Activation of a recombinant membrane type 1-matrix metalloproteinase (MT1-MMP) by furin and its interaction with tissue inhibitor of metalloproteinases (TIMP)-2

Hiroshi Sato[a,*], Takeshi Kinoshita[a], Takahisa Takino[a], Kazuo Nakayama[b], Motoharu Seiki[a]

[a]*Department of Molecular Virology and Oncology, Cancer Research Institute, Kanazawa University, 13-1 Takaramachi, Kanazawa 920, Japan*
[b]*Institute of Biological Sciences and Gene Experiment Center, University of Tsukuba, Tsukuba 305, Japan*

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Abstract **Membrane type 1-matrix metalloproteinase (MT1-MMP) initiates the activation of the zymogen progelatinase A/72-kDa type IV collagenase by cleavage of the Asn^{66}-Leu peptide bond. We previously pointed out that MT1-MMP possesses a unique amino acid sequence Arg-Arg-Lys-Arg^{111} which is a potential recognition sequence for furin-like proteases (*Nature*, 370 (1994) 61–65). Here, using a recombinant MT1-MMP expressed in *Escherichia coli* we demonstrated that furin specifically cleaves MT1-MMP between Arg^{111}-Tyr in vitro, which resulted in a stimulation of progelatinase A-activation function. Tissue inhibitor of metalloproteinases (TIMP)-2 inhibited activation of progelatinase A by forming a stable complex with activated MT1-MMP.**

Key words: Matrix metalloproteinase; Activation; MT1-MMP

1. Introduction

Membrane type-matrix metalloproteinase (MT-MMP) (recently renamed membrane type 1-MMP, MT1-MMP) is a new member of the matrix metalloproteinase (MMP) family which is associated with proteolytic activation of the zymogen progelatinase A/72-kDa type IV collagenase A [1–3]. Following the first discovery of MT1-MMP, three more MT-MMPs (MT2-, MT3-, MT4-MMP) were identified, and these MT-MMPs also possess the conserved Arg-Arg-Lys/Arg-Arg sequence sandwiched between the pro- and catalytic domains [4–6]. We deduced that this is a recognition sequence for Kex2-like proteases including furin [1,2]. Stromelysin-3 has a similar Arg-Gln-Lys-Arg sequence at the same position, which was since shown to be cleaved by furin [7,8]. We recently reconstituted progelatinase A processing using a recombinant MT1-MMP fusion protein containing the propeptide, catalytic and hinge domains, which indicated that MT1-MMP directly cleaves Asn^{66}-Leu peptide bond of progelatinase A to produce a 64-kDa intermediate form [9]. Here we demonstrate that the recombinant MT1-MMP is activated by furin, and that tissue inhibitor of MMP (TIMP)-2 makes a stable complex with the activated MT1-MMP, and thus inhibits progelatinase A-activation function.

*Corresponding author. Fax: (81) 762-60-7840.
E-mail: vhsato@kenroku.ipc.kanazawa-u.ac.jp

Abbreviations: MT1-MMP, membrane type 1-matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinases

2. Materials and methods

2.1. Expression of a recombinant MT1-MMP protein

A recombinant MT1-MMP protein was expressed as a fusion protein with glutathione-S-transferase [9]. The expression plasmid encoding GST-MT1-MMP (GST-MT) was constructed by inserting an MT1-MMP cDNA fragment encoding (Ser^{34} to Cys^{319}) at the *Eco*RI site of the pGENT2 vector [10]. The fusion protein was expressed in *E. coli* strain JM109 and purified using glutathione-Sepharose beads (Pharmacia Biotech). A purified sample of GST-MT protein absorbed on the beads was stored in TNC buffer (100 mM Tris-HCl, pH 7.5, 150 mM NaCl and 10 mM $CaCl_2$) containing 50% glycerol at −70°C.

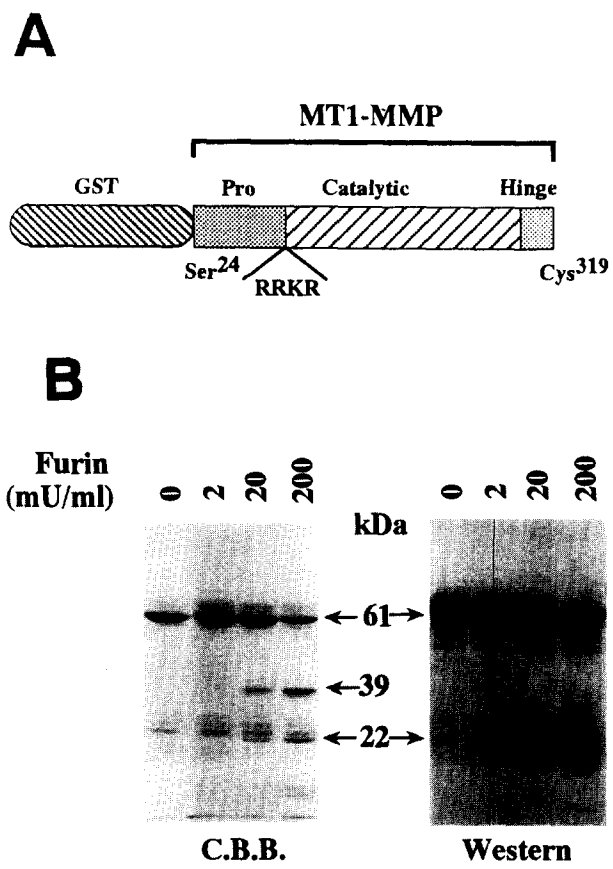

Fig. 1. Cleavage of GST-MT by furin. (A) Structure of GST-MT1-MMP fusion protein. (B) Cleavage of GST-MT with furin. GST-MT protein absorbed on the glutathione-Sepharose beads was incubated with furin at the indicated concentrations as described in Section 2, and then analyzed by SDS-PAGE and Coomasie Brilliant Blue staining (panel, C.B.B.). GST-MT treated with furin was separated as above and then analyzed by Western blotting using a monoclonal antibody against the catalytic domain of MT1-MMP (114-1F2) (panel, Western).

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2.2. Processing of GST-MT by furin

Furin truncated at the transmembrane domain was prepared as described previously [11,12]. A recombinant MT1-MMP protein absorbed on the beads was washed by centrifugation with 100 mM HEPES buffer (pH 7.0) containing 1 mM $CaCl_2$, 0.1% bovine serum albumin and then incubated with furin in a same buffer at 37°C for 1 h. MT1-MMP fragment was identified by Western blotting using a monoclonal antibody against the catalytic domain of MT1-MMP (114-1F2) [1]. To determine the cleavage site, 5 μg of GST-MT protein was incubated with 200 mU/ml of furin, subjected to SDS-polyacrylamide gel electrophoresis (PAGE), and electroblotted onto an Immobilon-Psq membrane (Millipore). A piece of the membrane containing the MT1-MMP fragment band was cut out and applied to a protein sequencer (Hewlett Packard, model G1005A system).

2.3. Interaction between GST-MT and TIMPs

Recombinant TIMP-1 and TIMP-2 proteins were supplied by K. Iwata (Fuji Chemical Industries Ltd., Takaoka, Japan). GST-MT or GST protein (20 ng) either treated or untreated with 200 mU/ml furin was incubated with about 50 pg of ^{125}I-labeled TIMP-1 or TIMP-2 for 10 min at 37°C, and then MT1-MMP protein was immunoprecipitated with anti-MT1-MMP monoclonal antibody (114-1F2) with the aid of protein A Sepharose beads. ^{125}I-labeled TIMP-1 or TIMP-2 coprecipitated with MT1-MMP was separated on 12% SDS-polyacrylamide gel and analyzed with the BAS 1000 scanner.

3. Results and discussion

A recombinant MT1-MMP fusion protein GST-MT, which contains the propeptide, catalytic and hinge domains of MT1-MMP, was incubated with a soluble form of furin for 1 h at 37°C and then the samples were subjected to SDS-PAGE. As shown in Fig. 1, incubation of GST-MT with furin resulted in cleavage of the 61-kDa protein into 39-kDa and 22-kDa fragments in a dose-dependent manner. Western blotting with a monoclonal antibody against catalytic domain of MT1-MMP, 114-1F2 indicated that the resultant 22-kDa fragment contains the catalytic domain. To locate the cleavage site for furin, the 22-kDa fragment was excised from the slot, and the amino-terminal amino acid sequence was determined. The resulting sequence, Tyr-Ala-Ile-Gln-Gly corresponded with residues 112–116 of MT1-MMP, indicating that furin cleaves MT1-MMP between Arg^{111} and Tyr^{112}, and thus removed the prodomain.

To examine whether the cleavage of MT1-MMP between Arg^{111} and Tyr^{112} facilitates enzymatic activation, progelatinase A-activating capacity was compared between furin-treated and untreated GST-MT. Treatment of the recombinant protein with furin enhanced progelatinase A-activation capability in parallel with the cleavage of prodomain (Fig. 2A). Incubation with furin-treated GST-MT processed progelatinase A depending on the concentration of GST-MT (Fig. 2B). The time course of progelatinase A activation by furin-

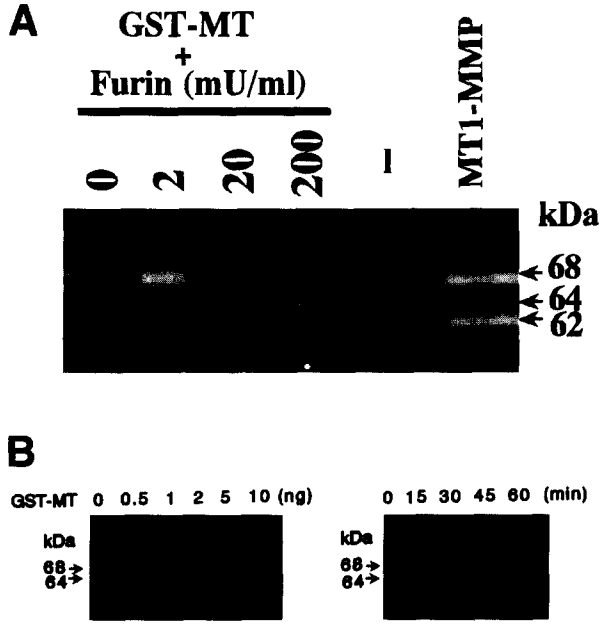

Fig. 2. Activation of MT1-MMP by furin. (A) stimulation of progelatinase A processing by furin. GST-MT recombinant protein (10 ng) was pre-treated with furin as described in the legend of Fig. 1, and then incubated with 10 ng of progelatinase A at 37°C for 1 h, which was then analyzed by gelatin zymography. Lane, —: progelatinase A. A culture supernatant from COS-1 cells co-transfected with gelatinase A and MT1-MMP plasmids was run as a control (lane, MT1-MMP). (B) GST-MT-dependent progelatinase A activation. Progelatinase A (10 ng) was incubated with the indicated concentrations of GST-MT treated with 200 mU/ml furin at 37°C for 1 h and analyzed by gelatin zymography (left panel). Progelatinase A (10 ng) was incubated with 5 ng of furin-treated GST-MT for the indicated periods and analyzed by gelatin zymography (right panel).

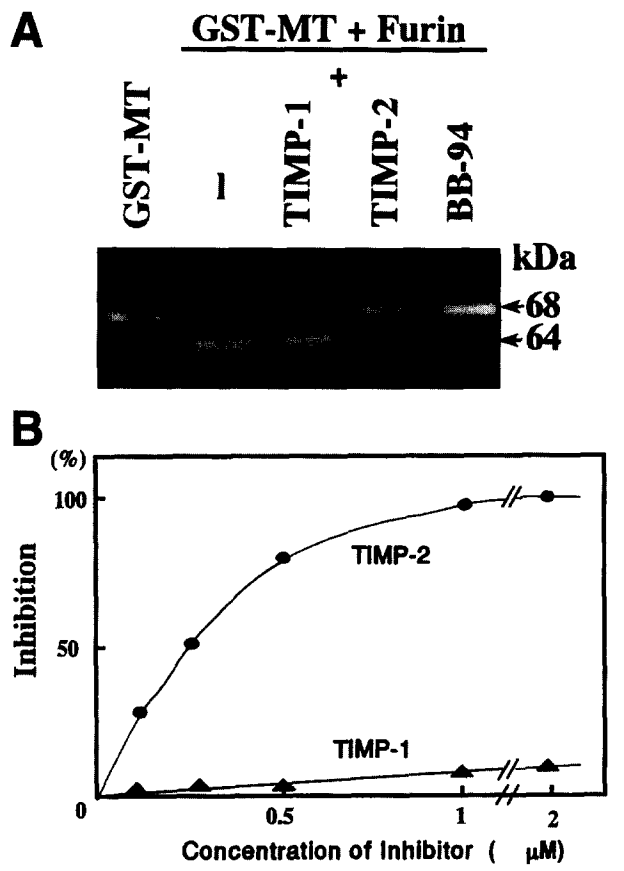

Fig. 3. Effect of inhibitors of MMP. (A) Inhibition of progelatinase A processing. Progelatinase A (10 ng) was incubated with 10 ng of GST-MT treated with 200 mU/ml furin in the absence (lane, —) or presence of 200 ng TIMP-1, TIMP-2 and 10^{-5} M BB-94 (lanes TIMP-1, TIMP-2 and BB-94, respectively) at 37°C for 1 h and analyzed by gelatin zymography. (B) Inhibition of progelatinase A processing by TIMP-1 or TIMP-2. Progelatinase A (10 ng) was incubated with 10 ng GST-MT treated with 200 mU/ml furin in the presence of various concentrations of TIMP-1 or TIMP-2 and analyzed by gelatin zymography. The ratio of processing was determined by densitometric analysis.

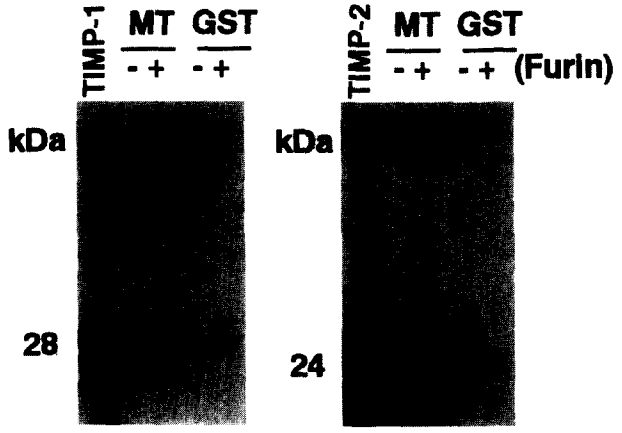

Fig. 4. Interaction between activated MT1-MMP and TIMPs. GST-MT or GST protein treated (lanes, +) or untreated (lanes, −) with furin was incubated with ^{125}I-labeled TIMP-1 (panel, TIMP1) or TIMP-2 (panel, TIMP-2), and then MT1-MMP protein was immunoprecipitated using an anti-MT1-MMP monoclonal antibody (114-1F2) with the aid of protein A sepharose beads. ^{125}I-labeled TIMP-1 or TIMP-2 co-precipitated with MT1-MMP was separated on 12% SDS-polyacrylamide gel and analyzed by BAS 1000 gel scanner.

treated GST-MT is also shown, where incubation of progelatinase A with activated MT1-MMP at a molar ratio of 2 to 1 caused complete processing within 1 h at 37°C.

To characterize the activated MT1-MMP, we examined the effects of known inhibitors of matrix metalloproteinases, TIMP-1, TIMP-2 and BB-94 on the activation of progelatinase A. As shown in Fig. 3, TIMP-2 effectively inhibited processing of progelatinase A by furin-treated GST-MT; however, TIMP-1 had only a slight effect. BB-94, a general inhibitor of MMP also inhibited processing by furin-activated GST-MT. These results were essentially same as that achieved with untreated GST-MT [9]. A direct interaction of MT1-MMP with TIMPs was examined using ^{125}I-labeled TIMP-1 and TIMP-2 (Fig. 4). ^{125}I-labeled TIMP-2 was co-precipitated with furin-treated, but not with untreated, GST-MT using a monoclonal antibody against MT1-MMP. However, TIMP-1 did not show a detectable interaction with either furin-treated or untreated GST-MT. TIMP-2-specific inhibition of progelatinase A processing by GST-MT is consistent with that observed using a membrane fraction containing activated MT1-MMP [13]. Thus, inhibition by TIMP-2 is due to a specific binding of TIMP-2 to activated MT1-MMP. We did not observe any stimulatory effect of low doses of TIMP-2 on progelatinase A processing in a reconstituted soluble system [14]. For cell surface activation, processing of progelatinase A must be preceeded by binding to the cell surface [15]. TIMP-2 may serve as a part of a progelatinase A receptor, collaborating with MT1-MMP to recruite progelatinase A to the cell surface, and this may accelerate the interaction between MT1-MMP and progelatinase A. Indeed, the carboxyl fragment of progelatinase A, through which TIMP-2 may bind, is essential for the binding and processing on the cell surface [13,14,16]. However, in a reconstituted soluble system MT1-MMP would directly interact with progelatinase A and thus even a truncated progelatinase A mutant lacking the carboxyl fragment was processed by the recombinant form of MT1-MMP (unpublished data).

Recently several mammalian Kex2-like proteases have been identified besides furin, and it remains to be seen whether these Kex2-like enzymes also process MT1-MMP [17–22]. However, most of these proteases are expresssed in specific cells, whereas furin is expressed in a wide variety of cells [23,24]. MT1-MMP is expressed in tumor cell lines, endothelial cells and normal fibroblast cells in the processed form ([14], unpublished data). Furin, with its wide distribution, may be responsible for this constitutive activation of MT1-MMP. Strongin et al. isolated an MT1-MMP–TIMP-2 complex from HT1080 cells and demonstrated an amino terminus at Tyr^{112} [14]. However, when we purified a form of MT1-MMP truncated at carboxyl transmembrane domain from the culture supernatant of CHO cells stably transfected with the expression plasmid, we found an amino terminus at Ala^{113} [25]. Mutation of Arg-Arg-Lys-Arg^{111} sequence of truncated MT1-MMP abolished processing in CHO cells, indicating the recognition of this sequence for the processing in cells. It still remains to be clarified whether an amino acid Tyr^{112} is removed after cleavage by furin or a protease other than furin cleaves MT1-MMP between Tyr^{112} and Ala^{113} in CHO cells.

During the course of our study, Pei and Weiss reported activation of MT1-MMP using a mutant protein lacking the transmembrane domain purified from MDCK cells [26]. Thus the same conclusion was drawn from independent experiments using different MT1-MMP samples.

The reconstituted system will be usefull not only for studying further the characteristics of MT1-MMP, but also for screening enzymes involved in the activation of MT1-MMP.

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