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MT4-(MMP17) and MT6-MMP (MMP25), A unique set of membrane-anchored matrix metalloproteinases: properties and expression in cancer

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Abstract

The process of cancer progression involves the action of multiple proteolytic systems, among which the family of matrix metalloproteinases (MMPs) play a pivotal role. The MMPs evolved to accomplish their proteolytic tasks in multiple cellular and tissue microenvironments including lipid rafts by incorporation and deletions of specific structural domains. The membrane type-MMPs (MT-MMPs) incorporated membrane anchoring domains that display these proteases at the cell surface, and thus they are optimal pericellular proteolytic machines. Two members of the MT-MMP subfamily, MMP-17 (MT4-MMP) and MMP-25 (MT6-MMP), are anchored to the plasma membrane via a glycosyl-phosphatidyl inositol (GPI) anchor, which confers these enzymes a unique set of regulatory and functional mechanisms that separates them from the rest of the MMP

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family. Discovered almost a decade ago, the body of work on GPI-MT-MMPs today is still surprisingly limited when compared to other MT-MMPs. However, new evidence shows that the GPI-MT-MMPs are highly expressed in human cancer, where they are associated with progression. Accumulating biochemical and functional evidence also highlights their distinct properties. In this review, we summarize the structural, biochemical, and biological properties of GPI-MT-MMPs and present an overview of their expression and role in cancer. We further discuss the potential implications of GPI-anchoring for enzyme function. Finally, we comment on the new scientific challenges that lie ahead to better understand the function and role in cancer of these intriguing but yet unique MMPs.

Keywords

Matrix metalloproteinases; Glycosyl-phosphatidyl inositol; Membrane anchor; Proteolysis; Cancer; Lipid raft; Protease inhibitors; Plasma membrane; Extracellular matrix

1 Introduction

Modifications and alterations of the immediate microenvironment are essential for cell survival in both physiological and pathological processes. Cancer cells, like normal cells, strongly depend on their ability to influence the structure and organization of the pericellular milieu so that they can display their malignant phenotype during cancer progression and fulfill their intended biological behavior. An important characteristic of malignant cells is their ability to detach from the primary tumor and to acquire migratory and invasive properties. To accomplish these goals, cancer cells hijack various proteolytic systems, which serve to modify the pericellular milieu. The MMP family of zinc-dependent endopeptidases is one of the major protease family involved in cancer progression; and their role in regulating tumor growth, metastasis and angiogenesis has been well established [1–6]. MMPs' role in cancer progression, however, is complex, involving both stimulatory and suppressive effects by MMPs produced by both the cancer cells and the tumor stroma [5, 7–9]. The complexity of MMPs' biological function in cancer is compounded by their multidomain structure, diverse substrate repertoire, differential tissue expression and regulation, interacting proteins and co-factors and their inhibitor profile, just to mention a few. Furthermore, analyses of the MMP domain structure reveal that these proteases also evolved to target substrates at multiple cellular and extracellular sites. This was achieved by the deletion or addition of accessory enzyme domains, whose main function is to direct the active protease to diverse cellular and tissue microenvironments in pursuit of their favorite substrate(s). Thus, the MMP family evolved to include secreted and membrane-anchored proteases, which as a whole can process multiple substrates at many locations.

The membrane-anchored MMPs (referred to as MT-MMPs), containing an anchoring motif at the C-terminal end, are perfectly suited for pericellular proteolysis [10]. Membrane anchoring facilitates these MMPs to reach key membrane and peripheral proteins as well as closely associated extracellular matrix (ECM) components thereby playing a pivotal role during alterations of the pericellular milieu in both physiological and pathological conditions [11–13]. The anchoring domains also facilitate diffusion and translocation of the active protease on the plasma membrane to specific membrane microdomains and its recruitment to the migrating front of motile cells [14]. Finally, membrane insertion confers MT-MMPs with a unique set of regulatory mechanisms that serve to control the pool of active protease at the cell surface including endocytosis, recycling, autocatalytic processing, and ectodomain shedding, [15]. Further specialization and refinement of the MT-MMPs was achieved by the presence of two distinct anchoring systems: a transmembrane domain and a glycosyl-phosphatidyl inositol (GPI)-anchor. Thus, the MT-MMPs are divided in

transmembrane MT-MMP (referred here to as TM-MT-MMPs) and GPI-anchored MT-MMPs (referred to as GPI-MT-MMPs). Since the discovery of the first TM-MT-MMP, MT1-MMP (MMP-14), more than 13 years ago [16, 17], a wealth of evidence has clearly shown that MT1-MMP as well as other TM-MT-MMPs are key players in cancer progression by promoting cell growth, migration, invasion, metastasis, and angiogenesis [10, 12, 14, 18]. A wealth of information also exists on the regulation, inhibition, and activity of TM-MT-MMPs. In contrast, the function and role of GPI-MT-MMPs in cancer remains largely elusive in spite of their identification in cancer tissues and their potential to impact cancer progression. Although there are only a limited number of studies, recent evidence suggest a unique role for GPI-MT-MMPs in cancer. Moreover, their unique structural organization has revealed distinct properties demanding new concepts in MMP regulation, substrate specificity, and inhibition. The purpose of this review is to provide a first overview of the properties, function, and regulation of the two GPI-MT-MMPs: MT4- and MT6-MMP. Although GPI-MT-MMPs have been implicated in several pathologies, our focus here is on their expression and role in cancer. We hope that this review will increase the awareness of these interesting and unique MMPs and open new avenues of research with potential clinical implications.

2 General properties of GPI-MT-MMPs

The MT-MMPs constitute a distinct set of proteases among the members of the MMP family in that they are anchored to the cell surface with their catalytic site exposed to the extracellular space. As such, the MT-MMPs are well positioned to attack many substrates located in the plasma membrane and in the immediate pericellular space [10]. The MT-MMPs subfamily is comprised of six members, four of which are tethered to the plasma membrane by a hydrophobic transmembrane domain of ~20 amino acids followed by a cytoplasmic tail, and two proteases that are tethered to the membrane via a GPI anchor. The transmembrane MT-MMPs are MT1-MMP (MMP-14) [16, 17], MT2-MMP (MMP-15) [19], MT3-MMP (MMP-16) [20] and MT5-MMP (MMP-24) [21, 22]. The GPI-MT-MMPs include MT4-MMP (MMP-17) [23, 24] and MT6-MMP (MMP-25) [25, 26]. With the exception of the anchoring region, the MT-MMPs share the same basic domain organization of most MMPs, including a signal sequence, a propeptide domain, a zinc-containing catalytic domain, a hinge region (linker 1), and a hemopexin-like domain. Both the transmembrane and the GPI-MT-MMPs also contain a RXR/KR motif at the end of the propeptide domain that serves as a recognition site for pro-convertases such as furin, which cleave the propeptide and activate the MT-MMP zymogen (Fig. 1). There are excellent reviews on the structure, function and regulation of transmembrane MT-MMPs, albeit mostly focused on MT1-MMP [10, 14, 27]. Here we will focus on the unique features of the GPI-MT-MMPs.

2.1 Identification and unique structural characteristics

MT4-MMP was first cloned from a human breast carcinoma cDNA library [24], while MT6-MMP was identified from peripheral blood leukocytes [25]. Due to the high expression of MT6-MMP in leukocytes, this MMP was also termed leukolysin [25]. Primary sequence alignment of the catalytic domain of MT4- and MT6-MMP shows they are 56% identical and 77% homologous. When compared to the MT1-MMP catalytic domain, MT4-MMP possesses 37% identity (50% similarity) while MT6-MMP possesses 42% identity (58% similarity). This suggests that GPI-MT-MMPs are structurally and functionally distant to TM-MT-MMPs. The hemopexin-like domain of the GPI-MT-MMPs is followed by a hydrophilic region (~35–45 amino acids) also called stem (linker 2) region and a hydrophobic tail of ~20–23 amino acids. The C-terminal hydrophobic tail of the GPI-MT-MMPs is removed a transamidase in the endoplasmic reticulum (ER) during incorporation of the GPI anchor, a process that is a unique characteristic of GPI-anchored proteins [28].

Biochemical studies using incorporation of [^{3}H] ethanolamine and release by phosphatidylinositol specific phospholipase C (PI-PLC) revealed that both MT4- and MT6-MMP are indeed GPI-anchored proteins [29–31].

The stem region of MT4- and MT6-MMP (residues A^{528}-A^{573} in MT4-MMP and residues A^{509}-A^{539} in MT6-MMP) exhibits unique features when compared to the stem of transmembrane MT-MMPs. The stem of MT4- and MT6-MMPs contains two and three cysteine residues, respectively, of unknown function (Fig. 1). Since cysteine residues are known sites for a variety of post-translational modifications including disulfide bond formation, palmitoylation, or prenylation, these cysteine residues may play an important role in regulation of protease function. For instance, cysteine residues may play a role in formation of homophilic interactions. We have recently shown that MT6-MMP [32] is found in reduction-sensitive high-molecular weight forms, which may represent dimeric and/or oligomeric forms of the protease. In the case of MT6-MMP, the protease is found at the cell surface as a major ~120-kDa species and minor ~180-kDa form that can be released from the membrane by PI-PLC [32]. The ~120 kDa form is also detected with natural MT6-MMP in extracts of human neutrophils [32]. Under reducing conditions, MT6-MMP is detected as a 57-kDa form, strongly suggesting that the ~120-kDa species represents a dimeric form of the enzyme, possibly held by a disulfide bond formed by cysteine residues at the stem region. Preliminary site-directed mutagenesis studies from our laboratory suggest that Cys^{532} in the stem region plays a key role in MT6-MMP homodimerization (A. Sohail, H. Zhao, Q. Sun and R. Fridman, manuscript in preparation). Furthermore, this Cys mutant exhibits enhanced enzyme degradation suggesting that homodimerization is important for protease stability. In TM-MT-MMPs, homophilic interactions have been reported with MT1-MMP, a membrane-tethered collagenase [11] and a major activator of pro-MMP-2 [16, 17]. MT1-MMP homodimerization has been suggested to play a role in collagenolysis and pro-MMP-2 activation [14]. However, the nature of the interactions and the domains involved in MT1-MMP dimeric/oligomeric forms remain unclear as both non-covalent [33, 34] and covalent [35, 36] interactions have been reported. Furthermore, as opposed to MT6-MMP, the high molecular weight forms of MT1-MMP are not readily detected by SDS-PAGE, suggesting a significant structural difference between transmembrane and GPI-anchored MMPs in regards to this unique supramolecular organization. The importance of MT6-MMP homophilic interactions for enzyme function remains to be elucidated, but it is tempting to speculate that these interactions may regulate diverse aspect of MT6-MMP including subcellular distribution, stability, endocytosis, catalysis, TIMP inhibition, and substrate specificity, just to mention a few. Recent studies have shown that oligomerization plays a key role in determining apical sorting and stabilization into rafts of GPI-anchored proteins [37, 38]. Consistent with these findings, MT6-MMP oligomers are also targeted to the lipid rafts [32]. However, the basal vs. apical sorting of MT6-MMP and its relevance for enzyme function remain to be determined.

2.2 Biosynthesis, trafficking, cellular distribution, and endocytosis

The detailed biosynthetic pathway of GPI-MT-MMPs has not yet been investigated. However, MT4- and MT6-MMP are likely to follow the biosynthetic pathway of typical GPI-anchored proteins. For a detailed review on the synthesis of GPI-anchored proteins see [28]. It is well established that GPI-anchored proteins are initially synthesized as transmembrane protein precursors. The hydrophobic tail of the precursor is cleaved by a transamidase during translation, and the protein is then linked to a pre-existing GPI-anchor on the ER membrane [28, 39] (Fig. 2). Currently, the transamidase cleavage site in MT4- and MT6-MMP is unknown. However, the C-terminal region of MT4- and MT6-MMP contain a putative transamidase cleavage site, referred to as ω, at Ala^{573} in MT4-MMP and Ala^{539} in MT6-MMP, as predicted by big-PI Predictor software [40–42]. In a typical GPI

anchor, phosphatidylinositol is glycosidically linked to an inositol ring of a non-acetylated glucosamine moiety that has three mannose residues attached to it. The terminal mannose is linked to phosphoethanolamine by a phosphodiester bond. The GPI-anchor is attached to the carboxy-terminal of the protein by an amide linkage to the amino group of phosphoethanolamine [43]. Variations in the GPI structure include the attachment of galactosyl side chains and additional phosphoethanolamine or mannosyl moieties to the first mannose, which depend both on the organism and cell type. Immediately after transfer to the protein, the GPI moiety goes through a deacylation process [44] followed by the replacement of unsaturated fatty chain in phosphatidylinositol with two saturated fatty acids chains. The fatty acid remodeling, an essential step for integration of GPI-anchored proteins into lipid rafts, most likely occurs in the Trans Golgi network [45]. In the Trans Golgi, GPI proteins also undergo post-translational modifications such as glycosylation. Our preliminary studies using an *O*-glycosylation inhibitor suggest that MT6-MMP is *O*-glycosylated (Q. Sun and R. Fridman, unpublished data).

The GPI-anchor confers the protein that is attached to it with important biological functions including localization to lipid raft microdomains, sensitivity to phospholipases, apical sorting, and a role in signal transduction. We have recently shown that successive detergent fractionation of cells expressing MT6-MMP results in the distribution of MT6-MMP into the Triton X-100-insoluble fractions, which are considered to be lipid rafts [32]. This distribution was further confirmed here by sucrose density gradient centrifugation as depicted in Fig. 3(a). These data show that MT6-MMP is enriched in light buoyant density fractions that also contain the raft-associated protein Caveolin-1. These results are consistent with a previous proteomic study that identified MT6-MMP as a major component of the lipid rafts in human neutrophils [46]. Lipid rafts have received considerable attention because they are thought to be involved in many biological functions, in particular, signal transduction [47], endocytosis [48], and apical sorting of GPI-anchored proteins [49]. The presence of MT6-MMP, and likely MT4-MMP, in lipid rafts may confer GPI-MT-MMPs with the unique ability to interact with and degrade specific components of the raft microenvironment. This fundamental cellular distribution needs to be considered in future degradomic studies aimed at defining the substrate profile of GPI-MT-MMPs by proteomic approaches [50].

GPI-anchored proteins also undergo raft-mediated endocytosis, which serves to recycle these proteins to the plasma membrane or to target the proteins to lysosomes for degradation [48, 51]. Although the pathway involved in the endocytosis of GPI-MT-MMPs needs to be elucidated, initial results from our laboratory indicate that MT6-MMP is endocytosed and recycled back to cell surface in MT6-MMP transfected colon cancer cells (J.-A. Cho and R. Fridman, unpublished results). These studies suggest that GPI-MT-MMP activity at the cell surface is also regulated by endocytosis and recycling, as reported for MT1-MMP [14, 52].

2.3 Inhibition of GPI-MT-MMPs

The members of the MMP family are specifically inhibited by tissue inhibitors of metalloproteinases (TIMPs), a family of four proteins (TIMP-1, −2, −3, and −4) that bind to the catalytic domain of the active protease terminating catalysis. For comprehensive reviews on the structure and function of TIMPs, the readers are directed to: [53–57]. Like all MMPs, the enzymatic activity of MT-MMPs is also inhibited by TIMPs. However, structural and functional studies revealed that MT-MMPs exhibit unique interactions with TIMPs. The TM-MT-MMPs are highly sensitive to inhibition by TIMP-2, TIMP-3, and TIMP-4, which behave as high-affinity, slow-binding, reversible inhibitors of these proteases. Interestingly, TIMP-1 is a very weak inhibitor of TM-MT-MMPs, and thus under physiological conditions TM-MT-MMPs can be regarded as resistant to TIMP-1. The presence of a threonine residue

at position 98 has been found to be responsible for the lack of activity of TIMP-1 against TM-MT-MMPs [58]. When the catalytic domains of GPI-MT-MMPs were examined for TIMP selectivity, it was found that both MT4- [59–61] and MT6-MMP [32, 62, 63] were efficiently inhibited by TIMP-1, TIMP-2, and TIMP-3. Thus, GPI- and TM-MT-MMPs exhibit a different TIMP inhibition profile. We showed that TIMP-1 is a more effective inhibitor of MT6-MMP than TIMP-2 (K_i values of 0.2 and 2 nM, respectively). Moreover, MT6-MMP is less sensitive to TIMP-2 inhibition than MT1- and MT3-MMP, mainly because of a slower association rate (an order of magnitude) of TIMP-2 to MT6-MMP [32]. In this regard, when we compared the association of exogenously administered TIMP-2 to cells expressing either MT6-MMP or MT1-MMP, we found that TIMP-2 could be readily detected only in MT1-MMP-expressing cells, consistent with the slow association of TIMP-2 to MT6-MMP. Interestingly, we also found that TIMP-1 failed to associate with MT6-MMP expressing cells [32]. PI-PLC-released MT6-MMP also did not form a co-precipitable complex with TIMPs. The presence of a GPI-anchor, and consequently localization in lipid rafts, was not the reason for the lack of TIMP association with MT6-MMP because a GPI-anchored MT1-MMP chimera readily bound TIMP-2 [32]. These data revealed a significant difference between MT1-MMP and MT6-MMP in their ability to form stable complexes with TIMPs when displayed at the cell surface. Therefore, we examined whether MT6-MMP activity is inhibited by TIMPs when the enzyme is present on the plasma membrane. As shown in Fig. 3(b), MT6-MMP dependent cleavage of α1-proteinase inhibitor (α1-PI) is inhibited by both TIMP-1 and TIMP-2, demonstrating the sensitivity of membrane-anchored MT6-MMP to TIMPs (Fig. 3(b)). Thus, although MT6-MMP/TIMPs complexes on the cell surface are not readily detected [32], MT6-MMP activity is sensitive to TIMP inhibition. Future studies will need to address in more detail the interactions between GPI-MT-MMPs and TIMPs in the lipid raft microenvironment.

In addition to TIMPs, a previous study showed that GPI-MT-MMPs can be inhibited by clusterin [63]. Clusterin (also known as apolipoprotein J) is a multifunctional and ubiquitously expressed protein that is involved in many physiological and pathological processes including cancer progression. Two isoforms of clusterin exist: a secreted 75– 80-kDa heterodimer present in many physiological fluids, and a truncated cytosolic/nuclear form of ~49 kDa. Clusterin plays a role in regulation of apoptosis, DNA repair, and cellular responses to stress conditions, just to mention a few [64]. The association of clusterin with MT6-MMP was identified when a recombinant soluble form of MT6-MMP, comprised of the catalytic domain and the hemopexin-like domain, co-purified with clusterin as a stable complex from the conditioned media [63]. These studies showed that clusterin binds to the hemopexin-like domain of MT6-MMP, resulting in inhibition of enzymatic activity. Clusterin inhibition of MT6-MMP activity was significantly weaker than that observed with TIMP-1 and TIMP-2 and was specific for MT6-MMP, since neither MMP-2 nor MT1-MMP were inhibited by or formed a complex with clusterin. However, a soluble MT4-MMP also formed a complex with clusterin, suggesting that this interaction is unique for the GPI-MT-MMPs. The MT6-MMP/clusterin complex was also identified with a membrane-anchored recombinant MT6-MMP and with natural MT6-MMP in human neutrophils [63]. In contrast to these findings, the studies of Sun et al. [32] showed no interactions of MT6-MMP with clusterin in colon cancer cells expressing recombinant MT6-MMP in spite of endogenous clusterin expression in these cells. These findings indicated that the high-molecular weight form of MT6-MMP (~120 kDa) detected in these cells is not a complex of the protease with clusterin but is consistent with homophilic MT6-MMP interactions [32]. At present, more studies are required to define the relevance of the interactions of GPI-MT-MMPs with clusterin and their significance in the regulation of their enzymatic activity.

2.4 Shedding of GPI-MT-MMPs

It is well established that GPI-anchored proteins can be released from the cells in membrane vesicles, referred to as exosomes [65, 66], resulting in the release of the protein with an intact GPI. Interestingly, the released GPI protein can be transferred to other cell types in a paracrine manner where it elicits biological effects [65, 67]. Previous studies have shown that both MT4- [29, 68] and MT6-MMP [30 and A. Sohail and R. Fridman, unpublished results] are shed in different cell types. Although the precise shedding mechanism of GPI-MT-MMPs remains to be elucidated, data with MT4- and MT6-MMP indicate that the molecular mass of the soluble enzyme is very similar to their membrane-anchored counterpart [29, 30], suggesting the possibility that they maintain the GPI anchor. Thus, it is tempting to speculate that GPI-MT-MMPs may also be shed via exosomes, as reported with other GPI-anchored proteins [69]. However, cleavage of the GPI anchor by a phospholipase-mediated cleavage cannot be ruled out. Indeed, shedding of MT4- and MT6-MMP was insensitive to various metalloproteinase inhibitors [29, 30]. Thus, both proteolytic and non-proteolytic shedding mechanisms may regulate the function and localization of GPI-MT-MMPs.

2.5 GPI-MT-MMP substrates

2.5.1 ECM proteins—A few studies reported the catalytic activities of GPI-MT-MMPs (Table 1). The emerging view from these studies is complex, and so far there is no clear consensus on the precise substrate repertoire of GPI-MT-MMPs (Table 1). It should be noted that most of the studies designed to define the substrate specificity of GPI-MT-MMPs utilized isolated recombinant catalytic domains expressed in bacteria [30, 59, 60, 62, 70], which cannot the address the influence of membrane insertion and other key enzyme domains on catalytic activity. In other studies, substrate identification was established by co-expression of the enzyme with a potential substrate in mammalian cells [59, 70, 71]. Nevertheless, the data so far suggest that GPI-MT-MMPs are multifunctional enzymes capable of cleaving ECM and non-ECM proteins. However, there are significant differences between MT4- and MT6-MMP in their substrate preferences. Whereas MT4-MMP exhibits minimal or no activity against many ECM proteins, MT6-MMP hydrolyzes a variety of ECM components. Specifically, soluble MT4-MMP was found to be active only against gelatin, fibrin, and fibrinogen [59–61]. However, expression of MT4-MMP failed to confer cells with the ability to invade a fibrin gel *in vitro* [72]. MT6-MMP, on the other hand, exhibits activity against gelatin, collagen IV, fibronectin and fibrin [30, 62]. In addition, MT6-MMP was shown to hydrolyze chondroitin and dermatan sulfate proteoglycans but showed no activity against laminin and collagen type I, II, and III [30]. The limited ECM degrading activity of the GPI-MT-MMPs is in accordance with their reported inability to support the *in vitro* invasion of cells through either Matrigel coated filters [32, 68] or three-dimensional fibrin gels [72]. In addition, neither MT4-MMP nor MT6-MMP played a role in invasion of basement membranes *in vitro* [12]. Although GPI-MT-MMPs are expressed in cancer cells [26, 32, 68, 73], these data highlight a significant functional difference among MT-MMPs in cancer cell behavior.

2.5.2 Non-ECM proteins—GPI-MT-MMPs have been shown to cleave several non-ECM proteins. For instance, MT4-MMP was found to possess ADAM (a disintegrin and metalloprotease)-17-like activity in that it can act as a sheddase of tumor necrosis factor (TNF)-α when co-transfected with pro-TNF-α in Cos-7 cells [59]. However, macrophages isolated from wild type or MT4-MMP null mice exhibited a similar extent of TNF-α in the medium when stimulated with lipopolysaccharide [74]. Thus, at least in macrophages, MT4-MMP does not appear to be a major TNF-α sheddase. The MT4-MMP catalytic domain also cleaves α_2-macroglobulin [59], suggesting that this cleavage may play a role in the control of proteolytic activity because α_2-macroglobulin is a major protease inhibitor. The catalytic

domain of MT4-MMP also cleaves the low density lipoprotein receptor related protein (LPR) [75], which is known to play a key role in the clearance of multiple ligands at the cell surface. The cleavage of LRP is not exclusive to MT4-MMP and is also mediated by MT1-, MT2-, and MT3-MMP [75]. MT6-MMP can also cleave α1-PI, a potent inhibitor of serine proteases [70 and Fig. 3(b)]. α1-PI cleavage by MT6-MMP results in loss of its inhibitory activity, suggesting that expression of MT6-MMP in leukocytes may aid the inflammatory response by facilitating the action of serine proteases [70]. We also found that crude plasma membranes of colon cancer cells transfected with human MT6-MMP can process α1-PI in a pattern similar to that reported by Nie and Pei [70], as shown in Fig. 3(b).

MT6-MMP, like other MMPs, also releases the D1 domain of urokinase plasminogen activator receptor (uPAR) by cleaving at the linker region connecting domains 1 and 2 of uPAR [76]. Our laboratory has also found that MT6-MMP can cleave galectin-3 (A. Sohail, Q. Sun and R. Fridman, unpublished data), a 31-kDa carbohydrate-binding protein known to play a key role in inflammation, apoptosis, and cancer progression [77]. Cleavage of galectin-3 by MT6-MMP is similar to that reported with gelatinases and MT1-MMP, which generate a 21-kDa degradation product [78–80].

2.5.3 Zymogens as substrates of GPI-MT-MMPs: Role of MT4- and MT6-MMP in pro-MMP-2 and pro-MMP-9 activation—It is well established that many proteolytic enzymes are produced in a latent zymogenic form devoid of enzymatic activity. To achieve catalytic competence, the zymogen needs to be activated in a process that usually involves a specific cleavage of a portion of the enzyme, which allows the exposure of the catalytic domain [81]. MMPs, including MT-MMPs, are known to be involved in the activation of zymogens, in particular activation of pro-MMPs. In this regard, the discovery of MT1-MMP was the result of studies designed to identify the surface activator of pro-MMP-2 [17]. Later studies confirmed the importance of MT1-MMP in the physiological activation of pro-MMP-2 [11]. MMP-2 (also known as gelatinase A), together with its closely homologue MMP-9 (gelatinase B), has long been recognized as a key protease in cancer progression and tumor angiogenesis [4, 5, 82, 83]. Thus, MT1-MMP together with MMP-2 constitute a potent proteolytic axis capable of accomplishing the hydrolysis of multiple substrates at the tumor cell periphery including collagen I and collagen IV, which helps cancer cells to invade basement membranes and navigate through the interstitial collagen-rich matrix [84–86].

Biochemically, the activation of pro-MMP-2 by MT1-MMP is initiated by the recruitment of the tissue inhibitor of metalloproteinase (TIMP)-2, which binds to the active site of MT1-MMP via the amino terminal region of the inhibitor. The MT1-MMP/TIMP-2 complex then recruits pro-MMP-2, which binds to the C-terminal region of TIMP-2. This ternary complex facilitates the activation of pro-MMP-2 by a neighboring TIMP-2-free MT-MMP [17, 87]. Thus, TIMP-2 significantly enhances the rate of pro-MMP-2 activation at the cell surface by clustering MT1-MMP with its proteolytic target. TIMP-2 also enhances the activation of pro-MMP-2 by MT3-MMP [88] but has no effect on MT2-MMP-dependent pro-MMP-2 activation [89]. While MT5-MMP activates pro-MMP-2 [21], the role of TIMP-2 in this process remains unknown. The process of pro-MMP-2 activation involves two sequential cleavage sites at the Asn^{37}–Leu^{38} and Asn^{80}–Tyr^{81} peptide bonds, leading to the formation of an intermediate form followed by the fully active species [90]. MT1-MMP only cleaves at the Asn^{37}–Leu^{38} site in the prodomain, while the second cleavage is an autocatalytic event [90]. Cell surface association of pro-MMP-2 enhances the efficiency of the reaction by promoting the second autocatalytic step.

The ability of TIMP-2 to efficiently inhibit both MT4- [59, 61] and MT6-MMP [62] raised the possibility that GPI-MT-MMPs are also involved in pro-MMP-2 activation in a

mechanism analogous to that of MT1-MMP. This possibility was examined in a number of studies using various approaches and cellular systems [26, 31, 32, 59–61, 68, 71, 91]. In the case of MT4-MMP, most studies agree that this protease is not an activator of pro-MMP-2 [59, 60, 68]. Moreover, exposure of MDA-MB-231 breast carcinoma cells transfected to express MT4-MMP to concanavalin A, which is known to induce MT1-MMP-dependent activation of pro-MMP-2, had no effect on pro-MMP-2 activation [68]. Our studies also support the inability of MT4-MMP to activate pro-MMP-2 (H. Zhao and R. Fridman, unpublished data). In contrast, Wang et al. [61], using conditioned media from HT1080 cells as a source of pro-MMP-2 and an MT4-MMP catalytic domain, showed generation of the intermediate form of MMP-2. However, when using conditioned media as a source of pro-MMP-2, it is difficult to determine whether the effects observed are a direct consequence of the interaction between pro-MMP-2 and the proposed “activator”.

In the case of MT6-MMP, Velasco et al. [26] showed that co-transfection of pro-MMP-2 with MT6-MMP in Cos-7 cells resulted in activation of pro-MMP-2. Similar results were obtained by Nie and Pei [71] by co-transfecting the cDNAs of both proteins in MDCK cells. However, in both studies the efficiency of MT6-MMP was significantly lower than that of MT1-MMP or MT5-MMP When a catalytic domain of MT6-MMP was incubated with pro-MMP-2, the zymogen was fully activated [71]. Interestingly, the MT6-MMP catalytic domain was found capable of cleaving pro-MMP-2 at both the Asn^{37}–Leu^{38} and Asn^{80} – Tyr^{81} sites as opposed to MT1-MMP, suggesting a unique interaction between MT6-MMP and pro-MMP-2 [71]. Contrary to these findings, Kojima et al. [31] found that expression of a FLAG-tagged MT6-MMP in Cos-1 cells did not activate exogenously administered pro-MMP-2. Likewise, Miyamori et al. [91] showed that co-transfection of pro-MMP-2 cDNA with either MT4- or MT6-MMP cDNAs did not result in activation. However, when claudin-5, a tight junction protein, was co-expressed in the cells, a modest activation of pro-MMP-2 was observed with both MT4- and MT6-MMP [91]. Although the precise mechanism by which claudin-5 stimulates pro-MMP-2 activation is unclear, the effect is not specific since both GPI-MT-MMP- and TM-MT-MMP-dependent pro-MMP-2 activation is stimulated [91]. Nevertheless, these studies suggest that GPI-MT-MMP-mediated pro-MMP-2 activation, in contrast to that accomplished by TM-MT-MMPs, may require stimulation by cooperating factors.

Extensive studies from our laboratory showed that MT6-MMP is unable to activate pro-MMP-2 under various experimental conditions including in the presence of various TIMP-2 concentrations, a factor that was not previously examined [32]. We also co-expressed pro-MMP-2 and MT6-MMP in mammalian cells, as performed by Nie and Pei [71], and found no pro-MMP-2 activation. Moreover, PI-PLC-released MT6-MMP was inactive against pro-MMP-2. Based on these negative findings [31, 32] and the modest activation reported in co-transfection systems [26, 71], we posit that MT6-MMP is unlikely to behave as an activator of pro-MMP-2 under physiological conditions. Furthermore, under conditions of MT1-MMP expression in cancer cells, pro-MMP-2 activation by GPI-MT-MMPs would not be expected to be significant.

The role of GPI-MT-MMPs in the activation of pro-MMP-9 was also investigated [32, 59, 62]. First, the primary sequence similarity of MT6-MMP with stromelysin-1 (MMP-3), a known pro-MMP-9 activator, suggested a possible role of MT6-MMP in pro-MMP-9 activation. Second, the fact that GPI-MT-MMPs are inhibited by TIMP-1 raised the question whether pro-MMP-9 activation can be promoted by GPI-MT-MMP/TIMP-1 complexes, analogous to the activation of pro-MMP-2 by MT1-MMP in the presence of TIMP-2. However, the data have shown that MT6-MMP is not a pro-MMP-9 activator regardless of the presence of TIMP-1 [32, 62]. Likewise, MT4-MMP cannot activate pro-MMP-9 [59, and unpublished data from our laboratory]. Taken together, the overwhelming evidence strongly

suggests that GPI-MT-MMPs did not evolve to accomplish pro-gelatinase activation at the cell surface.

2.5.4 Other zymogen substrate—Studies with ADAMTS (a disintegrin and metalloproteinase with thrombospondin-like motif)-4, a metalloprotease with ability to cleave proteoglycans such as aggrecan [92], have shown that MT4-MMP plays a role in ADAMTS-4 activation. Thus, MT4-MMP may indirectly contribute to ADAMTS-4-mediated degradation of cartilage in normal and pathological processes [93, 94]. In summary, although only a limited number of GPI-MT-MMP substrates have been identified, it appears that the substrate profile of GPI-MT-MMPs is very different from that exhibited by other MMPs, and in light of their membrane and extracellular distribution, may involve targets in both lipid rafts and non-lipid rafts.

3 Expression and role of GPI-MT-MMPs in cancer

Since their discovery, it became evident that the mRNA of GPI-MT-MMPs is highly expressed in various human cancers. Only recently, however, data have emerged showing protein expression and localization of MT4- [68] and MT6-MMP [32] in breast and colon cancer, respectively. Here, we will summarize the current information on the expression and role of GPI-MT-MMPs in human cancer tissues (Table 1).

3.1 MT4-MMP

MT4-MMP was originally cloned from a human breast carcinoma cDNA library [24] and consistently its mRNA was found to be expressed in breast cancer tissues [24, 68]. A study by Chabottaux et al. [68] examined the expression of MT4-MMP mRNA and protein in 63 breast adenocarcinomas and 21 samples of normal breast tissue. These investigators found no significant differences in MT4-MMP mRNA. However, immunohistochemistry studies revealed higher expression of MT4-MMP in cancer cells, while normal breast epithelial cells showed variable and overall moderate staining. MT4-MMP protein was particularly elevated in metastatic lymph nodes where both tumor and inflammatory cells displayed strong staining [68]. This is consistent with the reported expression of MT4-MMP in leukocytes [24, 95]. Overexpression of recombinant MT4-MMP in the breast cancer cell line MDA-MB-231 enhanced subcutaneous tumor growth when inoculated in RAG-1 immunodeficient mice [68]. Moreover, the MT4-MMP expressing MDA-MB-231 s.c. tumors developed lung metastases in 100% of the mice. Thus, MT4-MMP promotes both tumor growth and metastasis. Interestingly, functional studies failed to reveal an effect of MT4-MMP overexpression on *in vitro* cell migration and invasion, pro-MMP-2 activation, VEGF production, and angiogenesis [68]. These findings are similar to those obtained with MT6-MMP in colon cancer cells, in which expression of the protease did not confer a migratory and/or invasive phenotype or ability to activate pro-MMP-2 [32]. Together, these results suggest a unique role of GPI-MT-MMP in promotion of cancer progression.

While human breast cancer tissues express MT4-MMP [24, 68] where it appears to play a role in progression, the expression and function of MT4-MMP in mouse mammary cancinoma are unclear. An *in situ* hybridization study showed that MT4-MMP mRNA was not expressed in mouse mammary adenocarcinomas of transgenic mice expressing the polyoma virus T antigen under control of the MMTV promoter [96]. In fact, MT4-MMP mRNA was not expressed even in normal mammary glands regardless of their developmental stage [96]. The significance of these observations is unclear and need to be reproduced in other models of mammary cancer in mice before a definite conclusion can be made about the role of MT4-MMP in mouse mammary cancer development.

MT4-MMP mRNA expression was also examined in human gliomas [97] and was found to decrease with advancing tumor grade. In fact, MT4-MMP mRNA was highly expressed in normal brain tissue [97]. Although these studies involved a limited number of patients, the data suggest that the two GPI-MT-MMPs are differentially regulated in brain cancer. Furthermore, the studies in brain and breast tumors also suggest that the association of MT4-MMP expression with tumor progression may be cancer type specific.

3.2 MT6-MMP

Elevated MT6-MMP mRNA expression was found in several human cancers including brain (anaplastic astrocytomas and glioblastomas) [26, 97], colon [26], urothelial [98], and prostate [99] cancers. In gliomas, expression of MT6-MMP mRNA was suggested to contribute to disease progression [97]. The discovery of MT6-MMP mRNA expression in colon cancer [26] led to the first immunohistochemical study of MT6-MMP expression in samples of invasive colon cancer [32]. This study showed that while MT6-MMP protein was absent from normal colonic epithelium it was highly expressed in all invasive adenocarcinomas in the majority of the cases examined (50/60). In areas of *in situ* dysplasia, representing early stages of progression, staining for MT6-MMP was very weak albeit evident when compared to adjacent non-dysplastic epithelium. In contrast, invasive cancer cells showed high levels of MT6-MMP protein. In all the cases studied, MT6-MMP expression levels were independent of tumor differentiation and there was no association between intensity of staining and invasion of cancer cells into lymphovascular spaces [32]. Furthermore, other factors used to define tumor grade including architectural organization, cell polarity, mitotic rate, and extent of necrosis, as well as clinical stage, were not associated with differences in MT6-MMP expression. MT6-MMP staining in the tumor stroma was almost absent with the exception of strong expression in inflammatory-like cells [32], consistent with the known expression of MT6-MMP in leukocytes [25]. The high expression of MT6-MMP protein in the colon cancer cells is striking considering that many MMPs are known to be produced in the tumor stroma. However, the contribution of inflammatory cells, which produce MT6-MMP [25, 26], may also be critical in colon cancer.

Functional studies with colon cancer cell lines (HCT-116 and HT-29) overexpressing MT6-MMP showed that MT6-MMP expression correlated with enhanced tumor growth after subcutaneous inoculation in athymic mice [32]. Distant metastases to lungs or liver were not detected in MT6-MMP tumor bearing mice. Histopathological examination of the MT6-MMP subcutaneous tumors revealed an infiltrative tumor interface with a markedly desmoplastic stroma, suggesting that MT6-MMP promotes local tumor invasion. Whether MT6-MMP contributes to metastatic dissemination of colon cancer cells in mice needs to be investigated in relevant orthotopic models. However, *in vitro* studies showed no effects of MT6-MMP overexpression on tumor cell migration and invasion, and thus the precise role of MT6-MMP in colon cancer remains elusive. Although more studies are required to unveil the roles of GPI-MT-MMPs in cancer, the data so far suggest that these proteases influence cancer progression by mechanisms that are different from the TM-MT-MMPs. First, GPI-MT-MMPs do not act as progelatinase activators; second, their ECM degradation profile appears to be very limited; third, GPI-MT-MMPs do not promote tumor cell migration and invasion; and fourth, their inhibition profile appears unique.

4 Concluding remarks and future challenges

It is becoming evident that the GPI-MT-MMPs represent a distinctive subset of membrane-anchored MMPs that exhibit unique structural and functional characteristics, which separate them from the rest of the MMP family. Due to their unique characteristics and expression pattern, elucidating their regulation and role in malignant processes remains a major challenge and will require implementing a different set of biochemical and functional

strategies from those applied to the study of most MMPs. The evidence so far strongly suggests that GPI-MT-MMPs are involved in cancer progression, where they may function both in cancer and inflammatory cells. More studies are required to define the pattern of GPI-MT-MMPs expression in human cancers and their association with progression. Furthermore, mouse models of cancer are needed to unveil the impact of expression and downregulation of GPI-MT-MMPs in the cancer cells and in the tumor stroma on tumor development, growth, and metastasis. Assessment of GPI-MT-MMP-mediated proteolysis and function in cancer will require establishing quantitative activity-based and functional biological assays specific for GPI-MT-MMPs. Evaluation of GPI-MT-MMP specific activity in complex biological systems and how it affects cell behavior will be aided by identification of their unique substrates, likely in the raft environment, and generation of specific synthetic inhibitors. Additional studies are needed to elucidate the inhibition of GPI-MT-MMPs by TIMPs and by other potential inhibitors such as clusterin [63] and possibly RECK, a GPI-anchored protein with MMP inhibitory activity [100]. The regulation of GPI-MT-MMPs is still not fully understood, and future studies need to address their biosynthetic pathway, endocytosis, recycling, shedding, and the function and significance of dimerization/ oligomerization. The challenges are significant but surely worthwhile because they will shed light on these very unique MMPs. This is also an important task in light of the disappointments of clinical trials with MMP inhibitors and the lessons learned from these attempts. Today, it is evident that effective targeting of relevant MMP activity in cancer patients will demand a thorough understanding of the biology, function, expression, and substrate profile of all members of the MMP family and their precise roles in cancer progression.

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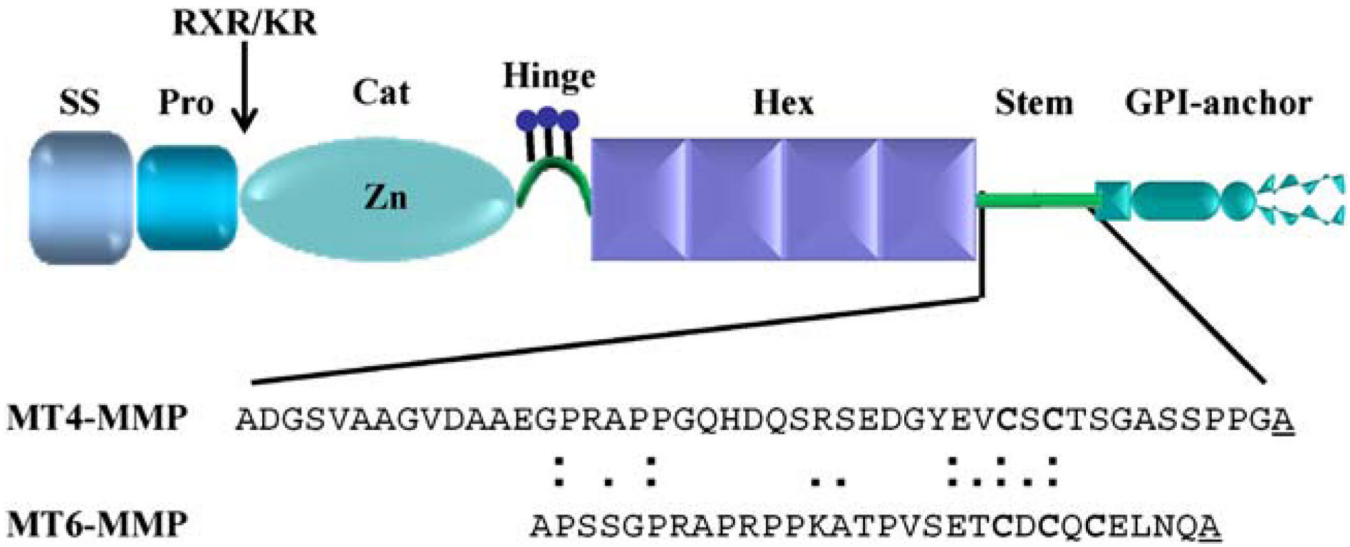

Fig. 1.

Structure of mature GPI-anchored MT-MMPs. *SS* signal-sequence, *Pro* prodomain, *RXR/KR* furin recognition motif, *Cat* catalytic domain, *Hex* hemopexin-like domain. The amino acid sequence of MT4- and MT6-MMP stem region (linker 2) is shown in detail. Cysteine residues in the stem region are indicated in *bold*. The homology between the stem regions of MT4- and MT6-MMP is indicated by *black squares*. The putative transamidase cleavage site, ω, is *underlined*

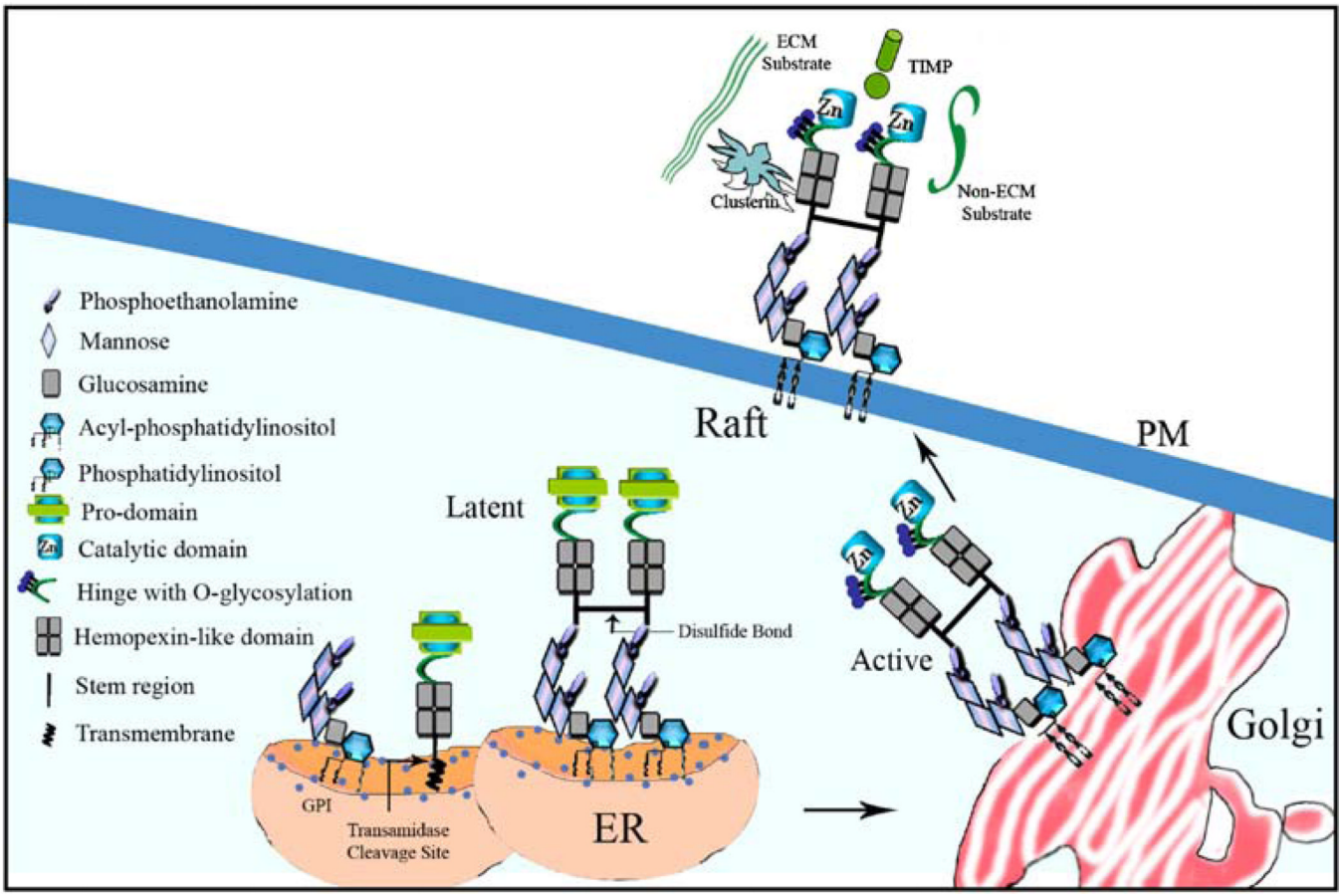

Fig. 2.

Hypothetical biosynthetic pathway of MT6-MMP. Pro-MT6-MMP is synthesized as a transmembrane precursor protein. This precursor protein is cleaved by a transamidase at the ω site releasing the carboxyl-terminal transmembrane domain and incorporating a preexisting GPI anchor in the endoplasmic reticulum (*ER*). Also in the ER, MT6-MMP undergoes homodimerization by formation of disulfide bonds between cysteine residues at the stem region (our unpublished data). At the Golgi network, MT6-MMP is activated by a furin-like pro-convertase and *O*-glycosylated (our unpublished data). In addition, unsaturated fatty chains in phosphatidylinositol are replaced with saturated fatty acids chains and MT6-MMP associates with lipid raft [32]. At the cell surface, MT6-MMP interacts with TIMPs and/or clusterin for regulation of enzymatic activity. Both ECM and non-ECM substrate are hydrolyzed by MT6-MMP at the cell surface. MT4-MMP biosynthetic pathway is likely to be similar to that of MT6-MMP

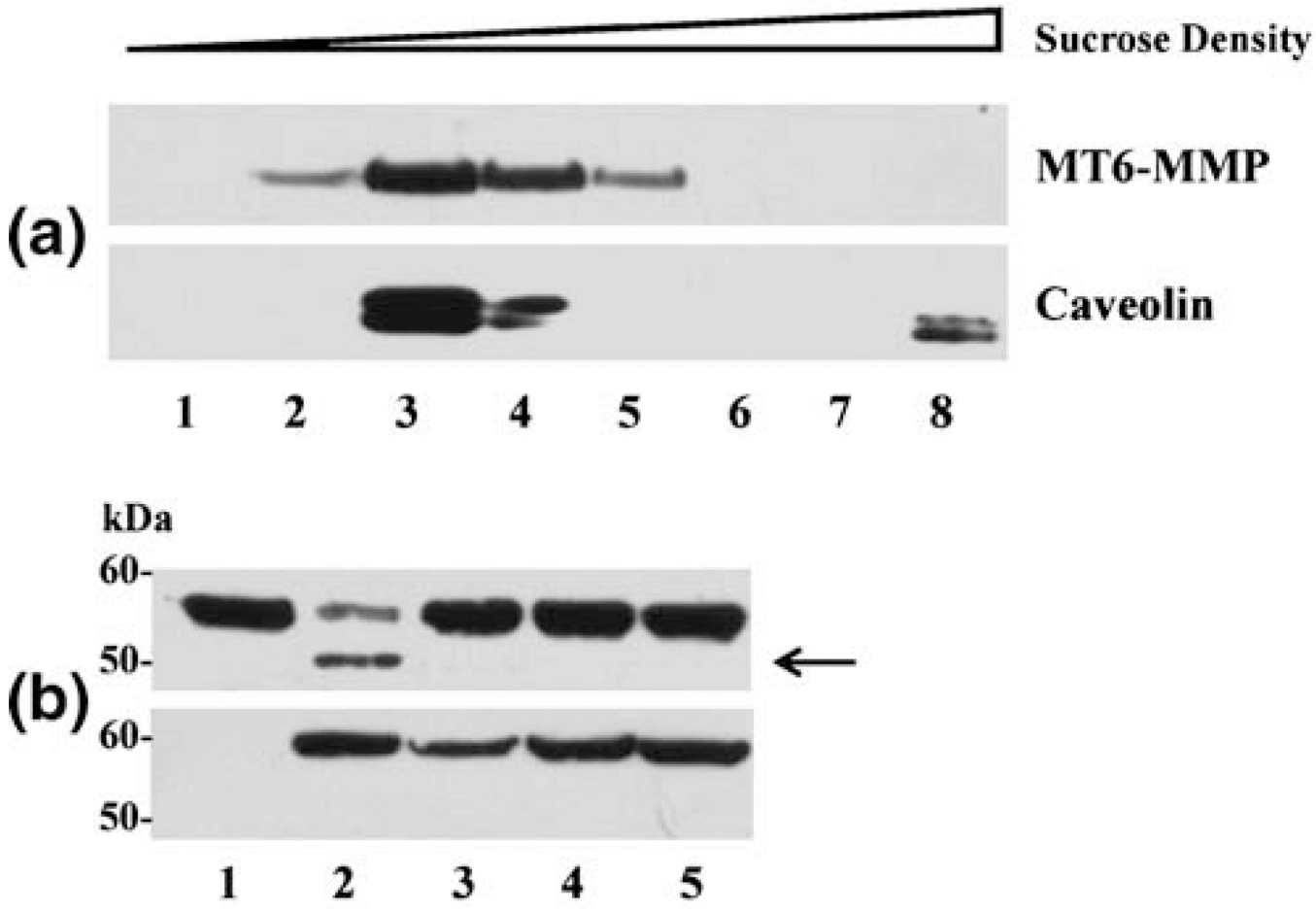

Fig. 3.

Lipid raft localization and activity of MT6-MMP. (**a**) Colon cancer HCT-116 cells expressing human recombinant MT6-MMP [32] were homogenized in 1% Triton X-100 containing Protease Inhibitor (Roche) mix and the homogenate was adjusted to 35% sucrose. The homogenate was placed at the bottom of a centrifuge tube and overlaid with two 4 ml layers of 30 and 5% sucrose. The samples were centrifuged at 100,000×g for 18 h at 4°C. Eight fractions (1 ml each) were collected sequentially from the top of the gradient and equal volume of each fractions were resolved by reducing 10% SDS/PAGE followed by immunoblot analyses using anti-human MT6-MMP monoclonal antibodies and a polyclonal antibody to human Caveolin, as a lipid raft marker. MT6-MMP, 57 kDa; Caveolin, 22 kDa. (**b**) Crude plasma membranes were isolated from HCT-116 cells transfected with empty vector (lane *1*) or with human recombinant MT6-MMP (lanes *2*– *4*) or with a catalytically inactive E/A MT6-MMP mutant (lane *5*) were incubated (18 h, 37°C in a 50 μl reaction volume with Proteinase Inhibitor cocktail without EDTA) with α1-PI in the absence (lanes 1, 2 and *5*) or presence of either 200 nM recombinant human TIMP-1 (lane *3*) or recombinant human TIMP-*2* (lane *4*). Equal amounts from each sample were mixed with Laemmli SDS-sample buffer and resolved by reducing 10% SDS–PAGE followed by immunoblot analysis with antibodies against human α1-PI (upper panel) and human MT6-MMP (lower panel) *Arrow* in (**b**) indicates the cleaved species of α1-PI

Table 1

Substrates, inhibitors and expression in cancer tissues of GPI-anchored MT-MMPs

		MT4-MMP	MT6-MMP
Substrates	Gelatin	Yes [59, 61]	Yes [62]
	Fibrinogen	Yes [59]	Yes [62]
	Fibrin	Yes [59]	Yes [62]
	Fibronectin	No [59]	Yes [30, 62]
	Collagen IV	No [59]	Yes [62]
	pro-TNFα	Ye s [59]	ND
	α2-macroglobulin	Yes [59]	No [101]
	Progelatinase-A	Yes [61, 91]	Yes [26, 71, 91]
		No [59, 60, 68]	No [31, 32]
	Progelatinase-B	No [59]	No [32, 62]
	ADAMTS-4 (aggrecanase-1)	Yes [93, 94]	ND
	Low density lipoprotein receptor related protein	Yes [75]	ND
	Chondroitin sulfate proteoglycan	ND	Yes [30]
	Dermatan sulfate proteoglycan	ND	Yes [30]
	α1 proteinase inhibitor	ND	Yes [30, 70]
	Urokinase plasminogen activator receptor	ND	Yes [76]
	Galectin-3	ND	Yes (our unpublished results)
Inhibitors	TIMP-1	Yes [59–61, 93, 102]	Yes [32, 62, 63]
	TIMP-2	Yes [59–61]	Yes [32, 62, 63]
	TIMP-3	Yes [59, 102]	Yes [62, our unpublished results]
	TIMP-4	ND	Yes (our unpublished results)
	Clusterin	Yes [63]	Yes [63]
			Ye s [32][a]
Expression in cancer tissues	Anplastic astrocytomas, glioblastomas	No[b] [97]	Yes [26, 97]
	Breast carcinoma	Yes [24, 68]	
	Colon cancer		Yes [26, 32]
	Urothelial cancer		Yes [98]
	Prostate cancer		Yes [99]

ND not determined

[a] No complex formation detected in HCT-116 colon cancer cells transfected with human MT6-MMP

[b] Expression decreased compared to normal brain