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Membrane-type 6 matrix metalloproteinase (MT6-MMP, MMP-25) is the second glycosyl-phosphatidyl inositol (GPI)-anchored MMP

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Abstract A recently identified membrane-type 6 matrix metalloproteinase (MT6-MMP) has a hydrophobic stretch of 24 amino acids at the C-terminus. This hydrophobicity pattern is similar to glycosyl-phosphatidyl inositol (GPI)-anchored MMP, MT4-MMP, and other GPI-anchored proteins. Thus, we tested the possibility that MT6-MMP was also a GPI-anchored proteinase. Our results showed that MT6-MMP as well as MT4-MMP were labeled with [^{3}H]ethanolamine indicating the presence of a GPI unit with incorporated label. In addition, phosphatidyl inositol-specific phospholipase C treatment released MT6-MMP from the surface of transfected cells. These results strongly indicate that MT6-MMP is a GPI-anchored protein. Since two members of MT-MMPs are now assigned as GPI-anchored proteinase, MT-MMPs can be subgrouped into GPI type and transmembrane type.

Key words: Membrane-type 6 matrix metalloproteinase; Membrane-type 4 matrix metalloproteinase; Glycosyl-phosphatidyl inositol anchor

1. Introduction

Matrix metalloproteinases (MMPs), also called matrixins, are a family of Zn^{2+}-dependent metalloendopeptidase that is involved in the degradation of extracellular matrix (ECM) in physiological and pathological conditions [1,2]. To date, 21 mammalian MMPs have been identified by cDNA cloning [3–8], and they can be sub-grouped into 15 soluble-type MMPs and 6 membrane-types (MT-MMPs). In MT-MMP subfamily, MT1-, MT2-, MT3-, MT5-MMPs are type I transmembrane proteins as they have transmembrane domain and about 20 amino acids cytoplasmic tail. On the other hand, MT4-MMP is a glycosyl-phosphatidyl inositol (GPI)-anchored proteinase [9]. GPI-anchored proteins attach to the plasma membrane via 2–3 fatty acids contained in the GPI molecule. Such a protein is synthesized as a precursor having a 15–20 hydrophobic amino acid stretch at the C-terminus (GPI-signal). The hydrophobic amino acid stretch is cleaved off in the ER lumen, and the ectodomain is transferred to the GPI moiety [10]. Thus, the mature GPI-anchored protein has neither transmembrane nor intracellular domains.

Recently, the sixth member of MT-MMP, MT6-MMP/leukolysin/MMP-25, has been identified [7,8]. The highest amino acid sequence identity to other MT-MMPs is with MT4-MMP (45–49%) followed by MT1-MMP (39%). MT6-MMP is expressed in leukocytes among normal tissue cells [7] and in colon carcinoma and astrocytoma among tumor cell lineage [8]. MT6-MMP was also reported to activate proMMP-2 (progelatinase A) [8]. However, the function of the enzyme remains to be determined.

The hydrophobicity plot of the deduced amino acid sequence at the C-terminus indicates that the putative transmembrane domain of MT6-MMP locates at the end of the C-terminus, similar to MT4-MMP. This suggests the possibility that MT6-MMP may be a GPI-anchored protein. In this paper, we report that MT6-MMP is the second GPI-anchored proteinase in the MMP family.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM) was from Nissui Pharmaceutical (Tokyo, Japan). Ham's F-12 medium was from Gibco Life Technologies (MD, USA). FuGENE6™ was from Roche Molecular Biochemicals (Basle, Switzerland). Phosphatidylinositol-specific phospholipase C (PI-PLC) was from Oxford Glyco Sciences (Abingdon, UK). Monoclonal mouse anti-FLAG epitope IgGs, M1 and M2, was from Sigma (MO, USA). Alexa488-conjugated goat anti-mouse IgG was from Jackson Immuno Research Laboratories (PA, USA).

2.2. Construction of FLAG-tagged human MT6-MMP

Partial sequence of MT6-MMP was screened from dbEST library, and the full-length human cDNA (accession no: AB042328) was obtained from placenta cDNA library of Marathon-Ready cDNA (Clontech, CA, USA) by 5′- and 3′-RACE method. To detect MT6-MMP protein by anti-FLAG antibody, FLAG epitope (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys)-tagged MT6-MMP expression constructs were generated. The FLAG epitope was inserted immediately downstream of the furin cleavage motif of MT6-MMP (following Arg^{107}) (MT6-F). MT1-F and MT4-F constructs were made as described previously [9]. All the mutant constructs were generated by polymerase chain reaction using overlap extension method of Ho et al. [11], and all polymerase chain reaction products were confirmed by DNA sequencing. All the mutated fragments were ligated into the original MT-MMP cDNA and whole cDNA was subcloned into the pSG5 expression vector (Stratagene, CA, USA).

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Abbreviations: MMP, matrix metalloproteinase; MT-MMP, membrane-type matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; ECM, extracellular matrix; GPI, glycosyl-phosphatidyl inositol; PI-PLC, phosphatidyl inositol-specific phospholipase C

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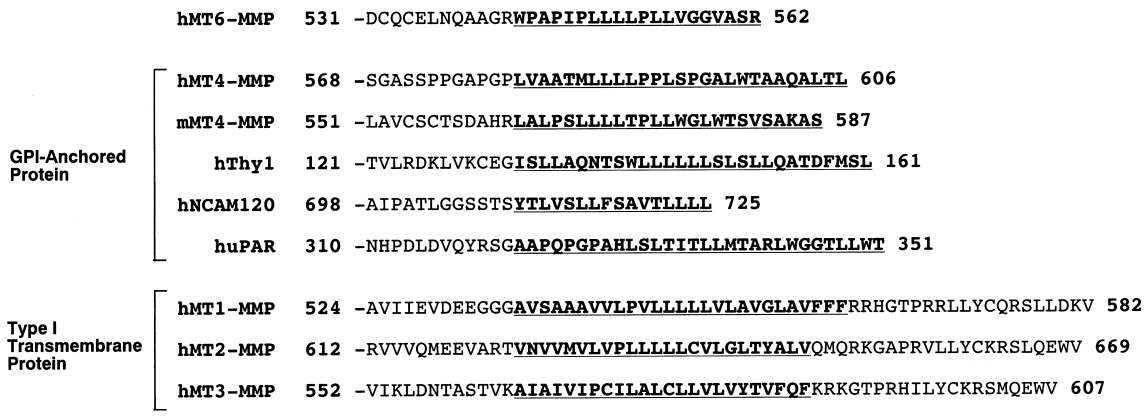

Fig. 1. Comparison of the C-terminal end of MT6-MMP to other GPI-anchored proteins. Amino acid sequences of the C-terminal end that include the transmembrane region of MT1–6-MMPs, human Thy1 (hThy1) (accession number 135830), GPI-anchor type human N-CAM (neural cell adhesion molecule, hNCAM120) (accession number 127861), and human urokinase receptor (huPAR) (accession number 465003) are listed and aligned. Amino acids in the expected transmembrane region according to hydrophobicity plots are indicated in bold and underlined.

2.3. Cell culture and transfection

COS1 cells and CHO-K1 cells were cultured in DMEM or Ham's F-12 medium supplemented with 10% FBS and Kanamysin in a humidified 37°C incubator. Sixteen hours before the transfection was performed, cells were re-seeded in 6-well plates at 2×10^5/well. Expression vectors for each protein were transfected using FuGENE6™ according to the manufacturer's instructions. After 48 h, the cells were harvested and subjected to each experiment.

2.4. Western blotting

Samples were separated by SDS/PAGE, and the proteins in the gel were transferred to a nitrocellulose membrane (Hybond-ECL, Amersham Life Science, Buckinghamshire, UK). After blocking with 10% fat-free dry milk in TBS (20 mM Tris–HCl, pH 7.5, 150 mM NaCl), the membrane was probed with anti-FLAG M2 monoclonal antibody (3 μg/ml) to detect FLAG-tagged MT-MMPs. The membrane was further probed with alkaline phosphatase-conjugated anti-mouse IgG to visualize FLAG-tagged MT-MMPs.

2.5. [^{3}H]Ethanolamine incorporation, immunoprecipitation and autoradiography

The experiments were performed as described previously [9]. Briefly, COS1 cells transfected with expression plasmids for MT6-F, MT4-F, MT1-F, or vector alone were incubated with [^{3}H]ethan-1-ol-2-amine (Amersham Life Science, Buckinghamshire, UK) (80 μCi/ml) for 24 h. Cells were lysed in the RIPA buffer (10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.1% sodium deoxycolicolate, 0.1% SDS, 10 mM EDTA, 2 mM PMSF, 10 μM E-64, 0.02% NaN_3) at room temperature (RT), and lysates were subjected to immunoprecipitation using anti-FLAG M2 conjugated beads (Sigma) at RT for 2 h. The immunoprecipitated samples were subjected to SDS–PAGE and ^{3}H-labeled materials were detected on X-ray film after reaction of the gel with EN^{3}HANCE (NEN™ Life Science Products, MA, USA). The FLAG-tagged MT-MMPs in the samples were visualized by Western blotting using anti-FLAG M2 antibody.

3. Results

3.1. Characterization of MT6-MMP as a GPI-anchored protein

The amino acid alignment of human MT6-MMP with other MT-MMPs and GPI-anchored proteins shows similar pattern of hydrophobicity at the C-terminus to MT4-MMP and GPI-anchored proteins (Fig. 1). Therefore, we addressed the possibility that MT6-MMP was expressed on the cell surface as a GPI-anchored protein. The synthesis of the GPI-membrane anchor unit includes the incorporation of ethanolamine for bridging the COOH-terminal amino acid and the glycan structure [10]. Thus, GPI-anchored proteins can be labeled metabolically with [^{3}H]ethanolamine [10]. A FLAG epitope of Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys was inserted downstream of the furin cleavage site of MT6-MMP (following Arg^{107}, MT6-F) as well as MT1- and MT4-MMP (MT1-F and MT4-F, respectively). COS1 cells were transfected with the expression plasmids for MT6-F, MT4-F, and MT1-F, and [^{3}H]ethanolamine was added to culture medium to metabolically label the cells. The cells were then lysed in RIPA buffer and subjected for immunoprecipitation using anti-FLAG M2 IgG-conjugated beads. As shown in Fig. 2, Western blotting analyses of these samples using anti-FLAG M2 antibody revealed major doublet bands at 62 and 58 kDa for MT6-F, a doublet of 71 and 67 kDa for MT4-F, and a 63- and 60-kDa band for MT1-F (WB). When these samples were analyzed by autoradiography, as expected, [^{3}H]ethanolamine was specifi-

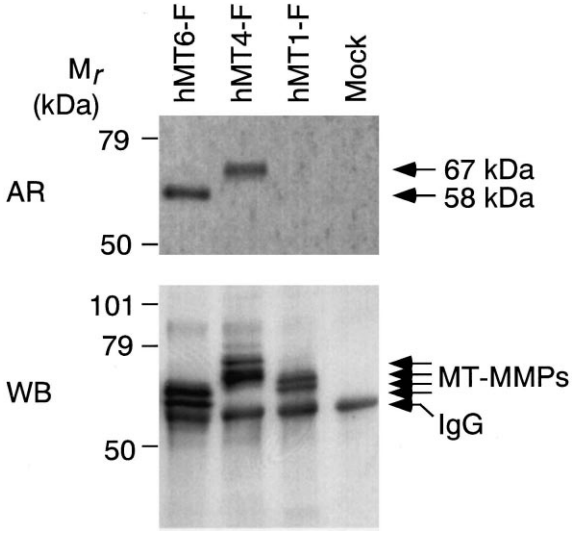

Fig. 2. Incorporation of [^{3}H]ethanolamine into MT6-MMP. COS1 cells, transiently transfected with the expression vectors for hMT6-F, hMT4-F, or hMT1-F, or with the vector alone were incubated with [^{3}H]ethan-1-ol-2-amine, and the cell lysates were subjected to immuno-precipitation using anti-FLAG M2 conjugated beads as described in Section 2. The samples were subjected to autoradiography (AR) or Western blotting (WB) using M2 antibody.

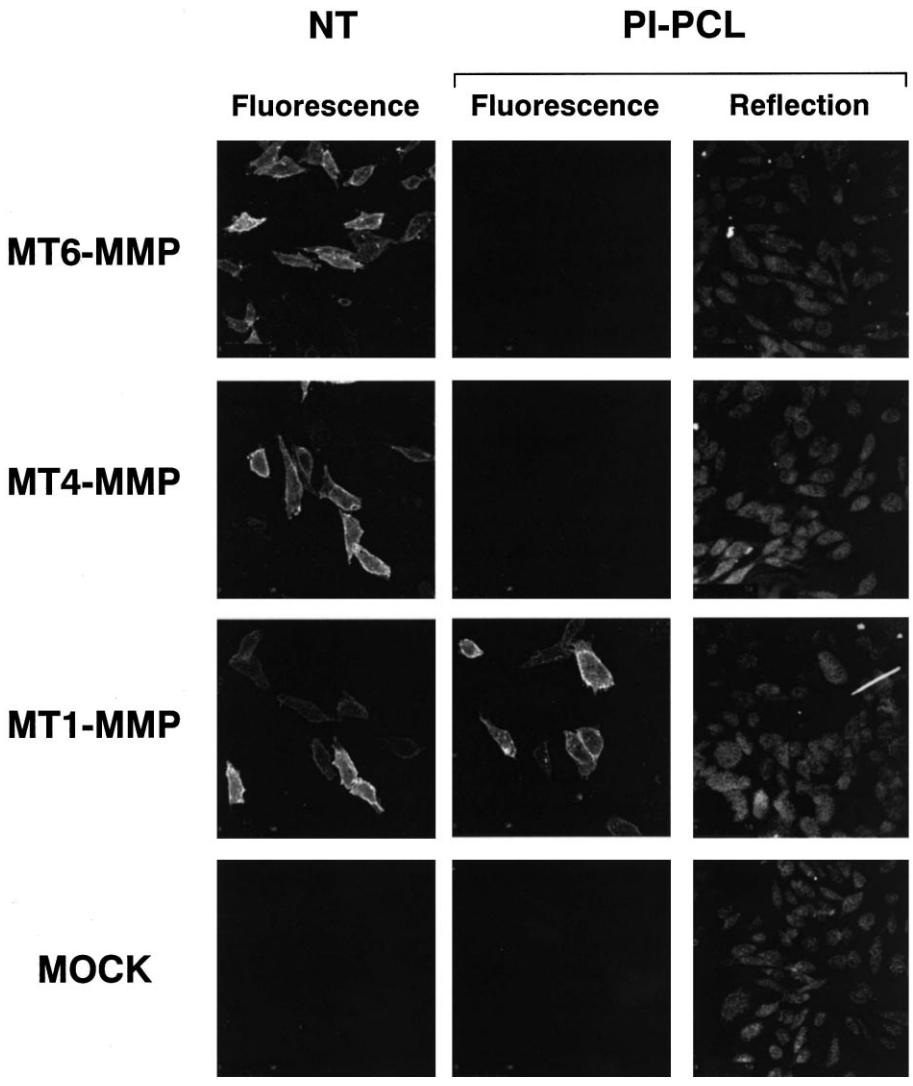

Fig. 3. Release of MT6-MMP from the cell surface by PI-PLC treatment. CHO-K1 cells were transfected with the expression plasmids for hMT6-F, hMT4-F, hMT1-F or the vector without insert. After 48 h, the cells in 8-well chamber slide were treated with or without PI-PLC (10 U/ml) in DMEM without FBS at 37°C for 1 h. The cells were then fixed, and MT-MMPs were immunolocalized using anti-FLAG M1 antibody without permeabilization of the cells. Cy3-conjugated anti-mouse IgG was used to visualize the signals and analyzed by confocal microscopy (Bio-Rad). The broad area of the staining pattern in each treatment is shown (×47). For PI-PLC treated cells, reflection images of same area were also shown.

cally incorporated into both MT6-F and MT4-F, but not into MT1-F (AR, lanes 1 and 2). The labeled band corresponds to one of the two bands at 60- or 67-kDa species for both MT6-F and MT4-F, respectively, detected by anti-FLAG M2 antibody (WB, lanes 1 and 2). We have previously shown that 71-kDa species of MT4-MMP that is not labeled with [^{3}H]ethanolamine is a precursor for GPI-anchoring likely to have C-terminal hydrophobic stretch intact [9]. Like MT4-MMP, the upper bands detected on the Western blot in MT6-MMPs (62 kDa) that are not labeled with [^{3}H]ethanolamine are likely to correspond to the precursor for GPI-anchoring, and the 58-kDa species is the GPI-anchored MT6-MMP.

We then tested the susceptivity of MT6-MMP expressed on the cell surface to the bacterial PI-PLC that is a hallmark of GPI-anchored protein [10]. As shown in Fig. 3, PI-PLC treatment of the transfected cells cleared off the cell surface located MT6-F as well as MT4-F. In contrast, the type I transmembrane proteinase MT1-MMP was insensitive to PI-PLC treatment. Taken together, we conclude that human MT6-MMPs is a GPI-anchored proteinase like MT4-MMP.

3.2. MT6-MMP does not activate proMMP-2

It has been shown that co-expression of proMMP-2 and MT6-MMP results in proMMP-2 activation [8]. However, co-expression of MT6-F with proMMP-2 in COS1 cells did not result in the activation of proMMP-2 at all as shown in Fig. 4 (lane 3). In contrast, MT1-F activated proMMP-2 into 65-kDa active species effectively (lane 2). The same result was obtained when purified proMMP-2 was added to the culture medium of transfected COS1 or CHO-K1 cells (data not shown). Therefore, we conclude that MT6-MMP, at least in our condition using COS1 or CHO-K1 cells, does not activate proMMP-2.

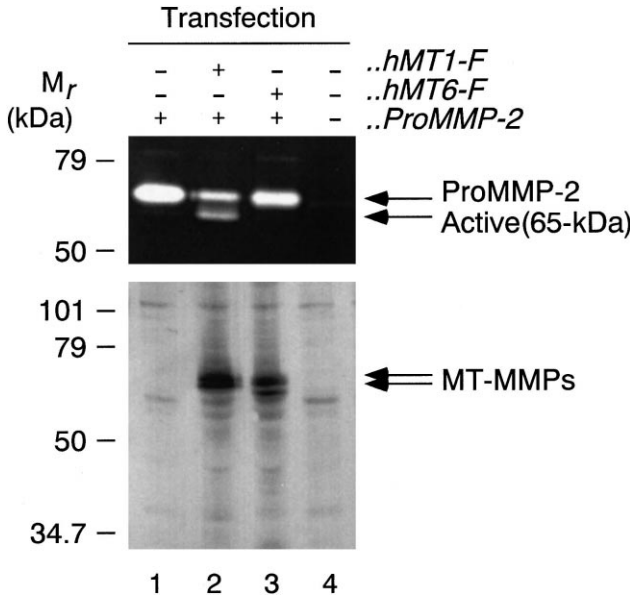

Fig. 4. MT6-MMP does not activate proMMP-2. COS1 cells were transfected with the expression plasmid of proMMP-2 together with the plasmid for MT1-MMP or MT6-MMP as indicated above. After 24 h, culture medium was changed to serum-free medium and further incubated for 36 h. Culture supernatant was then analyzed by zymography, and cell lysate by Western blotting using M2 antibody.

4. Discussion

In the present study, we have shown that MT6-MMP is a GPI anchored MMP. Among 6 MT-MMPs, MT4-MMP and MT6-MMP are GPI-anchored proteins, and thus MT-MMPs can be now further classified into two subgroups: transmembrane-type and GPI-type. The significance of the differences in the anchoring methods to the plasma membrane is still unknown, but it is likely that the cytoplasmic domain of the transmembrane-type MT-MMP may have a role in the regulation of the MT-MMPs' activity on the cell surface [12]. GPI-type MT-MMPs, on the contrary, utilize GPI-moiety to localize in microdomain structure of the plasma membrane which is rich in cholesterol and glycosphingolipids [9,13–15]. The enrichment of GPI-anchored proteins in microdomain was shown not to be ecto-domain dependent, but GPI-moiety-dependent manner [14]. Thus, different GPI-anchored proteins can be localized in the same site on the plasma membrane. In fact, MT4-MMP was shown to be colocalized with non-related GPI-anchored protein, uPAR [9], and may be localized with MT6-MMP as well. The uPAR has previously been shown to associate with β_2-integrin (CD18), and this interaction was shown to be important for leukocyte adhesion to the endothelial cells [16], local fibrin degradation [17] and cell migration [18]. The GPI-anchored proteinases, MT4-MMP and MT6-MMP, co-localized with uPAR in the same microdomain clusters. Thus, they may co-operate in conjunction with cell adhesion molecules, such as CD18.

GPI-anchored MMP in other species can be also found according to its amino acid sequence. One MT-MMP found in *Caenorhabditis elegans*, MMP-Y19 (GenBank accession number AB007817) [19], is likely to be a GPI-type because of its hydrophobicity pattern of the amino acid sequence of its C-terminal end (493-PRNEKLVLNSSSSH**FSLIYATITILI-LIF**-521). By searching *C. elegans* genome database, none of MT-MMPs other than MMP-Y19 was found. If MMP-Y19 is the only MT-MMP in *C. elegans*, GPI-type MT-MMP may be an ancestor of transmembrane-type MT-MMPs during evolution, and may suggest that GPI-anchored MMPs play an important role in certain fundamental biological phenomenon.

In contrast to the report by Velasco et al. [8], MT6-MMP did not activate proMMP-2 in this study. The difference between the results may be due to different experimental conditions used such as different cell types. Nevertheless, the MT6-MMP does not seem to activate proMMP-2 effectively. To activate proMMP-2 on the cell surface, a complex of MT-MMPs with TIMP-2 is required to attract proMMP-2 on the cell surface [20,21]. It also requires TIMP-2 free MT-MMPs near the MT-MMPs/TIMP-2/proMMP-2 complex [20]. Thus, inability of the activation of proMMP-2 by MT6-MMP may be explained by a defect in either steps. Activation of proMMP-2 by MT1-MMP [22], MT2-MMP [23], MT3-MMP [24], and MT5-MMP [6] has been reported. Thus activation of proMMP2 may be a common catalytic property of MT-MMPs. On the other hand, MT1-MMP gene knock out mice were deficient in proMMP-2 activation in vivo tissue [25]. Thus, it is likely that the major in vivo activator of proMMP-2 is MT1-MMP rather than the other MT-MMPs. In conclusion, our results indicate that MT6-MMP is the second GPI-anchored MMP in mammal. Anchoring MT4-MMP and MT6-MMP to the plasma membrane through GPI-anchor may indicate a unique biological character, regulation and function of these enzymes among MT-MMPs.

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