COS-1 cells by transient transfection of plasmids. As demonstrated by Western blotting using an antibody to the catalytic domain of MT1-MMP, expressed mutant proteins migrated predictably based on the truncated length of the propeptide domain and stained with similar intensity (measured by densitometry); MT1pro, lacking the entire N-terminal propeptide, appeared as a protein band of 53 kDa, whereas MT1 Ara AA was detected as a 63-kDa protein, identical to that of MT1-MMP (catalytic domain) were added to each reaction mixture to immunoprecipitate MT1-MMP and complexes. The mixtures were then incubated with protein A-coated Sepharose beads and then thoroughly washed in buffer. Beads were then added to SDS-PAGE sample buffer containing -mercaptoethanol. After boiling, samples were subjected to SDS-PAGE followed by autoradiography to identify bound to MT1-MMP as described previously (10).

Fig. 1. Schematic illustration of wild-type and mutant MT1-MMP. The domain structure of MT1-MMP is shown from the N terminus: 1) signal peptide, 2) propeptide (Pro-pep.) that contains a basic motif RRKR sequence attacked by PACE-like protease (furin), 3) catalytic domain that contains the zinc-binding site, 4) hinge region, 5) hemopexin-like domain, 6) transmembrane domain (TM), and 7) a 20-amino acid intracellular tail (Cyto.) at the C terminus. MT1-MMP lacking the entire propeptide domain (MT1pro), a chimeric protein with substitution of the N-terminal propeptide of MT1-MMP with the N-terminal propeptide of collagenase-3 (Col-3/MT), and a Ser34–Arg51 deletion mutant (MT1 34–51) were constructed by two-step PCR approaches. Wild-type and mutant MT1-MMPs were expressed by an eukaryotic expression vector (pcDNA3).

Fig. 2. Northern blot analysis of MT1-MMP. 10 μg of total cellular RNA from COS-1 cells transfected with pcDNA3 vector only and MT1-MMP cDNA was size-fractionated in a 1% denaturing agarose gel, transferred to a nylon membrane, and incubated with 0.8 kb of 32P-radiolabeled MT1-MMP cDNA as probe. The membrane was washed under stringent conditions. Blots were analyzed by autoradiography after 18 h of incubation. A single 4.5-kb mRNA transcript corresponding to the known MT1-MMP band was readily detected only in COS-1 cells transfected with MT1-MMP cDNA.
Propeptide Domain of MT1-MMP Is Required for Binding of TIMP-2

FIG. 3. Expression and function of mutant MT1-MMP (MTΔpro) lacking the entire N-terminal propeptide in transfected COS-1 cells. A, expression of mutant MT1-MMP (MTΔpro) and wtMT1-MMP protein in COS-1 cells. Cell lysates (left panel) and crude plasma membranes (right panel) isolated from COS-1 cells transfected with the expression plasmid pcDNA3 (Control), wtMT1-MMP, MTΔpro, and MTARAA (serving as a control protein for full-length MT1-MMP, because it has no furin-cleavage site) were analyzed by Western blotting using rabbit anti-MT1-MMP antibodies (left panel, using antibodies to the catalytic domain; right panel, using antibodies to the pro-domain of MT1-MMP). MTΔpro, appearing as a protein band of 53 kDa, was only detected by antibodies to the catalytic domain of MT1-MMP, whereas wtMT1-MMP in the cell lysate and membrane fraction was recognized by both antibodies at 63 kDa. The intensity of detected bands was comparable, indicating equivalent levels of protein synthesis. Nonspecific bands (present in Control lane) are noted in the right panel. B, mutant MT1-MMP (MTΔpro) lacking the entire N-terminal propeptide domain expressed in COS-1 cells does not activate progelatinase A. Recombinant progelatinase A (2 ng, measured by enzyme-linked immunosorbent assay) was incubated for 18 h at 37 °C with COS-1 cells transfected with pcDNA3 (Control), wtMT1-MMP, MTΔpro, or MTARAA. The conditioned medium was collected and analyzed by gelatin substrate zymography. In contrast to wtMT1-MMP and MTARAA, MTΔpro-transfected cells did not convert 72-kDa progelatinase A to 62-kDa activated gelatinase A. C, detergent-extracted membranes from MTΔpro-transfected cells induce progelatinase A activation when incubated with recombinant progelatinase A. Recombinant progelatinase A (Progel A, 2 ng) was incubated for 18 h at 37 °C with extracted plasma membranes (2 μg) from vector-transfected (Vector), MT1-MMP-transfected, Col-3/MT-transfected, and MTΔpro-transfected cells. Gelatin zymography was then performed. In contrast to intact cells, MTΔpro-extracted plasma membrane proteins activated progelatinase A. Extracted membrane proteins alone, at a concentration of 2 μg/well, exhibited no gelatinolytic activity (data not shown).

FIG. 4. N-terminal propeptide-deleted MT1-MMP (MTΔpro) expressed on the plasma membrane of COS-1 has gelatinolytic activity, but is not inhibited by TIMP-2. 3H-Methyl-labeled type I gelatin was incubated with plasma membrane fractions (2 μg total protein) isolated from vector control, MTΔpro, and wtMT1-MMP-transfected cells for 18 h at 37 °C. The digestion products were analyzed by SDS-PAGE (7% total acrylamide) under reducing conditions after termination of the reaction with SDS-sample buffer. The lanes are identified. Lanes 6 and 7 contain membranes that were co-incubated with 0.5 nM TIMP-2. The degradation of gelatin and inhibition of degradation by TIMP-2 are listed at the bottom.

right panel). Lower molecular weight bands (<50 kDa) present in vector-transfected and other transfected COS-1 cells are characterized as nonspecific bands.

Using the intact COS-1 cell system, the function of MTΔpro was examined for capacity to activate recombinant progelatinase A (monitored by gelatin zymography). Transfection of COS-1 cells with wtMT1-MMP or MTARAA plasmids resulted in processing of progelatinase A (72 kDa) into the activated form (62 kDa) via an intermediate form (64 kDa). Transfection of COS-1 cells with the MTΔpro plasmid failed to result in progelatinase A activation (Fig. 3B); progelatinase A activation was not induced even when the dose of transfected MTΔpro plasmid was increased 2-fold (data not shown). In a similar vein, nitrogen-cavitated plasma membranes (adjusted to 1 μg protein/sample) isolated from wtMT1-MMP-transfected cells readily activated progelatinase A; membranes isolated from MTΔpro-transfected COS-1 cells failed to activate progelatinase A (data not shown). In contrast to results achieved with intact plasma membranes, detergent-extracted membranes from MTΔpro-transfected cells induced progelatinase A activation (72 to >62 kDa) almost comparable with that achieved with wtMT1-MMP-extracted membranes (Fig. 3C). These data indicate that membrane-bound and membrane-solubilized MTΔpro differ in their capacity for progelatinase A activation.

The general proteolytic activity of MTΔpro was compared with wtMT1-MMP by incubating crude plasma membranes isolated from transfected COS-1 cells with [3H]gelatin substrate. As shown in Fig. 4, wtMT1-MMP had somewhat greater [3H]gelatin degrading capacity after 18-h incubation compared with MTΔpro; after 4 h of incubation, the degradation of [3H]gelatin was 17 and 14%, respectively. Addition of TIMP-2 at the initiation of the incubation with substrate (no preincubation) resulted in 56% inhibition of wtMT1-MMP membrane degradation of gelatin; TIMP-2 had a minimal effect on MTΔpro membranes. Membranes from vector-transfected cells lacked endogenous gelatinolytic activity. These results indicate that the deletion of the N-terminal propeptide domain of membrane-bound MT1-MMP does not impair the proteolytic activity of the enzyme against all substrates but is selective for impairing progelatinase A activation.
transfected with wtMT1-MMP cDNA for TIMP-2 binding properties. Whereas 125I-labeled TIMP-2 binding exceeded specific (NS) binding exceeded specific binding (9). Using 125I-labeled TIMP-2 as ligand, we have recently demonstrated that TIMP-2 binds to the catalytic domain of MT1-MMP in transfected COS-1 cells (10). Because propeptide-deleted MT1-MMP theoretically should be in the active form required for function or whether the pro-domain of other MMPs could function in this capacity, we constructed a substituted mutation in the pro-domain of MT1-MMP (Met1 to Arg95) using overlap extension PCR as described under "Materials and Methods" (Fig. 1). To confirm that transfected COS-1 cells synthesize chimeric MT1-MMP protein, Western blotting using an anti-MT1-MMP polyclonal antibody was performed with cell lysates and analyzed by SDS-PAGE followed by the ECL detection system. Compared with wild-type MT1-MMP, Col-3/MT chimeric protein was detected as a 65-kDa band (Fig. 7A). The chimeric protein was then examined for function in terms of pro-gelatinase A activation. As demonstrated by gelatin substrate zymography, Col-3/MT-transfected COS-1 cells (Fig. 7B), intact plasma membranes (data not shown), or membrane-extracted proteins.

Cell Surface Expression of N-Terminal Pro-domain-deleted MT1-MMP (MTΔpro)—To examine the localization of enzyme on intact cells, surface proteins from COS-1 cells transfected with MTΔpro or wtMT1-MMP plasmids were labeled by reacting the cells with sulfo-NHS-LC-biotin and immunoprecipitated using rabbit polyclonal anti-MT1-MMP antibodies. Biotinylated surface MT1-MMP was detected on nitrocellulose membranes by horseradish peroxidase-conjugated streptavidin as a 63-kDa protein band. Biotinylated MTΔpro was detected as a 53-kDa cell surface protein band (Fig. 5). Both relative mobility and staining intensity matched that of each protein detected by Western blotting of entire cell lysates. These data indicated that the loss of pro-gelatinase A activation induced by MTΔpro was not attributable to failure of insertion of the protein into COS-1 plasma membranes.

Failure of Binding of 125I-Labeled TIMP-2 to Propeptide-deleted MT1-MMP in COS-1 Cells—Current evidence suggests that cell surface activation of pro-gelatinase A by MT1-MMP requires an interaction with TIMP-2 as an intermediary (9). Using 125I-labeled TIMP-2 as ligand, we have recently demonstrated that TIMP-2 binds to the catalytic domain of MT1-MMP in transfected COS-1 cells (10). Because propeptide-deleted MT1-MMP theoretically should be in the active form based on general properties of secreted MMPs (1, 3, 4), COS-1 cells transfected with MTΔpro cDNA were compared with cells transfected with wtMT1-MMP cDNA for TIMP-2 binding properties. Whereas 125I-labeled TIMP-2 binds with a Kd of 4.9 nM to wtMT1-MMP-transfected cells (Fig. 6), binding of TIMP-2 to MTΔpro-transfected COS-1 cells was negligible (nonspecific binding exceeded specific binding). 125I-labeled TIMP-2 binding to MTΔpro-transfected COS-1 cells was not impaired (Kd, 3.7 nM; data not shown), further suggesting that furin cleavage of the propeptide domain of MT1-MMP is not required for functional activity of MT1-MMP. These data suggest that an intact N-terminal propeptide domain of membrane-bound MT1-MMP is required for binding of TIMP-2 at the cell surface and subsequent activation of pro-gelatinase A.

Sequence Specificity of the Propeptide Domain of MT1-MMP Required for Function—To address whether the amino acid sequence of the propeptide domain of MT1-MMP is unique in function or whether the pro-domain of other MMPs could function in this capacity, we constructed a substituted mutation in the pro-domain of MT1-MMP. Because the pro-domain of collagenase 3 (which classically is responsible for maintaining latency of this MMP) was determined to share minimal identity (<30% homology) with that of MT1-MMP, the signal and pro-domain of cDNA from Met1 to Arg92 (in the conserved sequence PRCGVPD) in MT1-MMP was replaced by the homologous cDNA of collagenase 3 (Met1 to Arg92) using overlap extension PCR as described under "Materials and Methods" (Fig. 1). To confirm that transfected COS-1 cells synthesize chimeric MT1-MMP protein, Western blotting using an anti-MT1-MMP polyclonal antibody was performed with cell lysates and analyzed by SDS-PAGE followed by the ECL detection system.
isolated from these cells (Fig. 3C) did not induce pro-gelatinase A activation. To investigate whether substitution of the N-terminal propeptide domain of MT1-MMP with that of collagenase 3 resulted in TIMP-2 binding activity, we performed 125I-TIMP-2 binding studies on COS-1 cells transfected with Col-3/MT compared with wild-type MT1-MMP cDNA. TIMP-2 binding was not demonstrated in COS-1 cells transfected with Col-3/MT plasmid or pcDNA3 vector alone (data not shown). Thus, we concluded that the TIMP-2 binding effect provided by the pro-domain of MT1-MMP is not replaceable by the pro-domain of secreted MMPs.

To further explore the unique property of MT1-MMP, a deletion mutant of MT1-MMP lacking the region from the beginning of pro-domain Ser34-Arg51 (MT34–51) was constructed. This mutation was expressed in COS-1 cells transiently transfected with the plasmid (Fig. 7A). As shown in Fig. 7B, no pro-gelatinase A activation (gelatin zymography) was noted in MT34–51-transfected COS-1 cells. These data further strengthen the concept that the N-terminal portion of the propeptide domain of MT1-MMP is necessary for MT1-MMP induced pro-gelatinase A activation.

Binding of 125I-Labeled TIMP-2 to Membrane-extracted MT1-MMP—Binding of 125I-TIMP-2 to membrane-extracted MT1-MMP, as depicted by an intense 22-kDa band (125I-TIMP-2-MT1-MMP complex dissociates after β-mercaptoethanol treatment and heating), was demonstrated with wtMT1-MMP and MTΔpro-transfected cell proteins (Fig. 8, lanes 2 and 4, respectively); minimal binding of 125I-TIMP-2 to vector-transfected and Col-3/MT-transfected cell membranes was noted (Fig. 8, lanes 1 and 3). These data are consistent with binding of TIMP-2 to soluble membrane MT1-MMP and MTΔpro followed by separation of the noncovalent complexes on SDS-PAGE. In the presence of 10-fold excess unlabeled TIMP-2 (competition with 125I-TIMP-2), a 22-kDa band was no longer detected with wtMT1-MMP- and MTΔpro-extracted proteins (data not shown). Omission of anti-MT1-MMP antibodies from the experiment resulted in the absence of 125I-TIMP-2 binding to all transfected cell types (data not shown).

DISCUSSION

The structure and function of secreted matrixins have been the subject of intense scrutiny for the past decade. The unique feature of MT-MMPs is their insertion into the plasma membrane of cells by a stretch of hydrophobic amino acids followed by a cytoplasmic sequence at the C-terminal tail of the molecule. Based on considerable homology with other members of the matrixin family, it has generally been assumed that other aspects of MT-MMP function, such as cleavage of the N-terminal propeptide domain during activation of the proenzyme, are analogous to secreted MMPs (1, 18, 19, 27, 28). Because purified MT-MMPs cannot readily be evaluated in their membrane-bound state, supportive data for the pro-domain cleavage hypothesis has been derived from experiments using secreted mutant forms of MT1-MMP lacking the transmembrane domain (29–32).

In the current study examining native membrane-bound MT1-MMP, we have demonstrated that the N-terminal pro-domain of MT1-MMP expressed in transfected COS-1 cells is required for function of the intact membrane-bound enzyme (function defined as the activation of pro-gelatinase A and binding of TIMP-2). The presence of an intact N-terminal propeptide in MT1-MMP was documented using a specific antibody to the pro-domain. These data are consistent with our hypothesis that conformational effects induced by the plasma membrane provide functional activity to membrane-bound MT1-MMP without cleavage of the molecule (13). In contrast, using detergent extracts of crude plasma membranes, we have confirmed that the pro-domain of MT1-MMP is not required for function of the soluble form of MT1-MMP. By transfecting MT1-MMP cDNAs into COS-1 cells, we have demonstrated that deletion of the entire N-terminal propeptide sequence of MT1-MMP resulted in loss of both 125I-TIMP-2 binding activity and pro-gelatinase A activation function. Loss of function of this mutein was not attributable to a detectable defect in protein synthesis or insertion into the plasma membrane, as studied by cell surface biotinylation (Fig. 5). As anticipated, MTΔpro reacted with an antibody generated against a peptide sequence contained within the
catalytic domain of MT1-MMP but did not react with an antibody against a pro-domain sequence of MT1-MMP. Based on the observation that cell membranes isolated from mutant MT1-MMP (MTΔpro)-transfected COS-1 cells exhibited gelatinolytic activity almost equivalent to that of wild-type MT1-MMP-transfected cells, it would appear that the mutant protein is properly folded and functional in the cell membrane. The fact that a chimeric mutant containing the N-terminal domain of collagenase 3 and the remainder of intact MT1-MMP (Col-3/MT) inserted into the plasma membrane was unable to bind TIMP-2 and activate pro-gelatinase A in intact COS-1 cells further emphasizes that the propeptide of MT1-MMP plays an essential role in the function of the molecule, presumably related to facilitating binding of TIMP-2. Our observation that TIMP-2 inhibited the gelatin-degrading activity of plasma membranes (nonsolubilized) isolated from MT1-MMP, but not membranes isolated from MTΔpro-transfected COS-1 cells, is consistent with TIMP-2 interacting with the propeptide domain of membrane-bound MT1-MMP. A deletion mutant of MT1-MMP lacking the region from Ser34 to Arg51 (MTΔ34–51) likewise lacked pro-gelatinase A activation function in transfected COS-1 cells. These data are consistent with the concept that the conserved N-terminal amino acid sequence following the signal peptide of MT1-MMP (E368WLQ-YGLPP47) is required for proper conformation and function of the enzyme in the plasma membrane. The high degree of homology in this propeptide sequence among MT1-MMP, MT2-MMP, MT3-MMP, and chicken MT-MMP (propeptide sequence among MT1-MMP, MT2-MMP, MT3-MMP, and chicken MT-MMP (28) lacking the transmembrane domain have been described previously with other MMPs (19, 34).

The issue of whether cells contain non-TIMP-2-related receptors for gelatinase A is disputed (9, 35–37). Recently Brooks et al. (38) suggested that the αvβ3-integrin functions as a cell surface receptor for pro-gelatinase A. Other investigators have been unable to reproduce this observation (12, 39). The role of MT-MMP in pro-gelatinase A activation in this scenario remains to be determined.

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Jian Cao, Michelle Drews, Hsi M. Lee, Cathleen Conner, Wadie F. Bahou and Stanley Zucker

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