Catalytic activities of membrane-type 6 matrix metalloproteinase (MMP25)

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Abstract This study describes the biochemical characterisation of the catalytic domain of membrane-type 6 matrix metalloproteinase (MT6-MMP, MMP25, leukolysin). Its activity towards synthetic peptide substrates, components of the extracellular matrix and inhibitors of MMPs was studied and compared with MT1-MMP, MT4-MMP and stromelysin-1. We have found that MT6-MMP is closer in function to stromelysin-1 than MT1 and MT4-MMP in terms of substrate and inhibitor specificity, being able to cleave type-IV collagen, gelatin, fibronectin and fibrin. However, it differs from stromelysin-1 and MT1-MMP in its inability to cleave laminin-I, and unlike stromelysin-1 cannot activate progelatinase B. Our findings suggest that MT6-MMP could play a role in cellular migration and invasion of the extracellular matrix and basement membranes and its activity may be tightly regulated by all members of the TIMP family.

Key words: Membrane-type matrix metalloproteinase; MT6-MMP; Tissue inhibitor of metalloproteinase; Extracellular matrix; Type-IV collagen

1. Introduction

Matrix metalloproteinases (MMPs) are a family of zinc dependent endopeptidases which are involved in connective tissue degradation in physiological and pathological events such as wound healing, angiogenesis, tumour growth and metastasis and arthritis to name but a few [1,2]. The MMPs can be broadly split into groups of enzymes termed stromelysins, gelatinases, collagenases and membrane-type MMPs based on differences in structure and substrate specificity [1]. The membrane-type MMPs can also be subdivided into two groups; the type-I transmembrane and the glycosylphosphatidylinositol (GPI)-anchored proteases.

MT1, 2, 3 and 5 (MMP14, 15, 16 and 24) are all type-I transmembrane proteins with a short cytoplasmic tail which has been shown in MT1-MMP to be required for localisation at invadopodia [3]. These enzymes have the ability to degrade many matrix components including type-I–IV collagen, gelatin, laminin, fibronectin and fibrin [4,5]. The phenotype of the MT1-MMP knock-out mouse suggests that it may play a major role in collagenolysis [6], which is supported by a recent in vivo study of Hotary et al. [7]. The MT-MMPs were able to activate progelatinase A and MT1-MMP was shown to activate procollagenase-3 (MMP2 and MMP13, respectively), either in vitro or in vivo [8–13]. These enzymes are inhibited by tissue inhibitor of metalloproteinase (TIMP)-2, which in complex with MT1-MMP also acts as a progelatinase A receptor, potentiating the activation process [14], and by TIMP-3 but largely not by TIMP-1 ([9], Fox and Murphy, unpublished observations).

The second sub-group of MT-MMPs consists of enzymes which are attached to the membrane via a covalent link to GPI. This sub-group includes MT4 and MT6-MMP (MMP17 and MMP25) [15,16]. The best characterised to date, MT4-MMP, has far less similarity with the other MT-MMPs at the structural and functional level and appears to be predominantly expressed in leukocytes as well as tumour cell lines [17–19]. MT4-MMP is very limited in its ability to degrade most ECM components examined to date [20]. Equally it does not have the ability to activate progelatinase A or procollagenase-3 [20,21]. Currently, the known substrates of MT4-MMP include fibrinogen, fibrin and proTNFα [20]. MT6-MMP or leukolysin (MMP25) is a recently identified member of the MT-MMPs expressed almost exclusively in the normal adult in peripheral blood leukocytes and many brain tumours [22,23]. Primary sequence alignments with the rest of the MMP family, in particular the catalytic domains, have shown that its closest sequence similarity is with MT4-MMP (MMP17, 65% identity, 77% similarity) followed by MT1-MMP (MMP14, 47% identity, 70% similarity) and stromelysin-1 (MMP3, 44% identity, 68% similarity) with almost equal similarity. This raises the interesting question to what extent it has similarity to these enzymes at the functional level. To date it has been shown that MT6-MMP, like MT4-MMP, is either very poor at, or unable to, activating progelatinase A and does not participate in ectodomain shedding of proTGF-α, HER-2 or proHB-EGF [16,22].

In this work we describe the expression and purification of recombinant active MT6-MMP catalytic domain which has enabled us to characterise its specificity towards peptides, extracellular matrix components and TIMPs. Our results suggest that MT6-MMP is much closer, although not identical to,

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Abbreviations: GPI, glycosylphosphatidylinositol; ECM, extracellular matrix; MMP, matrix metalloproteinase; MT-MMP, membrane-type matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; Mca, (7-methoxycoumarin-4-yl)acetyl; Dnp, 2,4-dinitrophenyl

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stromelysin-1 in function than MT4-MMP or MT1-MMP and may play a role in peripheral blood leukocyte invasion of the extracellular matrix and contribute to the metastasis and growth of brain tumours.

2. Materials and methods

2.1. Subcloning and isolation of the human proMT6-MMP catalytic domain

The proMT6-MMP catalytic domain was subcloned from the human cDNA clone PI-4 in pBluescript by polymerase chain reaction using primers 5′-AAAACTCGAGGGCGCAGGACGTGAGCCT-GGGCGTG-3′ and 5′-AAAAGAATTCAAGCCAGGGGTTTCCT-TGTGGGC-3′ which contained restriction sites for *Xho*I and *Eco*RI, enabling ligation in pRSET3b and the addition of a $[His]_6$ tag preceding the pro sequence, which was then sequenced to detect errors by dye-deoxy terminator sequencing. The proMT6-MMP catalytic domain consisting of residues 26–296 (where residue 1 is the initiating Met) was then expressed in *Escherichia coli* BL21 [DE3] pLysS by induction with 0.5 mM IPTG as described previously for the MT4-MMP pro-catalytic domain [20]. The enzyme was found to be expressed at high levels as insoluble inclusions and could be purified under denaturing conditions in an identical manner as described for the proMT4-MMP catalytic domain using DEAE-Sepharose [20]. The solubilised protein was then further purified by nickel affinity chromatography on Ni^+ NTA-agarose. Refolding of the purified enzyme was achieved by rapid dilution in 50 mM Tris–SO_4, 100 mM Na_2SO_4, 10 mM $CaSO_4$, 0.1 mM $ZnSO_4$, 10% glycerol, 0.1% Brij 35, pH 8.0, with the final protein concentration estimated to be 0.1 mg ml^{-1}, for 18 h at 4°C. To remove aggregates, the enzyme was then centrifuged at $10\,000\times g$ prior to reducing SDS–PAGE analysis and storage in aliquots at −80°C.

2.2. Characterisation of activity of proMT6-MMP catalytic domain

Activity of the MT6-MMP catalytic domain was measured in assay buffer (50 mM Tris–HCl, 150 mM NaCl, 10 mM $CaCl_2$, 0.05% Brij, pH 7.5) at 25°C at all times. The concentration of native, active enzyme after refolding was determined as described by Murphy and Willenbrock [24] by active site titration with TIMP-1, following inhibition of hydrolysis of 1 μM (7-methoxycoumarin-4-yl)acetyl (Mca)-PLGL-Dpa-AR-NH_2 in 2.5 ml assay buffer using a Perkin-Elmer LS50b fluorimeter. For calculation of k_{cat}/K_M for quenched fluorescent substrates, the activity of 0.5 nM MT6-MMP catalytic domain in 2.5 ml assay buffer was followed by monitoring hydrolysis of Mca-PLGL-Dpa-AR-NH_2, Mca-PLA-Nva-Dpa-AR-NH_2 and Mca-SPLA-QAVRSSSRK(2,4-dinitrophenyl (Dnp))-NH_2 where $[S] \ll K_M$ and k_{obs} was observed to follow an apparent linear relationship with respect to [S] ($[S] < 1.5$ μM). In the case of Mca-SPLAQAVRSSSRK(Dnp)-NH_2, C_{18} reversed phase-high performance liquid chromatography (RP-HPLC) analysis showed that there was more than one cleavage site. For identification of the cleavage sites the products were purified by RP-HPLC prior to analysis by matrix-assisted laser desorbing ionising time of flight mass spectrometry. For the calculation of the apparent K_I ($K_I^{(app)}$) for hydroxamic acid peptide analogue inhibitors Ro31-9790 (Roche Molecular Biochemicals, Wellwyn City Gardens, UK) and CT-1746 (CelltechChiroscience, Slough, UK), 0.5 nM MT6-MMP catalytic domain in 2.5 ml was incubated with 0–100 nM hydroxamate inhibitor for 30 min at 25°C, which allowed the enzyme–inhibitor complex to reach equilibrium, prior to the addition of 1 μM Mca-PLGL-Dpa-AR-NH_2. Analysis of the progress curves of hydrolysis allowed calculation of $K_I^{(app)}$ using the equation describing competitive tight binding inhibition [25]. As the catalytic domain of MT6-MMP was found to be unstable for prolonged periods of time, like that of MT4-MMP [20], $K_I^{(app)}$ for TIMPs could only be calculated for comparison of relative affinities with MT1-MMP, MT4-MMP and stromelysin-1. The MT6-MMP catalytic domain was incubated at 0.5 nM in 100 μl assay buffer at 25°C with TIMP-1, -2 or -3 at 0–50 nM for 2 h. This was then diluted to 2.5 ml with assay buffer for 2 h before activity monitored by the addition of 1 μM Mca-PLA-Nva-Dpa-AR-NH_2. $K_I^{(app)}$ was calculated using the equation describing competitive tight binding inhibition [25].

Macromolecular substrates for the studies of activity of MT6-MMP catalytic domain were obtained or prepared as follows: stromelysin-1, gelatinase B, collagenase-2 and MMP19 were prepared as described previously [26,27]. Fibrinogen purified from human plasma was obtained from Calbiochem. Type-IV collagen prepared from human placenta was a kind gift from Roche Diagnostics GmbH. Fibronectin from human plasma, thrombin, TPCK-treated trypsin and laminin-I were obtained from Sigma (Poole, UK). Mouse anti-human monoclonal antibodies to fibronectin were obtained from Gibco-BRL. Type-I gelatin was prepared from rat skin type-I collagen [4]. Controls were run without enzyme in assay buffer and the reactions were stopped by the addition of reducing SDS–PAGE buffer and storage on ice prior to analysis. Activity of MT6-MMP and stromelysin-1 could be inhibited by the addition of 1,10-phenanthroline. Fibronectin fragments generated by MT6-MMP and stromelysin-1 were further analysed by Western blot using monoclonal antibodies specific for the heparin, cell and gelatin binding domains [4]. Fibrin gel was prepared by clotting fibrinogen with thrombin as previously described [20]. Activation of recombinant progelatinase B or procollagenase-2 was followed by incubation of 5 μg pro-enzyme with 40 ng MT6-MMP catalytic domain or 80 ng stromelysin-1 before analysis on reducing SDS–PAGE.

3. Results and discussion

3.1. Expression and refolding of the recombinant proMT6-MMP catalytic domain

On induction with IPTG, the proMT6-MMP catalytic domain was found to be highly expressed in *E. coli* from pRSETB as insoluble inclusions, as reported for other MMP catalytic domains. Equally, on solubilisation with 8 M urea, the pro-catalytic domain could be purified under denaturing conditions on DEAE-Sepharose to give a major band at the expected mass with a number of degradation products. This material was further purified on a Ni^+ NTA-agarose affinity column and then refolded using the protocol established for the proMT4-MMP catalytic domain [20] (Fig. 1, lane 1). The refolded enzyme was found to show activity towards the quenched fluorescent substrate Mca-PLGL-Dpa-AR-NH_2, however, unlike the proMT4-MMP catalytic domain, incubation with 1 mM 4-amino phenylmercuric acetate or 10 μg ml^{-1} TPCK-treated trypsin did not result in an increase in activity of the refolded material. Examination by SDS–PAGE showed that the refolded material consisted of three forms approximating the expected mass of the pro, intermediate and active form of the enzyme and a smaller fragment at 17 kDa which is probably a degradation product (Fig. 1, lane 3). Gelatin zymography showed that only the 25 kDa band had any significant activity although clearance of gelatin was poor (data not shown). Treatment with trypsin did not

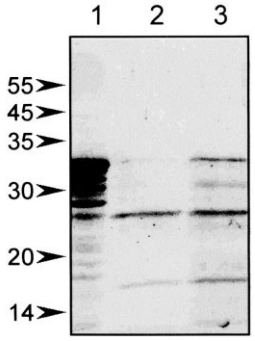

Fig. 1. Coomassie blue-stained reducing SDS–PAGE gel of: lane 1, $[His]_6$-proMT6-MMP catalytic domain purified from insoluble inclusions by DEAE-Sepharose and Ni^+ NTA-agarose under denaturing conditions. Lane 2, material from lane 1 refolded at a total protein concentration of 100 μg ml^{-1} and then centrifuged at $20\,000\times g$ for 30 min. Lane 3, material refolded but not centrifuged.

Table 1
Comparison of k_{cat}/K_M calculated at 25°C, pH 7.5 for MT6-MMP with other MMPs for quenched fluorescent peptide substrates

Substrate	$k_{cat}/K_M \times 10^4$ M^{-1} s^{-1}			
	Stromelysin-1 (MMP3)	MT1-MMP (MMP14)	MT4-MMP (MMP17)	MT6-MMP (MMP25)
Mca-PLGL-Dpa-AR-NH_2	0.16	2.1	2.4	3.8
Mca-PLA-Nva-Dpa-AR-NH_2	1.4	0.86	3.0	8.3
Mca-SPLAQAVRSSSRK(Dnp)-NH_2	NC	NH	3.5	NC

NH denotes 'not hydrolysed' and NC 'not calculated' as the substrate was not cleaved at a unique site.

alter the mobility of the three forms on SDS–PAGE (not shown). After centrifugation at $10\,000 \times g$ to remove aggregates, only the 25 kDa and 17 kDa forms were seen to remain on analysis by SDS–PAGE of the supernatant (Fig. 1, lane 2) and greater than 95% of activity towards the substrate Mca-PLGL-Dpa-AR-NH_2 was found to be retained. The remaining catalytic domain was also found to be resistant to trypsin hydrolysis on inspection by SDS–PAGE and activity towards Mca-PLGL-Dpa-AR-NH_2 did not alter (data not shown). This suggests that a large proportion of the pro-catalytic domain of MT6-MMP formed aggregates on folding and remained proteolytically protected from autocatalytic or trypsin processing to the active form, whereas the proportion which folded successfully underwent autocatalytic processing to yield a trypsin stable domain. This was not previously observed for the other pro-catalytic domains of MT-MMPs which can be refolded to yield stable pro-enzymes, although the stromelysin-like enzyme, MMP19, has also been reported to autoactivate during refolding [25]. For subsequent studies the autoactivated form of the catalytic domain was used.

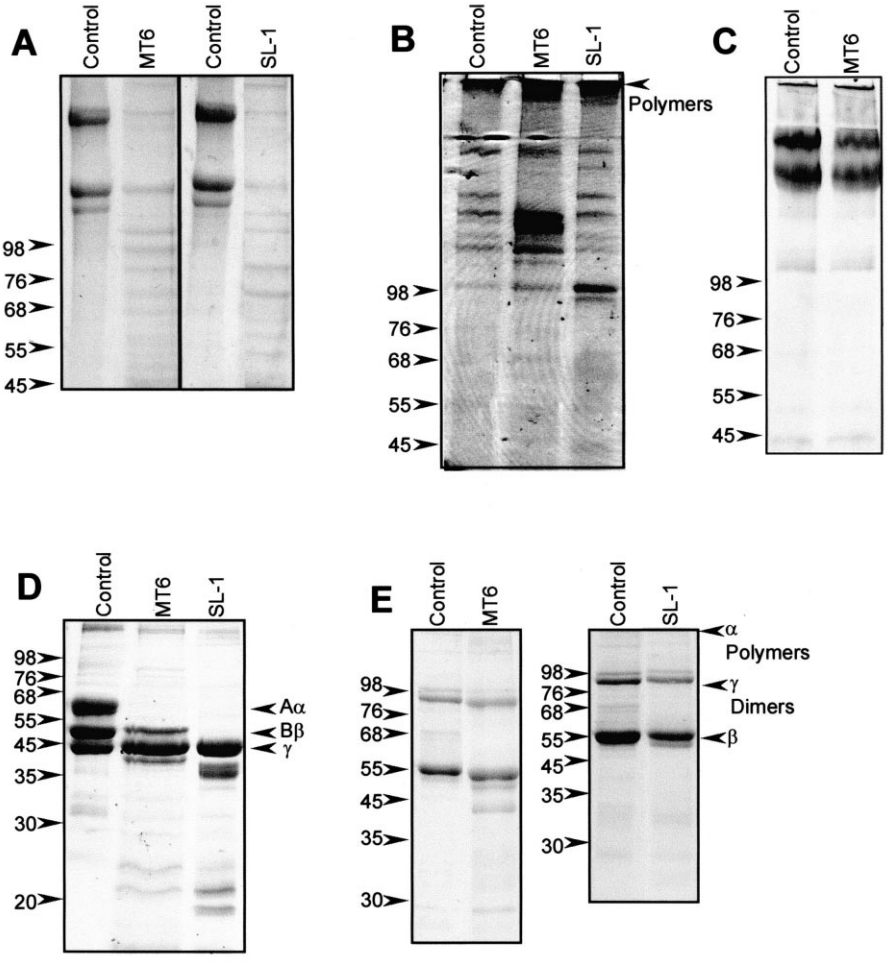

Fig. 2. Coomassie blue-stained reducing SDS–PAGE gels of ECM cleavage by MT6-MMP catalytic domain and stromelysin-1. All reactions were performed in assay buffer for 18 h at 25°C. A: 40 μg of type-I gelatin incubated in assay buffer alone (control) or with 250 ng active MT6-MMP catalytic domain (MT6) or 450 ng, i.e. equi-molar, stromelysin-1 (SL-1). B: 40 μg type-IV collagen purified form human placenta incubated in assay buffer alone (control), 250 ng MT6-MMP catalytic domain (MT6) or 450 ng stromelysin-1 (SL-1). C: 20 μg of laminin-I incubated with assay buffer or 250 ng MT6-MMP catalytic domain. D: 20 μg of fibrinogen incubated in assay buffer alone or with 250 ng MT6-MMP or 450 ng stromelysin-1. E: 500 μg of X-linked fibrin was incubated with 1 μg of MT6-MMP or 450 ng stromelysin-1.

Table 2
$K_I^{(app)}$ for MT6-MMP and other MMPs for hydroxamate peptide analogue inhibitors and TIMPs calculated at 25°C, pH 7.5

Inhibitor	$K_I^{(app)}$ ($\times 10^{-9}$ M)			
	Stromelysin-1 (MMP3)	MT1-MMP (MMP14)	MT4-MMP (MMP17)	MT6-MMP (MMP25)
CT-1746	10.9	1.1	4.7	0.3
Ro31-9790	119	3.3	2.3	6
TIMP-1	$\ll 0.2$	NI	$\ll 0.07$	0.02
TIMP-2	$\ll 0.2$	$\ll 0.002$	0.4	$\ll 0.002$
TIMP-3	$\ll 0.2$	0.06	0.2	$\ll 0.002$

Where $K_I^{(app)}$ was estimated to be significantly lower than [E] at which activity of uninhibited enzyme could be detected, and the inhibitor still titrated the enzyme, values have '$\ll$' in front to indicate the $K_I^{(app)}$ quoted is a maximal estimate. NI indicates 'not inhibited' at concentrations of [I] unless in the μM range or greater. Values for MT1 and 4-MMP are taken from [20]. Values for stromelysin-1 are taken from [20] for the hydroxamate inhibitors and [29] for the TIMPs.

3.2. Specificity of MT6-MMP towards peptide substrates and inhibitors

In order to establish whether MT6-MMP is closest to MT4-MMP, MT1-MMP or stromelysin-1 in substrate specificity, an initial investigation was made by examining peptide substrate specificity. Comparison of the efficiency of hydrolysis of Mca-PLGL-Dpa-AR-NH_2, Mca-PLA-Nva-Dpa-AR-NH_2 and Mca-SPLAQAVRSSSRK(Dnp)-NH_2 by MT6-MMP with MT1-MMP, MT4-MMP and stromelysin-1 indicates that MT6-MMP is closer in specificity to stromelysin-1. MT6-MMP shows a clear preference for the stromelysin substrate Mca-PLA-Nva-Dpa-AR-NH_2 over the general MMP substrate Mca-PLGL-Dpa-AR-NH_2 (Table 1). MT6-MMP only poorly hydrolysed a peptide consisting of the ectodomain cleavage site of proTNFα, Mca-SPLAQAVRSSSRK(Dnp)-NH_2, with an apparent k_{cat}/K_M of 1.3×10^4 M^{-1} s^{-1}, which was more efficiently hydrolysed by MT4-MMP at 3.5×10^4 M^{-1} s^{-1}. Subsequent examination of the cleavage of Mca-SPLAQAVRSSSRK(Dnp)-NH_2 by MT6-MMP by RP-HPLC indicated two cleavage sites, hence only an apparent rate of hydrolysis could be determined from the observed increase in hydrolysis by fluorescence. We have previously reported that MT1-MMP cannot hydrolyse this peptide [20]. This is unlike MT4-MMP which uniquely cleaves at Q↓A, as MT6-MMP instead cleaves at the same sites reported for MMP1, 9 and MT1-MMP (in a GST fusion), at SPLA↓QA↓VRSSSRK [4,28]. To further investigate the characteristics of the primed side of the active site of MT6-MMP, affinities for two competitive hydroxamic acid inhibitors were examined. Ro31-9790 is most potent towards gelatinases, MT-MMPs and collagenases but is a poor inhibitor of stromelysins. In contrast CT-1746 is a potent inhibitor of stromelysins, gelatinases and MT-MMPs but not collagenases [20]. The approximate 10-fold difference in affinity of MT6-MMP for these inhibitors is comparable to the difference seen with stromelysin-1 and therefore suggests that the primed side of the active site is more stromelysin-like in character (Table 2). In contrast MT1 and 4-MMP did not show any preference for this inhibitor, similar to the gelatinases (Table 2 and [20]).

3.3. Specificity of MT6-MMP for TIMPs

The catalytic domain of MT6-MMP was found to be well inhibited by TIMP-1, although it was effectively titrated by TIMP-2 and -3 where $K_I^{(app)}$ was estimated to be $\ll$[E] at which activity could be detected, hence the complex can be considered irreversible under the conditions of the experiment (Table 2). This again differs from MT1-MMP which is also inhibited by TIMP-2 and -3 but is very poorly inhibited by TIMP-1, and MT4-MMP which is titrated by TIMP-1 but has lower affinity for TIMP-2 and -3 (Table 2). These results

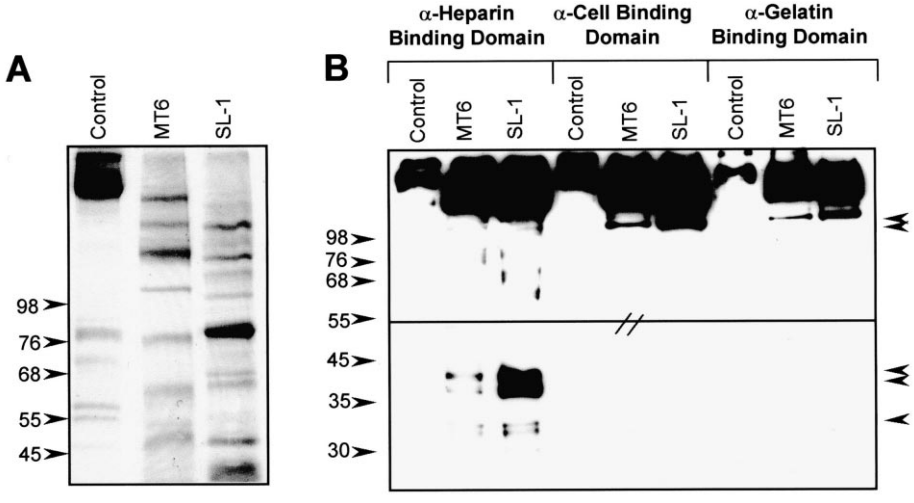

Fig. 3. Degradation of fibronectin. A: Coomassie blue-stained reducing SDS–PAGE analysis of the degradation of 20 μg fibronectin incubated in assay buffer overnight (18 h) at 25°C alone (control), with 250 ng MT6-MMP catalytic domain (MT6) or 450 ng stromelysin-1 (SL-1). B: Immunoblot of the degradation products produced in the reactions shown in A probed with mouse anti-human monoclonals to the heparin, cell and gelatin binding domains of fibronectin, visualised using the enhanced chemiluminescence method. The blot is split in two as indicated as different exposure times were required to detect the larger and smaller products. Arrows indicate the major products detected.

suggest that activity of MT6-MMP may require tight regulation in vivo.

3.4. Hydrolysis of extracellular matrix components by the MT6-MMP catalytic domain

As the peptide and hydroxamate inhibitor specificity of MT6-MMP suggested that it is a stromelysin-like enzyme, hydrolysis of ECM components known to be substrates for stromelysin-1 [1] by MT6-MMP was also examined. By following cleavage of ECM components using equal amounts of active MT6-MMP and stromelysin-1, as determined by active site titration by TIMP-1, approximate comparisons of the relative ability of each enzyme to cleave macromolecular substrates could be made. Exceptionally, MT6-MMP was able to cleave gelatin at a comparable rate to stromelysin-1 (data not shown). However, the products generated by MT6-MMP did not have the same mobility on SDS–PAGE as those generated by stromelysin-1 (Fig. 2A). Like stromelysin-1, MT6-MMP was able to hydrolyse type-IV collagen, although after 24 h at 25°C, the products were of a significantly larger size at the same enzyme:substrate ratio (Fig. 2B). A comparison of the relative abilities of the two enzymes to cleave this substrate showed MT6-MMP to be in the order of 2–3-fold faster at degrading type-IV collagen (data not shown). This activity of MT6-MMP may be of particular significance in view of the low temperature at which cleavage is seen. Recent studies have shown that gelatinase A or B (previously named 72 and 92 kDa type-IV collagenase) cleave type-IV collagen in a temperature sensitive manner, with little or no cleavage occurring at temperatures below 30°C, whereas stromelysin-1 efficiently cleaves type-IV collagen at 25°C. The suggestion is that solubilised type-IV collagen is not always wholly native at temperatures higher than 30°C and has led to the conclusion that the type-IV collagenase activity of gelatinase A and B may not be of such significance in vivo [30]. This makes the activity of MT6-MMP towards this component of the basement membrane particularly interesting. However, the activity of MT6-MMP does not extend to all components of the basement membrane as MT6-MMP was not able to show any significant activity towards laminin-I (Fig. 2C). MT6-MMP was able to cleave the Aα and Bβ chain of fibrinogen (Fig. 2D) and a comparison of the rate of cleavage with stromelysin-1 showed that they were producing the same cleavage pattern although MT6-MMP was approximately 3-fold slower (data not shown). In contrast, the catalytic domain of MT6-MMP appeared more effective at hydrolysing cross-linked fibrin than stromelysin-1, generating fragments of 50 and 40 kDa, whereas stromelysin-1 had only generated the 50 kDa fragment after 18 h (Fig. 2E and data not shown). On examining the cleavage of another ECM component, fibronectin, MT6-MMP and stromelysin-1 appeared to generate a variety of different fragments when visualised by Coomassie blue after SDS–PAGE (Fig. 3A). Analysis of the cleavage after an 18 h incubation, using monoclonal antibodies to the cell, gelatin and heparin binding domains of fibronectin, revealed that MT6-MMP and stromelysin-1 both predominantly release the heparin binding domain, although MT6-MMP appeared less effective over the same time scale (Fig. 3B). As a study by Hotary et al. [7] has recently shown that the MT-MMPs and not the soluble MMPs are the most effective at aiding cellular invasion of basement membranes, the ability of MT6-MMP to degrade components of the ECM and in particular the basement membrane protein type-IV collagen may aid the extravasation of neutrophils and the growth and metastasis of brain tumours.

3.5. Activation of progelatinase B (MMP9), procollagenase-2 (MMP8)

As stromelysin-1 has been shown to activate progelatinase B in vitro, the ability of MT6-MMP to activate progelatinase B was also investigated. MT6-MMP was not found to process progelatinase B, nor was it able to complete the processing of the intermediate form generated by MMP19 ([27], data not shown). Pei described that mRNA for MT6-MMP was found highly expressed in neutrophils [23], hence activation of neutrophil procollagenase-2 was also examined and again MT6-MMP was unable to process or activate this pro-enzyme (data not shown).

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