Membrane-Type Matrix Metalloproteinase 1 Is a Gelatinolytic Enzyme and Is Secreted in a Complex with Tissue Inhibitor of Metalloproteinases 2

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Abstract

The processing mechanism and gelatinolytic activity of the membrane-type matrix metalloproteinase 1 (MT-MMP-1) were examined by expressing in COS-1 cells a deletion mutant of MT-MMP-1 lacking the transmembrane domain (ΔMT1) and its site-directed mutant with a furin-resistant sequence in the propeptide domain (mutant ΔMT1). ΔMT1, but not mutant ΔMT1, was processed to an active form and exhibited gelatinolytic activity as seen using gelatin zymography. ΔMT1 isolated in a complex form with tissue inhibitor of metalloproteinases 2 (TIMP-2) from the stable transfectants demonstrated the NH2-terminal sequence of Ala18-Ile-Gln-Gly-Leu, indicating cleavage at one amino acid downstream from the furin recognition sequence. The ΔMT1/TIMP-2 complex formed a ternary complex with proMMP-2 through the COOH termini of TIMP-2 and showed gelatinolytic activity as seen using zymography. These results demonstrate for the first time that MT-MMP-1 is a gelatinolytic enzyme and secreted from cells in a complex form with TIMP-2, which can form a ternary complex of MT-MMP-1/TIMP-2/proMMP-2.

Introduction

MMPs are zinc endopeptidases consisting of at least 13 different members. They are classified into five groups according to their substrates and substrate specificities: interstitial collagenases, gelatinases, stromelysins, MT-MMPs, and others (1). Since they are synthesized as inactive zymogens (proMMPs), their activation is a rate-limiting step for the catalytic function. Among the MMPs, active MMP-2 (gelatinase A) is reported to be closely associated with cancer cell invasion and metastasis (2, 3). Although the activation mechanism of proMMP-2 in vivo had not been determined (4), many studies suggested that the enzyme is activated on cell surfaces (5, 6). Recently, we identified a new MMP possessing the transmembrane domain, i.e. MT-MMP-1, and found that it can activate proMMP-2 on the cell membrane (7). The expression of MT-MMP-1 in lung and gastric carcinomas is well correlated with the activation of proMMP-2 (8, 9). In addition, we purified a deletion mutant of MT-MMP-1 lacking the transmembrane domain (ΔMT1) and native MT-MMP-1 secreted from a human breast carcinoma cell line (MDA-MB-231) in soluble forms complexed with TIMP-2 and examined gelatinolytic activity and interaction with MMP-2. In addition, involvement of the RRKR sequence in the processing of ΔMT1 was studied by expressing a ΔMT1 in which the site was replaced by a furin-resistant sequence.

Materials and Methods

Construction of Plasmids. Plasmids encoding ΔMT1 lacking the COOH-terminal transmembrane and cytoplasmic domain of MT-MMP-1 (ΔAla336, Val382) was used as previously reported (11). A site-directed mutagenesis of N185VRKK of ΔMT1 to LVPRGS (mΔMT1) was performed using oligonucleotide PCR mutation primers CTTGTTCCCGTGGATCCTACGCCATC-CAGGGTCTCT (M1-1) and GGATCCACCGGAACGGCCTGTACCGACCATC-TACGCCCCC (M2-2). Nucleotide sequences (sense orientation in M1-1 and antisense in M2-2) encoding mutant sequence LVPRGS are underlined. The PCR-amplified cDNA fragment encoding mΔMT1 protein was subcloned into the pSG5 vector (Stratagene, La Jolla, CA). proMMP-2 cDNA isolated from the human placenta library (Clontech, Palo Alto, CA) was also subcloned into the pSG5 vector.

Cell Cultures and Transfection of Plasmids. COS-1 cells were obtained from the Japanese Cancer Research Resource Cell Bank (Tokyo, Japan). CHO cells lacking a dhfr gene and MDA-MB-231 human breast carcinoma cells were gifts from Dr. F. E. Baralle (United Nations Industrial Development Organization, International Center for Genetic Engineering and Biotechnology, Trieste, Italy) and Dr. M. Toi (Komagome Hospital, Tokyo, Japan), respectively. Plasmids were transiently transfected into COS-1 cells using calcium phosphate coprecipitation methods. A cell line constitutively expressing ΔMT1 was established in the dhfr-lacking CHO cells by two-step selection of the plasmid-containing cells after cotransfection of ΔMT1 cDNA/pSG5 plasmids and pK5 plasmids containing a neomycin-resistant gene and dhfr/pSV2 vector by modification of the methods described by Kaufman and Sharp (12).

Purification of the ΔMT1/TIMP-2 Complex and NH2-Terminal Sequence. In each purification step for ΔMT1 from CHO cells and native MT-MMP-1 secreted from concanavalin A-stimulated MDA-MB-231 cells, MT-MMP-1 and TIMP-2 were monitored by immunoblotting using mono-
COS-1 cells were analyzed under nonreducing conditions using gelatin substrate gel (10% the culture media from AMTI-transfected (Lane 1) and mAMTl-transfected (Lane 2) transfectcd COS-1 cells as described previously (9). Prestained standards are indicated. B. (Lane 4) is not clear, but it can sometimes be observed in the media of the transiently transfected cells. A gelatinolytic band of M, 56,000 (arrowhead) is detected only in the culture media of AMTl transfectants (Lanes 1 and 3) and mAMTl-transfected (Lane 2) COS-1 cells were analyzed under nonreducing conditions using gelatin substrate gel (10% acrylamide). A weak gelatinolytic band of M, 52,000 (arrowhead) is detected only in the sample from the AMTl transfectants. Gelatinolytic activity of M, 68,000 corresponds to proMMP-2 secreted by COS-1 cells.

clonal antibodies against MT-MMP-1 and TIMP-2 as described below. Since both AMTI and MT-MMP-1 were copurified with TIMP-2 and recovered only in the fractions containing TIMP-2, they appeared to exist in the complex forms with TIMP-2 in the culture media. Thus, the complexes were isolated from the corresponding media using a four-step protocol. The concentrated culture media were applied to DEAE-cellulose (2.5 × 8 cm; Whatman, England) and Green A Dymatex gel (2.5 × 8 cm; Amicon Corp., Beverly, MA). The fractions were eluted by a linear gradient of NaCl (0.15–2 M) and applied to an immunoaffinity column of anti-TIMP-2-IgG (clone 67–4H11, the antibody against the COOH-terminal tail domain of TIMP-2; 0.7 × 11 cm) (13). The bound complex was eluted with 3 M NaCl and dialyzed against 50 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 10 mM CaCl₂, 0.05% Brij 35, and 0.02% NaN₃. The samples were then subjected to anti-TIMP-2-IgG Sepharose (clone 68–6H4, the antibody against the first loop of the NH₂-terminal domain of TIMP-2; Ref.13) to remove free TIMP-2. The purified AMTI/TIMP-2 complex was applied to SDS-polyacrylamide gels, transferred onto polyvinylidene difluoride membranes, and the bands of interest were sequenced by 492 sequencer (Applied Biosystems, Foster, CA).

Immunoblot Analyses and Gelatinolytic Activity. Samples resolved by SDS-PAGE with reduction were transferred onto nitrocellulose filters and reacted with monoclonal antibodies against MT-MMP-1 (3). The monoclonal antibodies specific to the catalytic domain of MT-MMP-1 (clones 114–1F2, 114–10F2, and 113–18E4) were prepared using the synthetic peptides as described previously (7). The antibody to the NH₂-terminal propeptide domain (clone 152–16E5) was developed using a synthetic peptide corresponding to the amino acid sequence (L₁50RTHTQRSPQSL₄₄). For detection of gelatinolytic activity, gelatin zymography and gelatinase assay were performed using immunoblotting (under reduction) and gelatin zymography (under nonreduction). As shown in Fig. 1A, AMTI1 was recognized in the media as protein bands of M₄ 56,000 and 62,000, whereas only the M₄ 62,000 species was identified in the cell lysates. On the other hand, culture media and cell lysates from mAMTl-transfected cells contained only the M₄ 62,000 species and not the M₄ 56,000 form (Fig. 1A). Using gelatin zymography, the media from AMTI-transfected cells demonstrated gelatinolytic activity of M₄ 52,000, but no such activity was detected in the media from mAMTl-transfected cells (Fig. 1B).

One of the unique structures of the MT-MMP-1 protein is the insertion of an RRKR furin recognition sequence between the propeptide and catalytic domains (7). Pei and Weiss (14) have recently demonstrated that MMP-11 (stromelysin 3), which has a similar insertion containing the RQKR sequence, is intracellularly processed by furin to become an active species. The present study demonstrates that the RRKR furin recognition site is also essential for the processing of MT-MMP-1, since the expressed protein from mAMTl, which does not contain a furin recognition sequence, was not cleaved. Consistent with the processing, AMTI, but not mAMTl, exhibited gelatinolytic activity. These results suggest that MT-MMP-1 per se is a gelatin-degrading enzyme and requires removal of the propeptide domain for the enzymic activity as reported with the activation of other MMPs such as collagenases, gelatinases, and stromelysin (1).

Results and Discussion

Secretion and Gelatinolytic Activity of AMTI and mAMTl. AMTI and mAMTl were expressed in COS-1 cells by transient transfection, and the cell lysates and culture media were analyzed using immunoblotting (under reduction) and gelatin zymography (under nonreduction). As shown in Fig. 1A, AMTI1 was recognized in the media as protein bands of M₄ 56,000 and 62,000, whereas only the M₄ 62,000 species was identified in the cell lysates. On the other hand, culture media and cell lysates from mAMTl-transfected cells contained only the M₄ 62,000 species and not the M₄ 56,000 form (Fig. 1A). Using gelatin zymography, the media from AMTI-transfected cells demonstrated gelatinolytic activity of M₄ 52,000, but no such activity was detected in the media from mAMTl-transfected cells (Fig. 1B).

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Fig. 1. Immunoblotting (A) and gelatin zymography (B) of AMTI1 and mAMTl from the cDNA-transfected COS-1 cells. A, cell lysates (Lanes 1 and 2) and culture media (Lanes 3 and 4) harvested from AMTI (Lanes 1 and 3) and mAMTl (Lanes 2 and 4) transfectants were reacted with anti-MT-MMP-1 monoclonal antibody specific to the catalytic domain of MT-MMP-1 (clone 114–1F2; 8 µg/ml). Note that the processed form of M₄ 56,000 (arrowhead) is detected only in the culture media of AMTI transfectants while other samples contain the M₄ 62,000 form (arrow). The nature of the immunoreactive band of M₄ 68,000 detected in the culture medium from mAMTl-transfected cells (Lane 4) is not clear, but it can sometimes be observed in the media of the transiently transfected COS-1 cells as described previously (9). Prestained standards are indicated. B, the culture media from AMTI-transfected (Lane 1) and mAMTl-transfected (Lane 2) COS-1 cells were analyzed under nonreducing conditions using gelatin substrate gel (10% acrylamide). A weak gelatinolytic band of M₄ 52,000 (arrowhead) is detected only in the sample from the AMTI transfectants. Gelatinolytic activity of M₄ 68,000 corresponds to proMMP-2 secreted by COS-1 cells.

Fig. 2. SDS-PAGE (A) and gelatin zymography (B) of the purified AMTI1/TIMP-2 complex. A, the purified AMTI1/TIMP-2 complex was iodinated and subjected to SDSPAGE (12.5% acrylamide) under reduction and nonreduction (Lanes 1 and 2, respectively), and then the gels were autoradiographed. B, the complex was subjected to gelatin zymography without reduction (12.5% acrylamide). Note a gelatinolytic band of AMTI1 with M₄ 52,000 (arrowhead).
Purification of the ΔMT1/TIMP-2 Complex from CHO Cells. The ΔMT1/TIMP-2 complex was purified using a four-step protocol including an anti-TIMP-2 IgG-Sepharose column. The final product (145 μg) isolated from the medium (500 ml) migrated at the positions of Mr 52,000 and 23,000 under reducing conditions (Mr 52,000 and 23,000 under nonreducing conditions; Fig. 2A). Using immunoblotting, the Mr 56,000 species was recognized with an antibody against the catalytic domain of MT-MMP-1 (clone 114-1F2), but was not detected by an antibody specific to the NH2-terminal domain of MT-MMP-1 (clone 152-165E5; data not shown). The NH2-terminal sequence analyses demonstrated that the Mr 56,000 protein sequence A₁¹¹QGLKQWNH is identical to MT-MMP-1, indicating that the protein is an NH2 terminally truncated form of ΔMT1. The protein complexed with ΔMT1 was identified as TIMP-2, because the NH2-terminal sequence was WXSSPVXPPQQA, which matches the human TIMP-2 sequence (C5'SCSPVHPQQA; Ref. 13). When the purified ΔMT1/TIMP-2 complex was subjected to gelatin zymography, gelatinolytic activity of Mr 52,000 corresponding to ΔMT1 was demonstrated (Fig. 2B). However, the complex did not demonstrate gelatinolytic activity in a 14C-labeled gelatin solution assay or by the SDS-PAGE analysis of the digestion products of gelatin (data not shown), indicating complete inhibition of the activity in a complex form.

Strongin et al. (9) have reported that the NH2 terminus of MT-MMP-1 isolated from the cell membranes of proMMP-2-activated HT1080 cells is A₁¹¹QGLK, the sequence identical to that of ΔMT1. This suggests that a similar mechanism is involved in the NH2 terminus processing of ΔMT1 and native MT-MMP-1. Since the RRKR-furin recognition site is necessary for the processing as described above, furin may be a potential enzyme involved in the cleavage. However, NH2-terminal sequence data suggest that additional cleavage by another enzyme or autocatalysis at the Tyr₁₁²-, Ala₁₁³ bond is needed after cleavage of the Arg₁¹¹-Tyr₁₁² bond by furin. Another conceivable mechanism is, however, the direct cleavage by an unknown processing enzyme, which may also recognize the RRKR sequence.

Interaction of the ΔMT1/TIMP-2 Complex with MMP-2. Although the ΔMT1/TIMP-2 complex was bound to the immunoaffinity column of a monoclonal antibody specific to the COOH-terminal tail domain of TIMP-2 (clone 67-4H11), it did not bind with other antibodies against the first or third loop of the TIMP-2 NH2-terminal domain (clones 68-6H4 and 69-9E6, respectively; Ref. 13). On the other hand, ΔMT1 did bind to the column of the monoclonal antibodies specific to the catalytic domain of MT-MMP-1 (clones 114-10F2 and 113-18E4) only when the complex was dissociated by incubation with 20 mM EDTA (data not shown). These specific interactions with the monoclonal antibodies suggest that both the NH2-terminal domain of TIMP-2 and the catalytic domain of ΔMT1 are involved in the complex formation and that the COOH-terminal tail domain of TIMP-2 is free. Consistent with these findings is that the ΔMT1/TIMP-2 complex was unable to inhibit MMP-2.

The interaction of the ΔMT1/TIMP-2 complex with the MMP-2 species was examined by cross-linking experiments. When the ¹²⁵I-labeled ΔMT1/TIMP-2 complex was analyzed on an SDS-polyacrylamide gel after treatment with the cross-linker, a major cross-linked product of Mr ~80,000 was formed (Fig. 3A). Incubation of the complex with proMMP-2 generated a new band with a higher molecular weight and decreased the density of the Mr ~80,000 band (Fig. 3A), indicating the cross-linked trimolecular complex formation. On the other hand, the reaction of the complex with active MMP-2 was less efficient (Fig. 3A). This is most likely due to further degradation including cleavage of the COOH-terminal domain (15) during the cross-linking incubation. Very similar data were obtained when the ¹²⁵I-labeled proMMP-2 and active MMP-2 were incubated with the ΔMT1/TIMP-2 complex. A cross-linked form with higher molecular weight appeared in the sample incubated with proMMP-2, but only a very faint band was seen with active MMP-2 (Fig. 3C). The cross-linked complex formation of ΔMT1/TIMP-2 with GST-C was also observed, although no cross-linking was seen with ΔMT1/TIMP-2 (Fig. 3B). These data indicate that the purified ΔMT1/TIMP-2 complex can form, at least in part, the trimolecular complex with proMMP-2, and that the interaction is mediated through the COOH termini of both TIMP-2 and proMMP-2. It has been reported that the TIMP-2 and proMMP-2/TIMP-2 complex bind to HT1080 cells through the NH2-terminal domain of TIMP-2 (10). Strongin et al. (9) have also demonstrated that binding of TIMP-2 to HT1080 cells is essential to proMMP-2 activation by MT-MMP-1 on the cell membranes. Cell surface events of MT-MMP-1 might be different from that of soluble secreted ΔMT1. However, the present data suggest that when MT-MMP-1 is expressed and processed to an active species on the cell surfaces, MT-MMP-1 may function as a receptor for TIMP-2 and/or the proMMP-2/TIMP-2 complex, facilitating MT-MMP-1-mediated activation of proMMP-2.

Secretion and Purification of MT-MMP-1 from MDA-MB-231 Cells. Human metastatic breast carcinoma cells, MDA-MB-231 cells, bind proMMP-2 on the cell surfaces and activate the zymogen into the active MMP-2 species (16). A recent study (17) demonstrated further that concanavalin A treatment of the carcinoma cells stimulates the secretion of MT-MMP-1. The secretion of MT-MMP-1 can be monitored by immunoblotting using a polyclonal antibody against the catalytic domain of MT-MMP-1.
production of MT-MMP-1. Therefore, we investigated in the present study the ability of the MDA-MB-231 cells to secrete MT-MMP-1 into the culture medium. With immunoblot analyses using the anti-MT-MMP-1 antibody specific to the catalytic domain (clone 114-1F2), protein bands of M, 60,000 and 56,000 were recognized in the plasma membrane fractions and culture media of MDA-MB-231 cells, respectively (Fig. 4A). However, both forms failed to react with a monoclonal antibody specific to the propeptide domain of MT-MMP-1 (clone 152-165E5; data not shown), suggesting that they lack the NH2-terminal propeptide domain. The secreted form of MT-MMP-1 was also purified in a complex form with TIMP-2 during purification from the culture media which contained both TIMP-1 and TIMP-2 measured by corresponding sandwich enzyme immunoassays (Refs. 13 and 18; data not shown). Purified MT-MMP-1 migrated as a band of M, 56,000 under reduction (Fig. 4B) and M, 52,000 under nonreducing conditions (data not shown).

Gelatin zymography of the culture medium and purified complex demonstrated a M, 52,000 band of lysis (Fig. 4C). These results indicate for the first time that native MT-MMP-1 capable of digesting gelatin is also secreted from the concanavalin A-stimulated MDA-MB-231 cells in a complex form with TIMP-2.

The mechanism and biological significance of MT-MMP-1 secretion from the cells are not yet clear. However, the data demonstrating that MT-MMP-1 is localized to the cell membrane of HT1080 and MDA-MB-231 cells in a propeptide-domain-deleted form (9) suggest that sequential processing of proMT-MMP-1 occurs prior to secretion from the cell surfaces, i.e., NH2-terminal truncation and then cleavage of the COOH-terminal domain including the transmembrane region. Itoh et al. (19) recently reported that the proMMP-2/TIMP-2 complex becomes activated by transposition of TIMP-2 to the active MMPs from the complex when the ternary complex with active MMP-2 or MMP-3 is incubated with APMA or the cell membranes. Thus, it is possible to speculate that the MT-MMP-1/TIMP-2 complex is generated and released as a result of the activation of the proMMP-2/TIMP-2 complex by MT-MMP-1 on the cell surface. The present study, however, does not preclude the possibility that the complex is formed in the culture media after release of free MT-MMP-1 from the cell membranes. Endowment of MT-MMP-1 with gelatinolytic activity suggests that in addition to proMMP-2 activation, the protease functions as an extracellular matrix-degrading enzyme since MT-MMP-1 may exist, at least transiently, in a TIMP-2-free form, especially on the cell membranes. The role of MT-MMP-1 as an extracellular matrix-degrading enzyme remains to be elucidated through additional work on the substrates and tissue distributions of the free MT-MMP-1.

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References

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