Membrane Type 4 Matrix Metalloproteinase (MT4-MMP, MMP-17) Is a Glycosylphosphatidylinositol-anchored Proteinase*

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Among the five membrane-type matrix metalloproteinases (MT-MMPs), MT1-, MT2-, MT3-, and MT5-MMPs have about a 20-amino acid cytoplasmic tail following the transmembrane domain. In contrast, a putative transmembrane domain of MT4-MMP locates at the very C-terminal end, and the expected cytoplasmic tail is very short or nonexistent. Such sequences often act as a glycosylphosphatidylinositol (GPI) anchoring signal rather than as a transmembrane domain. We thus examined the possibility that MT4-MMP is a GPI-anchored proteinase. Our results showed that [3H]ethanolamine, which can be incorporated into the GPI unit, specifically labeled the MT4-MMP C-terminal end in a sequence-dependent manner. In addition, phosphatidylinositol-specific phospholipase C treatment released the MT4-MMP from the surface of transfected cells. These results indicate that MT4-MMP is the first GPI-anchored proteinase in the MMP family. During cultivation of the transfected cells, MT4-MMP appeared to be shed from the cell surface by the action of an endogenous metalloproteinase. GPI anchoring of MT4-MMP on the cell surface indicates a unique biological function and character for this proteinase.

Matrix metalloproteinases (MMPs), also called matrixins, are a family of Zn2+-dependent metalloendopeptidases that is involved in the degradation of extracellular matrix (ECM) in physiological and pathological conditions (1, 2). To date, 20 mammalian MMPs have been identified by cDNA cloning (3–6), and they can be subgrouped into 15 soluble-type and 5 membrane-type MMPs (MT-MMPs). With the exception of stromelysin 3 (MMP-11), soluble-type MMPs are secreted as zymogens and can act at distant sites following activation (3). Thus, they can participate in the degradation of a broad area of ECM, for example during tissue resorption. On the other hand, MT-MMPs have a hydrophobic amino acid stretch that is embedded in the plasma membrane at the C terminus and expressed on the cell surface (6–10). Thus, MT-MMPs are thought to be responsible for the degradation of ECM at the cell periphery, and this may modulate various cellular functions in tissue such as proliferation, apoptosis, differentiation, and migration, etc. (11).

Cell surface proteins attach to the plasma membrane in at least two different ways, via transmembrane domains that are comprised of a stretch of hydrophobic amino acids or through a glycosylphosphatidylinositol (GPI) anchor (12). The former includes cytokine receptors, G-proteins, and integrins. Because they have signaling domains on both sides (11), these types of proteins act as interfaces connecting the outside and inside of the cells. In the case of GPI-anchored proteins, attachment to the plasma membrane is via a GPI molecule that contains 2–3 fatty acids. The 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 endoplasmic reticulum lumen, and the ectodomain is transferred to the GPI moiety (12). Thus, the mature GPI-anchored protein has no transmembrane or intracellular domains. Examples of such proteins include the urokinase receptor (uPAR) (13) and neural cell adhesion molecule (12, 14).

The MT4-MMP (MMP-17) gene was originally cloned from a cDNA library derived from breast carcinoma cells (15). Among the MT-MMPs, MT4-MMP is unique in the following criteria. First, it is distantly related in the amino acid sequence to the other members (less than 40% sequence identity in the catalytic domain); the sequence identity among other members is more than 65%. Second, a putative transmembrane domain locates at the end of the C terminus. Thus, MT4-MMP lacks a cytoplasmic tail, whereas all the other MT-MMPs have a short cytoplasmic tail (20–22 amino acids) (6–9). Its C-terminal sequence suggests that MT4-MMP is a GPI-anchored enzyme. However, its characterization has been hampered to date because the reported cDNA (15) does not direct expression of the enzyme in transfected cells (16). A comparison of the reported cDNA sequences for MT-MMPs revealed that the cDNA for MT4-MMP does not have the region encoding a signal peptide common to all MMPs. On the other hand, we recently isolated cDNAs derived from new transcripts for human and mouse MT4-MMP that have a signal peptide coding sequence (16, 17). Both human and mouse cDNAs for MT4-MMP can direct expression of the gene products when the cDNAs are expressed in the cells. Thus, newly obtained MT4-MMP cDNAs enabled us to examine whether MT4-MMP is a GPI-anchored protein. In this report, we demonstrated that both the human and mouse MT4-MMP proteins are expressed on the cell surface of transfected cells as a GPI-anchored protein.

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1 The abbreviations used are: MMP, matrix metalloproteinase; MT-MMP, membrane-type matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; ECM, extracellular matrix; GPI, glycosylphosphatidylinositol; PI-PLC, phosphatidylinositol-specific phospholipase C; uPAR, urokinase-type plasminogen activator receptor; E-64, trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; anti-MT4PEX, polyclonal anti-mouse MT4-MMP hemopexin-like domain antibody; CHO-K1, Chinese hamster ovary cells; hMT-MMP, human MT-MMP; mMT-MMP, mouse MT-MMP; hMT-F1, human MT-F1; mMT-F1, mouse MT-F1.
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EXPERIMENTAL PROCEDURES

Materials—Dulbecco's modified Eagle's medium was from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). Ham's F-12 medium was from Life Technologies, Inc. FuGENE6™ and Pefabloc® Sc (4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride) were from Roche Molecular Biochemicals. Phosphatidylinositol-specific phospholipase C (PI-PLC) was from Oxford Glyco Sciences (Abingdon, UK). Monoclonal anti-FLAG epitope IgGs M1 and M2, and trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E6-4) were from Sigma. Cy3-conjugated goat anti-mouse IgG was from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Alexa488-conjugated goat anti-rabbit IgG was from Molecular Probes, Inc. (Eugene, OR). Polyclonal anti-human urokinase receptor (anti-uPAR) IgG was from American Diagnostica Inc. (Greenwich, CT). Peptidyl hydroxamate MMP inhibitor, BB94 was kindly provided by Dr. Peter D. Brown at British Biotechnological Pharmaceuticals Ltd. (Oxford, UK).

Construction of FLAG-tagged Human and Mouse MT4-MMP, hMT4/MT1TMC1, and Catalytically Inactive mMT4-MMP (E248A)—Full-length mouse (GenBank® accession number AB021224) and human MT4-MMP cDNAs (GenBank® accession number AB021225) were subcloned into pSGS expression vector (Stratagene, La Jolla, CA). To detect MT4-MMP protein by anti-FLAG M1 and M2 antibody, FLAG epitope (Asp-Tyr-Lys-Asp-Asp-Asp-Lys)-tagged MT4-MMP expression constructs were generated. The FLAG epitope was inserted either immediately downstream of the furin cleavage motif (following Arg124 for mouse and Arg128 for human) (MT4-F1) or one amino acid downstream of the motif (following Gin125 for mouse and Gin129 for human) (MT4-F2) (see Fig. 1). Anti-FLAG M1 antibody recognizes the epitope (MT4-F1) or is located at the N-terminal end of the protein in the presence of 1 mM Ca2+. Thus, M1 antibody is able to recognize the epitope only when MT4-F1 is processed at the furin cleavage motif. On the other hand, anti-FLAG M2 antibody recognizes the FLAG epitope at the N terminus or anywhere else in the molecule, thus, it recognizes both MT4-F1 and MT4-F2 even without cleavage by a furin-like enzyme.

To test sequence specificity of MT4-MMP, the C-terminal portion after the hemopexin-like domain of hMT4-MMP (Glu325-Leu356) was swapped with the sequence of hMT1-MMP (Pro996-Val1022) (hMT4/MT1TMC1). Also, a catalytically inactive mutant of mMT4-MMP was generated by site-directed mutagenesis (Glu248 to Ala).

All the mutant constructs were generated by polymerase chain reaction using the overlap extension method of Higuchi et al. (19). The antibody used to detect MT4-MMP is a polyclonal antibody that contains 2-mercaptoethanol. The samples were subjected to SDS-polyacrylamide gel electrophoresis, and3H-labeled materials were detected on x-ray film after reaction of the gel with EN3HANCE (NEN Life Science Products). The FLAG-tagged MT4-MMP, chimera protein, and MT1-MMP in the samples were visualized by Western blotting using anti-FLAG M2 antibody.

Indirect Immunofluorescence Staining—CHO-K1 cells transfected with expression plasmids for the FLAG-tagged MT4-MMPs were reseeded on coverslips and cultured for 24 h. Cells were then fixed with 3% paraformaldehyde in phosphate-buffered saline. After blocking with 5% goat serum and 3% bovine serum albumin in Tris-buffered saline for 1 h at room temperature, cells were reacted with anti-FLAG M1/M2 antibodies (10 or 3 mg/ml), respectively, polyclonal rabbit anti-MT4PEX antibodies (1:500) to detect FLAG-tagged MT-MMPs or anti-MT4PEX serum (1:200) according to the manufacturer's instructions. After 48 h, the cells were harvested and analyzed.

Western Blotting—To detect the protein in the culture supernatant, the protein in the medium was concentrated by treatment with 10% trichloroacetic acid. Cell lysates or trichloroacetic acid-concentrated protein samples were separated by SDS-polyacrylamide gel electrophoresis, and the proteins in the gel were transferred to a nitrocellulose membrane (Hybond-ECL, Amersham Pharmacia Biotech). After blocking with 10% fat-free dry milk in Tris-buffered saline (20 mM Tris-HCl, pH 7.5, 150 mM NaCl), the membrane was probed with anti-FLAG M2 monoclonal antibody (5 mg/ml) to detect FLAG-tagged MT-MMPs or anti-MT4PEX serum (1:200) to detect MT4-MMP.

α[3H]Ethalonamime Incorporation, Immunoprecipitation, and Autoradiography—CHO-K1 cells in 6-well plates transfected with expression plasmids for FLAG-tagged MT4-MMPs were reseeded on coverslips and cultured for 24 h. Cells were then fixed with 3% paraformaldehyde in phosphate-buffered saline. After blocking with 5% goat serum and 3% bovine serum albumin in Tris-buffered saline for 1 h at room temperature, cells were reacted with anti-FLAG M1/M2 antibodies (10 or 3 mg/ml), respectively, polyclonal rabbit anti-MT4PEX serum (1:200), and polyclonal rabbit anti-human uPAR IgG (5 mg/ml) at room temperature for 2 h. CaCl2 (1 mM) was included throughout the procedure of washing and, in the case of the M1 antibody, incubation. Cy3-conjugated goat anti-mouse IgG and Alexa488-conjugated goat anti-rabbit IgG were used to visualize the antigen signal. The signals were analyzed by confocal microscopy (Bio-Rad).

RESULTS

Structural Characteristics of the C-terminal Region of MT4-MMP—The amino acid alignment of mouse and human MT4-MMP with other MT-MMPs shows distinct differences in hydrophobicity at the C terminus (Fig. 1A), and this hydrophobicity pattern at the C-terminal end is rather similar to that of GPI-anchored proteins such as human Thy-1, human NCAM120, and human uPAR (Fig. 1A). Therefore, we have addressed the possibility that MT4-MMP is 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 C-terminal amino acid and the glycan structure (see Fig. 1B) (12). Thus, GPI-anchored proteins can be labeled metabolically with [3H]ethanolamine (12).

Incorporation of [3H]Ethanolamine into MT4-MMP—To detect MT4-MMP protein effectively, we have constructed FLAG epitope-tagged mouse and human MT4-MMP expression plasmids. A FLAG epitope of Asp-Tyr-Lys-Asp-Asp-Asp-Lys was inserted downstream of the furin cleavage site (following Arg128 for hMT4-MMP and Arg124 for mMT4-MMP) (F1) or one
amino acid after the cleavage site (following Gln 129 for hMT4-MMP and Gln125 for mMT4-MMP) (F2) (Fig. 1A). The mutant constructs include a MT4-MMP/MT1-MMP chimera (MT4/MT1TMCP) in which the downstream of the hemopexin-like domain of MT4-MMP (Gly527–Leu606) is swapped with the one in MT1-MMP (Pro 509–Leu582). Transfection of the expression plasmids for mMT4-F1, hMT4-F1, hMT4-F1/MT1TMCP, and MT1-F1 into COS-1 cells resulted in the expression of these enzymes in the cells. Western blotting analyses using anti-FLAG M2 antibody revealed major doublet bands at 68 and 67 kDa for mMT4-F1, a doublet of 71 and 67 kDa for hMT4-F1, a 67-kDa band for MT4-F1/MT1TMCP, and a 63-kDa band for MT1-F1 (see also Fig. 2A, WB). As shown in Fig. 2A, [3H]ethanolamine was specifically incorporated into both mMT4-F1 and hMT4-F1 but not into hMT4-F1/MT1TMCP or hMT1-F1. The labeled band corresponds to one of the two bands (67-kDa species) for both mouse and human MT4-F1 detected by anti-FLAG M2 antibody (Fig. 2A, WB, lanes 1 and 2). The incorporation of [3H]ethanolamine into hMT4-MMP is C-terminal sequence-dependent, because the chimeric hMT4-F1/MT1TMCP was not labeled at all (lane 3). Thus, MT4-MMP was labeled with the [3H]ethanolamine in association with a sequence-specific modification in the C-terminal region most likely by the GPI moiety. Therefore, the upper band detected on the Western blot in MT4-MMPs (68 kDa in mMT4-F1 and 71 kDa in hMT4-F1) that is not labeled with [3H]ethanolamine is likely to correspond to the precursors for GPI anchoring, and the 67-kDa species is the GPI-anchored protein.

**PI-PLC Treatment Releases MT4-MMP from the Cell Surface—**Another indication of a GPI-anchored protein is the sensitivity for bacterial PI-PLC that cleaves the GPI moiety and releases the protein from the cell surface (see Fig. 1B) (12). Thus, the transfected COS-1 cells were treated with PI-PLC. As shown in Fig. 3A, some levels of mMT4-F1 and hMT4-F1 were spontaneously released into the supernatant during incubation of the cells (Sup, lanes 1 and 3), PI-PLC

**FIG. 1.** Schematic representation of the mutants, comparison of the C-terminal end region of MT4-MMP and other MT-MMPs, and GPI anchoring. A, a FLAG epitope DYKDDDDK was inserted immediately after the furin cleavage motif (F1) or one amino acid after the motif (F2) in human and mouse MT4-MMP and human MT1-MMP. The case of hMT4-MMP is shown at the top. The chimeric hMT4-MMP (hMT4/MT1TMCP) in which the sequence after the hemopexin-like domain of hMT4-MMP was swapped with the one in hMT1-MMP is indicated at the bottom. Amino acid sequences of the C-terminal end that include the transmembrane region of MT1–4-MMPs, human Thy1 (hThy1) (GenBank™ accession number 135830), GPI anchor-type human neural cell adhesion molecule (hNCAM120) (GenBank™ accession number 127861), and human urokinase receptor (huPAR) (GenBank™ accession number 465003) are also listed and aligned. Expected transmembrane region amino acids, according to hydrophobicity plots, are indicated in bold and are underlined. WT, wild type; PEX, hemopexin-like domain. B, schematic representation of a GPI anchoring is shown. Characterization of the GPI anchor includes the incorporation of ethanolamine (EtN) (*1) and the sensitivity of phosphatidylinositol-specific phospholipase C (PI-PLC) (*2). P, phosphate; GlcNH2, glucosamine.
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Shedding of MT4-MMP from the Cell Surface—During cultivation of the transfected COS-1, MT4-MMP appeared to be shed into the culture supernatant (Fig. 4A, lane 1). To test the possibility that proteinases release MT4-MMP from the cell surface, various proteinase inhibitors were tested for the inhibition of the shedding. The shedding of mMT4-MMP was not affected by mutation of the active site (Glu248 to Ala) (Fig. 3B). In contrast, the type I transmembrane proteinase MT1-MMP was insensitive to PI-PLC treatment (Fig. 3A, lanes 5 and 6, and Fig. 3B). Taken together, we conclude that both mouse and human MT4-MMPs are GPI-anchored proteinases, and the GPI anchor signal is located in the C-terminal portion of the molecule, similar to other GPI-anchored proteins.

Cell Surface Localization of the Active form of MT4-MMP—We next examined the localization of MT4-MMP in a Chinese hamster ovarian cell line (CHO-K1) that is more suitable than COS-1 cells for analyzing subcellular localization. CHO-K1 cells transfected with the hMT4-F1 expression plasmid were co-immunostained with anti-FLAG M2 antibody and polyclonal rabbit anti-MT4PEX antibody without permeabilization. As shown in Fig. 5, MT4-F1 expressed on the cell surface was stained with anti-FLAG M2 antibody (F2/M2) and anti-MT4PEX antibody (F2/PEX) but not with M1 (F2/M1). This suggests that the signal detected in hMT4-F1-expressing cells with M1 antibody is the specific signal-detecting the furin cleavage site processed form. Unfortunately, the M1 antibody was ineffective for Western blotting to confirm the size of the processed species (data not shown).

Co-localization of MT4-MMP with uPAR on the Cell Surface—GPI-anchored proteins are thought to exist in microdomains formed by lipid rafts in the cell membrane that are rich in cholesterol and glycosphingolipids (20–22). Therefore, we tested whether MT4-MMP can exist in the same microdomains with another GPI-anchored protein, uPAR (Fig. 6). CHO-K1 cells were co-transfected with hMT4-F1 or hMT1-F1 and uPAR expression vector and stained with anti-FLAG M1 (M1) and anti-human uPAR (huPAR) antibody (uPAR). As shown in Fig. 6, the hMT4-F1 (hMT4) and uPAR signals both showed a mixture of small and large dotlike staining patterns. When these images were merged, it became evident that MT4-F1 and uPAR signals co-localize in the bigger dots, showing a yellow color (Merge). Small dotlike signals on the cell body did not co-localize, as red (hMT4-F1) and green (uPAR) signals can be seen independently. On the other hand, the signal of MT1-MMP does not co-localize well with the major signal of huPAR, shown by numerous independent localizations of green and red signals (Fig. 6). The data suggest that these two nonrelated GPI-anchored proteins are likely to be present in the same microdomain on the cell surface.

DISCUSSION

In this paper, we have presented evidence that MT4-MMP is a GPI-anchored proteinase. The other four MT-MMPs are type...
I transmembrane proteins with a short cytoplasmic tail comprised of about 20–22 amino acids (Fig. 1). Thus, MT4-MMP is unique in the MT-MMP subfamily not only in amino acid sequence homology but also in the method used for anchoring to the plasma membrane. Recent studies indicate that pericellular ECM degradation is regulated not only by the bulk levels of proteinases on the cell surface but also by localization of proteinases to the specific site on the cell surface to increase local concentration of the enzyme. In the case of MT1-MMP, swapping the transmembrane/cytoplasmic domain with that in the interleukin-2 receptor \(\alpha\) chain abolished the localization of the enzyme to the invadopodium structure of melanoma cells (23), suggesting that the transmembrane/cytoplasmic domain contains the signal to locate MT1-MMP to the specific site on the cell surface. On the other hand, MT4-MMP is tethered to the plasma membrane through the lipid and was expected to scatter uniformly on the cell surface because it lacks a cytosolic apparatus that may regulate localization. However, MT4-MMP-expressing CHO-K1 cells showed a dotlike staining pattern on their surface. Recent studies showed that GPI-anchored proteins are concentrated into the microdomains, or lipid rafts, on the living cell surface that are enriched for cholesterol and glycosphingolipids (21, 22). Because microdomain-enriched GPI-anchored proteins can form clusters comprised of at least 15 molecules (21), some of the immunostained dotlike clusters may correspond to such microdomains. The enrichment of proteins in microdomains was shown not to be ectodomain-dependent but rather to be GPI moiety-dependent (21). Supporting this notion, our data also showed that hMT4-MMP co-localized with a nonrelated GPI-anchored protein, uPAR, on the cell surface of CHO-K1 cells. This also indicates that the cellular localization of MT4-MMP may be regulated in its GPI moiety-dependent manner. The GPI-anchored uPAR has previously been shown to associate with \(\beta\) 2 integrin (CD18), and this interaction was shown to be important for leukocyte adhesion to the endothelial cells (24), local fibrin degradation (25) and cell migration (26). Because MT4-MMP co-localizes with uPAR in the same microdomain clusters, these different types of proteinase systems (uPA/uPAR and MT4-MMP) may co-operate in conjunction with cell adhesion molecules such as CD18.

All the MT-MMPs, including MT4-MMP and many ADAM family members, have a furin cleavage motif at the end of the propeptide (7–9, 15, 27). Thus, they are thought to be activated...
intracellularly by furin or related proteinases and expressed on the cell surface as an active form. This feature is particularly important and effective for membrane-bound proteinases because membrane proteinase activity is likely to be associated with cell behavior and function (11). The active enzyme appearing on the cell surface can be used immediately or removed from the site by shedding when it is not needed. The MT4-F1 on the cell surface was detected as an active form using M1 antibody (Fig. 5). Because we could not quantitate the pro and active forms of MT4-MMP on the cell surface, it is still possible that some fraction of pro MT4-MMP is transported to the cell surface without processing. In relation to this, it is of interest to know whether the 67-kDa GPI-anchored MT4-F1 retained the propeptide. However, the poor reactivity of M1 antibody in Western blotting prevented us from answering this question.

Several membrane proteins are known to be cleared from the cell surface by shedding. Shedding of the surface proteins, such as tumor necrosis factor receptor (28–30), Fas ligand (31), receptor (28–30), Fas ligand (31), a receptor (31), and CD44 (32), is mediated by metalloproteinases as it is inhibited by broad spectrum hydroxamate metalloproteinase inhibitors such as BB94. Similarly, the shedding of MT4-MMP is also at least in part due to metalloproteinases that are insensitive to TIMPs. The shedding of MT4-MMP is not specific to COS1 cells because transfected CHO-K1 cells also shed the enzyme (data not shown). The shedding of MT4-MMP is not specific to pro MT4-MMP is transported to the cell surface as an active form. This feature is particularly important and effective for membrane-bound proteinases because membrane proteinase activity is likely to be associated with cell behavior and function (11). The active enzyme appearing on the cell surface can be used immediately or removed from the site by shedding when it is not needed. The MT4-F1 on the cell surface was detected as an active form using M1 antibody (Fig. 5). Because we could not quantitate the pro and active forms of MT4-MMP on the cell surface, it is still possible that some fraction of pro MT4-MMP is transported to the cell surface without processing. In relation to this, it is of interest to know whether the 67-kDa GPI-anchored MT4-F1 retained the propeptide. However, the poor reactivity of M1 antibody in Western blotting prevented us from answering this question.

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One GPI-anchored proteinase, membrane dipeptidase, was shown to be released from the cell surface by phospholipase C (34), and the shedding was induced by insulin stimulation in 3T3-L1 adipocytes. This shedding is not likely to be ectodomain-dependent but rather GPI moiety-dependent, because cleavage occurred in the GPI moiety (34). Therefore, it is interesting to speculate that other GPI-anchored proteinases including MT4-MMP may also be released from the cell surface in response to certain stimuli that activate the responsive phospholipase for shedding in vivo.

In conclusion, we have shown that MT4-MMP is a GPI-anchored protein. This anchoring method is unique and thus indicates a unique biological function and character of MT4-MMP as a GPI-anchored proteinase.

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