Membrane type matrix metalloproteinase 1 Activates Pro-gelatinase A without Furin Cleavage of the N-terminal Domain

(Received for publication, June 24, 1996, and in revised form, August 28, 1996)

Jian Cao‡, Alnawaz Rehemtulla§, Wadie Bahou‡, and Stanley Zucker¶

From the ‡Departments of Medicine and Research, Department of Veterans Affairs Medical Center, Northport, New York 11768, the §Department of Medicine, the State University of New York, Stony Brook, New York 11794, and the ¶Department of Radiotherapy, University of Michigan, Ann Arbor, Michigan 48109

Membrane type matrix metalloproteinase 1 (MT-MMP1), a novel 63-kDa member of the matrix metalloproteinase family, is a membrane-anchored enzyme and an activator for gelatinase A. In addition to its C-terminal hydrophobic transmembrane domain, MT-MMP1 has an insertion of 11 amino acids between its propeptide and catalytic domain encrypted with a RRKR recognition motif for the paired basic amino acid cleaving enzyme, furin. In this report, we investigated whether the cleavage of the RRKR motif of MT-MMP1 by Golgi-associated furin is analogous to a similar enzyme activation mechanism observed with stromelysin-3. Mutant forms of MT-MMP1 were cotransfected into COS-1 cells with cDNAs for pro-gelatinase A and/or furin. Immunoprecipitation and immunoblotting using specific antibodies were employed to characterize cell proteins. Whereas furin readily cleaved soluble MT-MMP1 lacking the transmembrane domain (ΔMT-MMP1), a soluble stromelysin-1/ΔMT-MMP1 chimera without the RRKR basic motif was resistant to furin-induced cleavage. COS-1 cells cotransfected with wild type MT-MMP1 cDNA and furin cDNA demonstrated a 63-kDa protein (latent enzyme) on SDS-polyacrylamide gel electrophoresis rather than the anticipated lower molecular weight activated enzyme. Inhibition of furin activity with α₁-protease inhibitorPittsburgh (a furin inhibitor) did not affect the pro-gelatinase A activation mechanism in COS-1 cells cotransfected with MT-MMP1 and pro-gelatinase A cDNAs. Furthermore, substitution of the RRKR motif of MT-MMP1 with alanine residues by site-directed mutagenesis resulted in the same 63-kDa protein without loss of pro-gelatinase A activation function. These data indicate that furin-induced activation of MT-MMP1 is not a prerequisite for pro-gelatinase A activation. The mechanism of activation of cell-bound MT-MMP1 remains to be elucidated.

Matrix metalloproteinases (MMPs) play an important role in remodeling of the extracellular matrix in numerous physiologic processes. A delicate balance among the control of synthesis, activation of MMPs, and inhibition by tissue inhibitors of metalloproteinases (TIMPs) is required to maintain tissue integrity. Disruption of this balance, resulting in excessive extracellular matrix breakdown, occurs in diseases such as cancer and rheumatoid arthritis (1, 2).

In contrast to most MMPs, pro-gelatinase A (MMP-2, 72-kDa type IV collagenase) is produced constitutively in relatively high concentrations by many types of cells and is not induced by cytokines that regulate other MMPs (1). The final activation step may therefore exert a more important influence in controlling tissue gelatinase A activity than with other MMPs. Activation of pro-gelatinase A differs from that of other MMPs by involving a cell surface activation mechanism (3–6), which requires the participation of a recently described integral plasma membrane MMP. A complementary DNA encoding a 63-kDa MMP with a putative transmembrane domain (MT-MMP1) has recently been isolated and cloned. Expression of the gene product of MT-MMP1 on cell surfaces induces the activation of pro-gelatinase A and enhances cellular invasion in vitro (7). Although the potential transmembrane domain of MT-MMP1 deduced from the amino acid sequence functions as a membrane linker when fused to a secretory protein, the specific sequence of the transmembrane domain is not essential for functional activity (8). Strongin et al. (4) proposed that TIMP-2, in stoichochemical concentrations, serves as an intermediate in pro-gelatinase A activation by binding to activated MT-MMP1 in the plasma membrane; this bimolecular complex then binds and activates pro-gelatinase A. Homology screening for MMPs has revealed other membrane-spanning MMPs that share considerable homology with MT-MMP1 (9–11).

Based on the classical concept that MMPs are synthesized as latent enzymes requiring the disruption of the amino terminus of the molecule for activation, the mechanism of propeptide cleavage in MT-MMP1 has been the subject of considerable speculation. Analogous to the activation mechanism described for stromelysin-3 activation (12), MT-MMP1 has a paired basic amino acid cleaving enzyme (PACE, synonymous with furin) recognition motif (RXKR) sandwiched between the propeptide and catalytic domains of the latent molecule (7). The Arg¹¹¹-Tyr¹¹² peptide bond has been proposed as the site of post-translational endoproteolysis (7, 13) leading to the activation of MT-MMP1 in the trans-Golgi network. This hypothesis is supported by the data of Pei and Weiss (14), which demonstrated that secreted forms of transmembrane deletion mutants of MT-MMP1 are efficiently processed to active proteinases following post-translational endoproteolysis immediately down-celling enzyme; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; ST, stromelysin; WT, wild type.

This paper is available online at http://www-jbc.stanford.edu/jbc/
stream of the RRKR motif by a PACE pathway; the secreted deletion mutants of MT-MMP1 directly mediated pro-gelatinase A activation via a two-step proteolytic cascade indistinguishable from the process observed with intact cells.

In the current study we have examined the role of furin in the processing of wild type (WT) pro-MT-MMP1 required for the activation of pro-gelatinase A. Based on the difficulty in purifying WT MT-MMP1 and reconstructing the molecule as an integral plasma membrane protein, we have constructed a cDNA encoding a soluble form of MT-MMP1 lacking the transmembrane-spanning domain (ΔMT-MMP1). Cotransfected COS-1 cells with cDNA for wild type or ΔMT-MMP as well as cDNA for gelatinase A and cDNA for furin have permitted us to perform molecular modeling experiments to examine the role of these proteins in activation of pro-gelatinase A. Whereas furin readily cleaved soluble ΔMT-MMP1, WT MT-MMP1 (membrane-bound) was resistant to furin-induced cleavage. Inhibition of furin activity did not affect the pro-gelatinase A activation mechanism in COS-1 cells cotransfected with WT MT-MMP1 and gelatinase A cDNAs.

**MATERIALS AND METHODS**

**Chemicals and Proteins**—Recombinant pro-gelatinase A, C-terminal gelatinase A, N-terminal gelatinase A, TIMP-2, inactive mutant gelatinase A, and TIMP-1 were purified as described previously (5). EDTA, nylfluoride, soybean trypsin inhibitor, and L-transepoxysuccinylleucyl-tinase A, and TIMP-1 were purified as described previously (5). EDTA, gelatinase A, N-terminal gelatinase A, TIMP-2, inactive mutant gelatinase A, and a 20-amino acid intracellular tail at the C terminus. MuMT-MMP1 has substituted alanine for Arg111, Lys110, Arg111 by a sequential PCR-based strategy. A truncated mutant MT-MMP (ΔMT-MMP1) and chimeric protein (ST-1/ΔMT-MMP1) were constructed by cDNA manipulations as described under "Materials and Methods."

**Fig. 1. A schematic illustration of wild type and mutant MT-MMP1.** The domain structure of MT-MMP1 is shown from the N terminus: signal peptide (Sig); propeptide (Pro-pep) that contains a basic motif RRKR sequence (capital letters) attacked by PACE-like protease; catalytic domain (catalytic) that contains zinc-binding site (Zn2+); hinge (Hinge); hemopexin-like domain (Hemopexin); transmembrane domain (TM); and a 20-amino acid intracellular tail at the C terminus. MuMT-MMP1 has substituted alanine for Arg111, Lys110, and Arg111 to alanine (MuMT-MMP1) were used as follows (underlined nucleotides indicate the altered codons): the mutagenic primer (forward primer, 5'-3': TT GAC GAG GCA GCA TAC GCC ATC CAG GGT) was paired with MT-MMP 3'-primer (5'-3': CAC GAA TTC TCA GAC CCT GTC CAG CAC GGA AC) (5) to generate the C-terminal mutant MT-MMP fragment carrying the desired mutations by employing PCR using a cDNA template. A PCR fragment coding for the signal and propeptide domains of MT-MMP1 (Met1-Arg111) containing the desired mutations was generated by amplifying the MT-MMP1 cDNA template with the 5'-MT-MMP1 forward primer (5'-3': CAC GAA TTC CGG ACC ATG TCT CCC GCC CCA AGA) described previously (5) and the reverse primer (5'-3': TA TGC TGC CCT TGC AAC ATT GCC CTT GAT), which complemented the mutated C-terminal MT-MMP fragment generated above. PCR amplification using the 5' and 3' MT-MMP1 primers to generate full-length MT-MMP1 mutant was employed as described in Fig. 1. The resulting PCR fragment was cloned into pcDNA3 expression vector. Similarly, a chimera between stromelysin-1 and ΔMT-MMP1 (ST-1/ΔMT-MMP1) was constructed using the introduced stromelysin-1 fragment encoding signal and propeptide domains (from Met1 to His98) in ΔMT-MMP1 plasmid, which lacks N-terminal signal and propeptide domains (from Met1 to Arg111). All of the mutants were confirmed by extensive restriction analysis and partial sequence analysis using an automated DNA sequencer (ABI PRISM Dye Terminator Cycle Sequencing Core kit, Perkin-Elmer). The predicted products were 57 kDa for ΔMT-MMP1, 56 kDa for ST-1/ΔMT-MMP, and 63 kDa for MuMT-MMP1. Furin transfections were performed as described previously (17). Gelatinase A and mutant gelatinase A (employing cDNA from an inactive mutant gelatinase A) with a glutamic acid residue (Glu175) replaced by alanine (Glu175→Ala) (18) were cloned in a pcDNA3 expression vector employing a cytomegalovirus promoter as described above. α1-protease inhibitor (Pitasulbarg (α1-PI_ins)) cDNA was cloned into the EcoRI/SauI site of the pMT2 expression vector as described previously (19).  

**Cell Culture and Transfection**—COS-1 cells were cultured in DMEM (Life Technologies) containing 10% fetal calf serum (Atlanta Biologicals) and 2 mM glutamine under 5% CO2 atmosphere. On the day of transfection, COS-1 cells were washed with phosphate-buffered saline, pH 7.4, followed by the addition of DMEM containing 10% NuSerum, 300 mg/ml DEAE-dextran, 100 μM chloroquine, and 1.25 mg/ml DNA (20). The cells were then incubated for 4 h at 37°C in an atmosphere of growth medium.
cells were labeled with 35S-methionine for 8 h. Supernatants were collected and immunoprecipitated with anti-gelatinase A polyclonal antibodies. Antigen-antibody complexes were precipitated with protein A-agarose beads as described under “Materials and Methods.” Precipitates were separated on SDS-PAGE, and radioactivity was detected by means of autoradiography. The bands in the pcDNA3 control lane represent nonspecifically labeled proteins.

Preparation of Cell Membrane Fractions—Confluent MT-MMP1-transfected and vector-control-transfected COS-1 cells (~2 × 10⁷ cells) were harvested following treatment with trypsin-EDTA and washed thoroughly. Cells suspended in 25 mM sucrose, 5 mM MgCl₂ in Tris base, pH 7.4, were subjected to nitrogen cavitation at 1000 p.s.i. N₂ for 30 min at 4 °C, as modified from a previously described method (21). Whole cells and nuclei were removed by centrifugation at 770 × g for 10 min, and the postnuclear supernatant was centrifuged at 100,000 × g for 60 min, and plasma membranes, Golgi, and ribosomes were recovered in the pellet. The 100,000 × g supernatant was designated as cytosol. Cell fractions were incubated with recombinant human pro-gelatinase A for 18 h at 37 °C with or without inhibitors and examined by zymography to evaluate enzyme activation.

Immunoprecipitation—Twenty-four hours following transfection, the cells were labeled with 35S-methionine (DuPont NEN) in methionine-free DMEM (Life Technologies) and incubated for 5 h at 37 °C as described previously (8). Thereafter, the conditioned medium was collected and clarified by centrifugation. Cells were then lysed in lysis buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM EDTA, 0.02% NaN₃, 1% Nonidet P-40, and 0.125 mM dithiothreitol. Both the conditioned media and the cell lysates were incubated with anti-TIMP-2 polyclonal antibody (4), mouse monoclonal antibody (kindly provided by Dr. Iwata of Fuji Chemical Industries, Ltd., Takaoka, Japan in collaboration with Dr. Seiki) (5), or rabbit polyclonal antibody against MT-MMP1 (22). The immunogen for MT-MMP1 was the synthetic peptide CDGNFDTVAMLGREG. As described previously, immunoprecipitation using the rabbit polyclonal antibody to MT-MMP1 provides virtually identical results as the mouse monoclonal antibody to the same synthetic peptide (22). Antigen-antibody complexes were precipitated with protein A-agarose beads followed by brief centrifugation, washing, and then electrophoresis in a 10% SDS-polyacrylamide gel (8). Radioactive polypeptides were detected by means of autoradiography.

To distinguish between activated and latent proteinases, excess α₁M was used to form a stable complex with activated, but not latent, MMPs prior to performing immunoprecipitation; the characteristic migration on SDS-PAGE of radiolabeled activated MMPs complexed with α₁M is disrupted (23). Briefly, COS-1 cells were incubated with 35S-methionine for 6 h at 37 °C; excess α₁M (100 μg/ml) was added for 1 h at 37 °C; and conditioned medium was collected and immunoprecipitated as described above. Cell lysates were prepared and also incubated with α₁M (100 μg/ml) along with anti-MT-MMP1 for 1 h at 37 °C before immunoprecipitation, SDS-PAGE, and autoradiography as described above.

Pulse-Chase Analysis—Transient transfected cells were labeled with 35S-methionine (100 μCi/ml) for 20 min and chased with DMEM containing 10% fetal calf serum for 0, 30, 60, 120, and 240 min. Cells were lysed in lysis buffer and immunoprecipitated with polyclonal antibodies to MT-MMP1 as described previously (8). Immunoblotting—Immunoblotting was performed using monoclonal antibodies to MT-MMP1 (5, 7) and protein A affinity-purified rabbit polyclonal antibodies to human gelatinase A, TIMP-2 (5), and furin as previously reported (24). Transient transfected COS-1 cell-conditioned medium was precipitated with 10% trichloroacetic acid, and the precipitated proteins were dissolved in SDS sample buffer. The transfected cells were lysed by 2 × SDS gel-loading buffer. The samples were resolved by 10% polyacrylamide gel electrophoresis, and proteins were transferred to nitrocellulose membranes and probed with antibodies. After extensive washing with TBS-T (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.1% Tween), the membrane was probed with anti-rabbit and anti-mouse IgG as described previously (5). Molecular weight was determined using prestained protein standards.

Gelatin Zymography—Zymography was performed in 10% polyacrylamide gels that had been cast in the presence of 0.1% gelatin as described previously (5). After electrophoresis, SDS was replaced by Triton X-100, thus renaturing gelatinases, followed by incubation in a Tris-based buffer for 24 h. Gels were stained with Coomassie Brilliant Blue, and gelatinolytic activity was detected as a clear band in the background of uniform staining.

RESULTS AND DISCUSSION

Functional and Structural Properties of MT-MMP1—Expression plasmids encoding cDNA for WT MT-MMP1 were cotransfected in COS-1 cells along with the expression vector for WT pro-gelatinase A. This cotransfection resulted in activation of COS-1-secreted pro-gelatinase A as demonstrated by gelatin zymography (Fig. 2A) or immunoprecipitation (Fig. 2B). In contrast, transfection of pro-gelatinase A cDNA alone in COS-1 cells resulted in secretion of pro-gelatinase A exclusively. Cotransfection in COS-1 cells of WT MT-MMP1 cDNA along with cDNA from an inactive mutant gelatinase A (13) with a glutamic acid residue (Glu³⁷⁵) replaced by alanine (Glu³⁷⁵ → Ala) resulted in cleavage of 72-kDa Glu³⁷⁵ → Ala to a 64-kDa protein (data not shown), thus excluding a gelatinase A autoactivation mechanism as being the primary activation mechanism (25). Atkinson et al. (25) presented similar data and suggested
MT-MMP1 Activates Pro-gelatinase A

that the binding of pro-gelatinase A to the cell membrane increases its local concentration such that an intermolecular processing reaction is promoted in which the activated enzyme is involved in the activation of the intermediate form (conversion of 64- to 62-kDa gelatinase A). 

Crude plasma membranes (in low protein concentration) isolated from COS-1 cells transfected with MT-MMP1 DNA but not from vector-transfected COS-1 cells (even at high concentration) activated recombinant human pro-gelatinase A during an 18-h incubation in a dose-dependent fashion (Fig. 3A). Co-incubation of COS-1 membranes with the metal chelator, 1,10-phenanthroline, the nonfunctional C-terminal component of gelatinase A, low dose TIMP-2 (0.1 nM), and high dose TIMP-1 (1800 nM, data not shown), inhibited membrane-induced activation of pro-gelatinase A, but 450 nM TIMP-1, N-terminal pro-gelatinase A, the serine proteinase inhibitors, soybean trypsin inhibitor and phenylmethylsulfonyl fluoride, and the cysteine proteinase inhibitor, E-64 did not (Fig. 3B). The cytosoisolated from MT-MMP1 cells did not activate pro-gelatinase A (data not shown). These data indicate that a pro-gelatinase A activation mechanism is localized to the plasma membrane of MT-MMP1-transfected cells and coincides with the plasma membrane insertion of MT-MMP1. Inhibition of pro-gelatinase A activation by the addition of C-terminal gelatinase A and low dose TIMP-2 is consistent with membrane binding of pro-gelatinase A through the C-terminal component of the latent enzyme (3, 5).

To further explore structure-function relationships, MT-MMP1 protein localization was examined in COS-1 transfected cells. Whereas WT MT-MMP1 (63 kDa) was not secreted and was detected primarily in cell membrane fractions (7, 8), ΔMT-MMP1 was secreted into conditioned media as a furin-processed 54-kDa enzyme (see below), while intracellular ΔMT-MMP1 (cell lysate) was demonstrated solely as the latent 57-kDa species (Fig. 4A and B). If MT-MMP1 is processed at an RXXR furin consensus sequence intracellularly or on the cell surface, it is anticipated that a lower molecular weight product (deletion of the N-terminal 79 amino acids besides the 32-amino acid signal peptide) would be visible in cell lysates; this was not the case. Pei and Weiss (14) also identified MT-MMP1 on the cell surface as the unprocessed zymogen and speculated that the MT-MMP1 transmembrane domain may “shield” the proteinase from rapid intracellular degradation by furin.

ΔMT-MMP1 Mutant, but Not MT-MMP1 or Chimeric ST/ΔMT-MMP1, Is Processed to the Activated Form by Furin—Among MMP family members, only stromelysin-3 and MT-MMPs (7, 10, 11, 14) contain the specific sequence RXKR between the propeptide and catalytic domains. Pei and Weiss (12) reported that stromelysin-3 was processed directly to its mature active form by furin at an RXXR site and secreted as an active enzyme. Therefore, it was proposed that proΔMT-MMP1 is processed to the mature form by the same mechanism (7). To confirm this deduction, we prepared a fusion protein using the N terminus of stromelysin-1 containing signal and propeptide domains from Met3 to His3 instead of that of ΔMT-MMP1 by manipulating the cDNAs of both proteins (Fig. 1); this plasmid construct (chimeric ST-1/ΔMT-MMP1) lacked the RXXR sequence. ΔMT-MMP1 and fusion protein were expressed in COS-1 cells by transient transfection of plasmids. After 24 h of incubation in serum-free media, the proteins in conditioned media were precipitated, and cells were lysed. Immunoblotting of secreted proteins using anti-MT-MMP1 antibody (which reacts equally with ΔMT-MMP1 and ST-1/ΔMT-MMP1) demonstrated that the fully mature form of ΔMT-MMP1 was detected as a 54-kDa protein (presumably activated), whereas the chimeric protein was expressed as a 64-kDa molecule2 rather than as the 57-kDa native intracellular protein, presumably reflecting specific processing or modification of the chimeric protein associated with secretion as previously noted (8) from the cells (Fig. 4A). In cell lysates, both ΔMT-MMP1 and ST-1/ΔMT-MMP1 were present as 57-kDa proteins. Therefore, lacking the RXXR sequence (and susceptibility to attack by the paired basic amino acid cleaving enzyme furin), ST-1/ΔMT-MMP1 was unable to be converted into its mature form. In contrast, ΔMT-

1 The calculated molecular mass of the ST-1/ΔMT-MMP1 secreted protein varied between 61 and 64 kDa under different conditions of SDS-PAGE; nonetheless, the secreted protein uniformly had a higher mass than the chimeric intracellular protein (57 kDa) identified in the cell lysate. To avoid confusion between publications, we will use the original 64-kDa mass calculated by Cao et al. (8) to describe the secreted ST-1/ΔMT-MMP1 chimeric protein.
MMP1 was processed and secreted as the mature form but present only in the higher molecular mass latent form (57 kDa) intracellularly. The latter data are consistent with the expectation that many proteins processed by furin in the trans-Golgi network are rapidly secreted and are not retained within cells (25).

To directly examine whether furin can induce the maturation of DMT-MMP1 intracellularly, the plasmid encoding DMT-MMP1 was cotransfected into COS-1 cells along with the furin expression plasmid; the DMT-MMP1 cDNA containing plasmid was transfected alone as a control. The products, both in culture media and in cell lysates, were examined by immunoprecipitation using monoclonal antibodies to MT-MMP1. Under conditions of coexpression with furin after 5 h of incubation, DMT-MMP1 was detected extracellularly exclusively as the mature 54-kDa product, while the transfection with DMT-MMP1 alone produced both extracellular forms (64 and 54 kDa) of DMT-MMP1 (Fig. 4B); the latter observation is due to endogenous production of low levels of furin (26) by COS-1 cells (confirmed by immunoblotting; data not shown). Intracellular DMT-MMP1 was not present in the mature form even with furin coexpression in COS-1 cells. These experiments are consistent with the concept that the N terminus of DMT-MMP1 destined for secretion is processed at the RRKR furin consensus sequence in the trans-Golgi network.

To simplify comparison between publications, the original 64-kDa calculation made by Cao et al. (8) is retained in this figure. B, immunoprecipitation of COS-1 cells cotransfected with DMT-MMP1 and furin. COS-1 cells were cotransfected with the DMT-MMP1 plasmid together with pcDNA3 or furin cDNA plasmid. COS-1 cells transfected with pcDNA3 alone represent the negative control. Cells were labeled with [35S]methionine, and after 5 h, proteins in the media or cell lysates were immunoprecipitated with anti-MT-MMP1 antibody. A major single band for DMT-MMP1 was detected as a 57-kDa product in cell lysates, while two bands (64 and 54 kDa) were detected in the medium of DMT-MMP1-transfected cells (as previously reported) (8). Only the mature 54-kDa form of DMT-MMP1 (appearing as a broad 51–54-kDa band) was detected in the medium from COS-1 cells cotransfected with DMT-MMP1 and furin plasmids.

FIG. 4. Processing of DMT-MMP1 at the RRKR sequence but not the ST-1/ΔMT-MMP1 chimera lacking the RRKR sequence. A, Western blot analysis of mutant MT-MMP1-transfected cells. Both serum-free conditioned media (collected at 24 h) and cell lysates from COS-1 cells transiently transfected with pcDNA3 (control), ΔMT-MMP1, or ST-1/ΔMT-MMP1 expression vectors were analyzed by immunoblotting with anti-MT-MMP1 antibody. The first three lanes represent conditioned media, and lanes 4–6 represent the cell lysates. Molecular weights were determined using prestained protein standards. The calculated molecular mass of processed ST-1/ΔMT-MMP1 protein in the media has varied between 61 and 64 kDa in different gels. To simplify comparison between publications, the original 64-kDa calculation made by Cao et al. (8) is retained in this figure. B, immunoprecipitation of COS-1 cells cotransfected with ΔMT-MMP1 and furin. COS-1 cells were cotransfected with the ΔMT-MMP1 plasmid together with pcDNA3 or furin cDNA plasmid. COS-1 cells transfected with pcDNA3 alone represent the negative control. Cells were labeled with [35S]methionine, and after 5 h, proteins in the media or cell lysates were immunoprecipitated with anti-MT-MMP1 antibody. A major single band for ΔMT-MMP1 was detected as a 57-kDa product in cell lysates, while two bands (64 and 54 kDa) were detected in the medium of ΔMT-MMP1-transfected cells (as previously reported) (8). Only the mature 54-kDa form of ΔMT-MMP1 (appearing as a broad 51–54-kDa band) was detected in the medium from COS-1 cells cotransfected with ΔMT-MMP1 and furin plasmids.

FIG. 5. Furin is not required for MT-MMP1 processing. COS-1 cells were transfected with either pcDNA3 expression vector (control) or MT-MMP1 cDNA or cotransfected with both MT-MMP1 and furin cDNA or with MT-MMP1 and α-1PI cDNA. Cells were labeled with [35S]methionine, and the cell lysates were immunoprecipitated with anti-MT-MMP1 antibody. 63-kDa MT-MMP1 was detected as the unprocessed protein under all conditions.

FIG. 6. MT-MMP1 is stable over 4 h in transfected cells as demonstrated by pulse-chase analysis. COS-1 cells transiently transfected with the MT-MMP1 expression plasmid were pulse-labeled with [35S]methionine for 20 min and chased with DMEM containing 10% fetal calf serum for 0, 30, 60, 120, and 240 min. The lysates were immunoprecipitated with anti-MT-MMP1 polyclonal antibody. The (+) lane represents COS-1 cells transfected with pcDNA3 plasmid.
complete conversion of latent to mature ΔMT-MMP1 (54 kDa) occurring after 24 h of incubation (Fig. 4A), may, in part, be due to extracellular processing by secreted furin.

To further examine the role of furin in MT-MMP1 processing, MT-MMP1 cDNA (wild type) was coexpressed with furin cDNA in COS-1 cells and the products in the cell lysate were immunoprecipitated with monoclonal antibodies against MT-MMP1. Coexpression of furin with MT-MMP1 did not alter the molecular weight of MT-MMP1 (Fig. 5) or the magnitude of gelatinase A activation as examined by gelatin zymography (Fig. 2). Furthermore, cotransfection of COS-1 cells with MT-MMP1 and α1-PIpHsa, a reactive site variant of α1-PI that inhibits furin activity in situ (17), did not interfere with MT-MMP1-induced activation of progelatinase A or affect the molecular weight of MT-MMP1 (Figs. 2 and 5). Since the level of furin expression in transfected cells did not affect progelatinase A activation or cleavage of latent WT MT-MMP1 in COS-1 cells, this implies that furin is not required for either WT MT-MMP1 activation or progelatinase A activation.

In other experiments, we have demonstrated that transfection of α1-PIpHsa plasmid into COS cells very efficiently inhibits processing of von Willebrand factor by endogenous COS cell furin as well as transfected furin (27).

The MT-MMP1 activation process was further explored by incubating [35S]methionine-labeled COS-1 cells (cotransfected with MT-MMP1 or ΔMT-MMP1 and the furin expression vector) with α2M (100 μg/ml). Conditioned media and cell lysates were then examined following immunoprecipitation. After the addition of α2M, ΔMT-MMP1 (54 kDa) in conditioned media was no longer visible on autoradiography, indicating that 54-kDa ΔMT-MMP1 is an activated protease that became entrapped by the α2M bait mechanism (23). In contrast, the mobility of radiolabeled 64-kDa ΔMT-MMP1 in conditioned medium and 63-kDa WT MT-MMP1 in cell lysate was unchanged following α2M treatment (data not shown). These data confirm that WT MT-MMP1 is not attacked and converted to an activated protease by furin.

Stability of MT-MMP1: Lack of Intracellular Processing—Pulse-chase analysis demonstrated that the major intracellular 63-kDa MT-MMP1 was quite stable even after a 4-h chase and was still readily visible after 24 h (data not shown) (Fig. 6). Lower molecular weight cleavage products were not visible intracellularly.

RXKR Sequence of MT-MMP-1 Is Not Required for MT-MMP1 Function—To directly assess the relationship between the RXKR sequence of MT-MMP1 and the function of the molecule, a substitution mutant was constructed. Depending on the specific proprotein convertase involved, critical roles have been established for basic residues at position −1, −2, or −4 relative to the scissile bond. Therefore, MuMT-MMP1 was constructed for Ala substitutions at each of these positions (Fig. 1). MuMT-MMP1 and MT-MMP1 plasmids were transfected into COS-1 cells together with furin cDNA. After labeling with [35S]methionine, cell lysates were prepared and examined by immunoprecipitation using anti-MT-MMP1 antibodies. MuMT-MMP1 was detected as a 63-kDa protein (Fig. 7A),
identical to that of wild type MT-MMP.

To investigate whether the Arg\textsuperscript{108}-Arg\textsuperscript{109}-Lys\textsuperscript{110}-Arg\textsuperscript{111} sequence of MT-MMP1 is critical for pro-gelatinase A activation, transient expression of MuMT-MMP1 and pro-gelatinase A in cotransfected COS-1 cells were examined; MuMT-MMP1 induced pro-gelatinase A activation as efficiently as MT-MMP1 (Fig. 7B). Thus, although the basic amino acids in the RRKR sequence of MT-MMP were changed by substitution with the nonpolar amino acid alanine to Ala\textsuperscript{108}-Arg\textsuperscript{109}-Ala\textsuperscript{110}-Ala\textsuperscript{111}, the MuMT-MMP1 did not lose the functional capacity for pro-gelatinase A activation. These data provide additional evidence to support the concept that furin-induced activation of MT-MMP1 is not a prerequisite for function of MT-MMP1 in the activation of pro-gelatinase A. These experiments in COS-1 cells emphasize the importance of the intracellular environment in the function of proprotein converting enzymes (28) and suggests a mechanism limiting furin accessibility intracellularly for interaction with MT-MMP1 in the protein-processing pathway.

Role of TIMP-2 in MT-MMP1-induced Activation of Pro-gelatinase A—Based on the identification in cell extracts of TIMP-2 cDNA (1) of MT-MMP1 and TIMP-2 cDNA-precipitation with anti-TIMP-2 antibodies, we demonstrated a specific role of TIMP-2 in this process, we examined whether receptor and activator for pro-gelatinase A. To investigate the activated MT-MMP1-TIMP-2 complex in turn acts as a receptor and activator for pro-gelatinase A, To investigate the specific role of TIMP-2 in this process, we examined whether COS-1 cells produce endogenous TIMP-2. Employing immunoprecipitation with anti-TIMP-2 antibodies, we demonstrated that COS-1 cells synthesize a small amount of TIMP-2 (Fig. 8A). Transient transfection into COS-1 cells with a low concentration of TIMP-2 cDNA (1 \mu g) together with MT-MMP1 and gelatinase A plasmids resulted in enhanced activation of pro-gelatinase A. Transfection of COS-1 cells with a higher concentration of TIMP-2 cDNA (5 \mu g) completely inhibited activation of pro-gelatinase A (Fig. 8B). This result is consistent with the Strongin et al. (4) that stoichiometric amounts of TIMP-2 are necessary for MT-MMP1-induced pro-gelatinase A activation; higher concentrations of TIMP-2 interfere with this process.

Conclusion—In conclusion, we have demonstrated that MT-MMP1 is identified solely as a 63-kDa protein in transfected COS-1 cells; nonetheless, MT-MMP1-cotransfected cells activate pro-gelatinase A. Although furin processes secreted \Delta MT-MMP1 lacking a transmembrane domain, furin does not attack the RRKR sequence of wild type MT-MMP1 or intracellular \Delta MT-MMP1, thereby indicating that the furin consensus sequence in MT-MMP1 is not involved in pro-gelatinase A activation in COS-1 cells.

One aspect of transfection studies in COS cells requires further discussion. COS cells have the capacity to express proteins at very high levels, which may overwhelm the protein processing machinery; i.e. MT-MMP1 cDNA-transfected COS-1 cells may be making more MT-MMP1 than endogenous furin is capable of processing. In that case, cotransfection of COS-1 cells with the furin expression vector should enhance these processing events as was demonstrated by augmented cleavage of secreted \Delta MT-MMP1 (Fig. 4B). The fact that intracellular MT-MMP1 was not cleaved in COS-1 cells cotransfected with the furin expression vector indicates that this processing event does not occur in COS-1 cells. Therefore, another mechanism for MT-MMP1 activation needs to be invoked in these cells. Based on our data, it appears that the 63-kDa MT-MMP1 protein identified in COS-1 cells is functionally active and responsible for activation of pro-gelatinase A. We hypothesize that conformational effects induced by the plasma membrane localization of MT-MMP1 may provide functional activity to this MMP without cleavage of the molecule; receptor-bound TIMP-2 may participate in this process. An alternative hypothesis is that MT-MMP1 is activated by a non-furin mechanism to a lower molecular weight species that has a very short half-life in cells and is thereby not detected by immunoprecipitation. The isolation from tumor cell lysates by Strongin et al. (4) of MT-MMP1 with Tyr\textsuperscript{112} at the N terminus suggests that furin may be functionally active in MT-MMP1 processing and activation in these specific cells or, alternatively, that furin processing may have occurred in vitro following detergent extraction of cell proteins. Additional experiments are required to clarify this issue.

Acknowledgments—We thank Cathleen Conner and Michelle Drews for technical assistance, Dr. A. Docherty (Slough, United Kingdom) and D. Strickland (Washington, D.C.) for providing reagents, and Dr. S. Weiss (Ann Arbor, MI) for helpful discussions.

REFERENCES
1. Birkedal-Hansen, H., Moore, W. G. I., Bodden, M. K., Windsor, L. J., Birkedal-Hansen, H., DeCarlo, A., and Engler, J. A. (1993) Crit. Rev. Oral Biol. Med. 42, 197–250
2. Liotta, L. A., Steeg, P. S., and Stetler-Stevenson, W. G. (1991) Cell 64, 327–336
3. Murphy, G., Willenbrock, F., Ward, R. V., Cockett, M. I., Eaton, D., and Docherty, A. J. P. (1992) Biochem. J. 283, 637–641
4. Strongin, A. Y., Collier, I., Bannikov, G., Marmer, B. L., Grant, G. A. and Goldberg, G. I. (1995) J. Biol. Chem. 270, 5331–5338
5. Zucker, S., Conner, C., DiMassimo, B. I., Ende, H., Drews, M., Seiki, M. and Ibaou, W. F. (1995) J. Biol. Chem. 270, 23730–23736
6. Overall, C. M., and Sodek, J. (1990) J. Biol. Chem. 265, 21141–21151
7. Sato, H., Takino, T., Okada, Y., Cao, J., Shinagawa, A., Yamamoto, K. and Seiki, M. (1994) Nature 370, 61–65
8. Sato, H., Takino, T., and Seiki, M. (1996) J. Biol. Chem. 271, 801–805
9. Nisai, X. S., Peru, A. M., Liano, E., Velasco, G., and Lopez-Otin, C. (1996) Cancer Res. 56, 944–949
10. Will, B. and Hinzmann, B. (1995) Eur. J. Biochem. 231, 602–608
11. Takino, T., Sato, H., Shinagawa, A., and Seiki, M. (1995) J. Biol. Chem. 270, 23013–23020
12. Pal, D. and Weiss, S. J. (1995) Nature 375, 244–247
13. Imai, K., Osue, E., Aoki, T., Nomura, H., Fujii, Y., Sato, H., Seiki, M. and Okada, Y. (1996) Cancer Res. 56, 2707–2710
14. Pal, D. and Weiss, S. J. (1995) J. Biol. Chem. 271, 9135–9140
15. Strickland, D. K., Steiner, J. P., Migliorini, M., and Battey, F. D. (1988) Biochemistry 27, 1458–1466
16. Boshart, M., Weber, F., Jahn, G., Dorsch-Hasler, K., Fleckenstein, B. and Schaffner, W. (1985) Anal. Biochem. 145, 538–545
17. Wise, R. J., Barr, P. J., Cheng, K. A., and Kaufman, R. J. (1990) Biochem. J. U. S. A. 115, 41378–9380
18. Crabbe, T., Zucker, S., Cockett, M. I., Willenbrock, F., Tickie, S., O'Connell, J. P., Scathern, J. M., Murphy, G. and Docherty, A. J. P. (1994) Biochemistry 33, 6684–6690
19. Walsley, L. C., Rehentall, A., Bristol, J. A., and Kaufman, R. J. (1995) J. Biol. Chem. 264, 8458–8465
20. Kluexen, F. W. and Lubbert, H. (1993) Anal. Biochem. 208, 352–356
21. Zucker, S., Wieman, J. M., Lysak, R. M., Wilkie, D., Ramamurthy, N. S., Golub, L. M., and Lane, D. (1995) Cancer Res. 47, 1608–1614
22. Foda, H. D., George, S., Conner, C., Drews, M., Tompkins, D. C., and Zucker, S. (1996) J. Biol. Chem. 271, 538–545
23. Ogata, Y., England, J. J., and Nagase, H. (1992) J. Biol. Chem. 267, 3581–3584
24. Rehentall, A., and Kaufman, R. J. (1992) Blood 79, 2349–2355
25. Sato, H., Takino, T., Okada, Y., Shinagawa, A., Yamamoto, K., Sato, H., and Seiki, M. (1995) J. Biol. Chem. 270, 30479–30485
26. Miheret, S. E., Chevalier, S., Borel, D., Seidah, N. G., and Boileau, G. (1995) Biochem. J. 309, 683–688
27. Rehentall, A., Barr, P. J., Rhodes, J. C., and Kaufman, R. J. (1993) Biochem. J. 292, 1583–1590
28. Benjannet, S., Reudelhuber, T., Mercure, C., Rondeau, N., Cockett, M. I., and Seidah, N. G. (1992) J. Biol. Chem. 267, 11417–11423