Cellular activation of proMMP-13 by MT1-MMP depends on the C-terminal domain of MMP-13

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Abstract Procollagenase-3 (proMMP-13) can be activated by soluble or cell associated membrane type matrix metalloproteinase 1 (MT1-MMP). In this study we show that the cell based activation of proMMP-13 by MT1-MMP was dependent on the C-terminal domain, as $\Delta_{249-451}$ proMMP-13, which lacks the haemopexin domain, and a chimaera from N-terminal MMP-13 and C-terminal MMP-19 (proMMP-13/19) were not processed by MT1-MMP expressing cells. Only the initial cleavage at Gly^{35}–Ile^{36} was dependent on MT1-MMP activity, as conversion to the active enzyme (Tyr^{85} N-terminus) required a functional MMP-13 active site. Unlike proMMP-2 activation, this process was independent of tissue inhibitor of metalloproteinase-2 (TIMP-2) as MT1-MMP expressing cells from the *TIMP-2−/−* mouse efficiently activated proMMP-13.
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Key words: Activation; Procollagenase-3; Membrane type matrix metalloproteinase 1; TIMP-2 null mouse; Progelatinase A; Tissue inhibitor of metalloproteinase-2

1. Introduction

Human procollagenase-3 (proMMP-13) is a member of the collagenase subfamily of matrix metalloproteinases (MMPs) and is expressed in breast tumours, hypertrophic chondrocytes and skin fibroblasts in vivo [1–3]. One important mechanism for the regulation of the collagenolytic activity of MMP-13 in vivo is the activation of the respective proenzyme by extracellular events. MMP-13 is an inactive proenzyme [4,5], therefore the unravelling of physiologically relevant activation pathways represents an important topic of investigation. We have shown that active MMP-3, gelatinase A (MMP-2) and membrane type MMP (MT1-MMP) mediate proMMP-13 activation in vitro and in cell model systems [6]. It is, however, not clear to date which domains of proMMP-13 contribute to the MT1-MMP driven activation of the proenzyme at the cell surface and whether there is a contribution of tissue inhibitor of metalloproteinase-2 (TIMP-2) in this process. The activation of proMMP-2 by cell bound MT1-MMP involves the establishment of a complex between TIMP-2 and MT1-MMP that forms a 'receptor' which binds the C-terminal domain of proMMP-2 to the C-terminal domain of TIMP-2 [7]. In this study we assess the role of the C-terminal domain of proMMP-13 and of TIMP-2 in regulating MT1-MMP dependent activation of proMMP-13 at the cell surface. In conjunction with data evaluating the significance of autolytic proteolysis of proMMP-13 in this process we extend our knowledge of cellular activation of proMMP-13 by MT1-MMP.

2. Materials and methods

2.1. Expression and purification of recombinant MMPs and TIMPs

proMMP-13, the C-terminal deletion mutant of MMP-13 ($\Delta_{249-451}$ proMMP-13), proMMP-2, TIMP-1 and TIMP-2 were purified as published [4,8–10]. A chimaeric enzyme was constructed from N-terminal MMP-13 and C-terminal MMP-19 (proMMP-13/19) by ligating the *Xcm*I to *Eco*RI C-terminal domain fragment of MMP-19 into the pEE12 vector carrying the coding sequences of the N-terminal domain of proMMP-13. Stable NS0 cell lines were generated and proMMP-13/19 was purified using SP-Sepharose [4].

2.2. Generation of pEE12 E^{205}-A proMMP-13

A pEE12 expression vector for an inactive mutant of proMMP-13 (E^{205}-A) was generated by extension mutagenesis. Two PCR products were generated using the mutagenic primers: 5′-GTGGCCGAATGCATGCGCAGCAACAAGAAACAAG-3′ and 5′-CTGCGCATGCATTCGGCCACTCCTTAGGTCTTG-3′, in conjunction with two vector primers and proMMP-13 cDNA as the template. The PCR products were overlap extended using vector primers and the product was cleaved with *Hin*dIII and ligated into pEE12. The cDNA sequence was verified by dideoxy sequencing.

2.3. Cellular model systems to investigate the activation of proMMP-13 by MT1-MMP overexpressing cells

2.3.1. HT1080 cells constitutively overexpressing MT1-MMP. HT1080 cells transfected with MT1-MMP in the HCMV/gpt vector, pGWIH9, were from British Biotechnology [7]. They make 1.5 pmol MT1-MMP per mg of membrane protein or vector control cells (0.13 pmol MT1-MMP per mg membrane protein) and were cultured as described [7].

2.3.2. Inducible MT1-MMP overexpressing HTC75 fibrosarcoma cells. The HTC75 cell line, which carries the pTET off control element, was transfected with the human MT1-MMP cDNA in pTRE and the pSV2neo plasmid as a selective marker [11,12]. proMMP-13

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Abbreviations: MMPs, matrix metalloproteinases; TIMPs, tissue inhibitor of metalloproteinases; proMMP-13, procollagenase-3; E^{205}-A proMMP-13, inactive point mutant of proMMP-13; $\Delta_{249-451}$ proMMP-13, C-terminal deletion mutant of MMP-13; proMMP-13/19, chimaeric MMP-13 constructed from N-terminal proMMP-13 and C-terminal MMP-19; MMP-2, gelatinase A; MT1-MMP, membrane type matrix metalloproteinase 1

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activation was performed following induction of MT1-MMP expression for 24 h in doxycycline free medium. Cells produce >10 times the endogenous level of MT1-MMP [12].

2.3.3. MT1-MMP overexpressing fibroblasts from the TIMP-2−/− mice. Immortalised *TIMP-2−/−* mouse fibroblasts [13,14] were transfected with *Pvu*I linearised MT1-MMP cDNA in pcDNA3.1 zeo(+) and stable cell lines were established following selection with 250 μg/ml zeocin in Dulbecco's modified Eagle's medium (DMEM), 10% foetal calf serum and 2 mM glutamine.

2.4. Cellular activation experiments

Cells were seeded into 24 well plates at a density of 5×10^4 cells/well and grown for 24 h in each appropriate medium. Each well was supplemented with 100 ng purified proMMP-13 in 300 μl serum-free DMEM and, where desired, some wells were complemented with TIMP-1 and TIMP-2, or the serine proteinase inhibitor aprotinin in order to analyse their role in cellular activation. Alternatively, activation experiments were also performed by transfection of pEE12 proMMP-13 and E^{205}-A proMMP-13 (0.5 μg/well) into the MT1-MMP overexpressing cell lines using FuGENE 6. After 24 h, the medium was replaced with 300 μl/well serum free medium and incubated for a further 24 h.

2.5. Western blotting

A 20 μl aliquot of medium was removed and analysed on 10% SDS–PAGE followed by Western blotting. Blots were incubated with an antiserum to proMMP-13 and visualised using a peroxidase conjugated secondary antibody and chemiluminescent substrate.

3. Results

3.1. Characterisation of proMMP-13 activation by HT1080 and HTC75 cells expressing MT1-MMP

HT1080 cells expressing MT1-MMP were supplemented with recombinant proMMP-13 and incubated for 24 h to analyse proMMP-13 activation. proMMP-13 was activated via an intermediate 56 kDa to the final active 48 kDa form (Fig. 1, lane 5), while vector control cells were unable to activate proMMP-13 (Fig. 1, lane 1). Activation was not affected by the serine proteinase inhibitor aprotinin (lane 6), and partially inhibited by TIMP-1 (lane 7). TIMP-2 inhibited activation efficiently since it is a potent inhibitor of MT1-MMP (lane 8). Coexpression of proMMP-13 in HTC75 cells expressing MT1-MMP also resulted in efficient activation (Fig. 2, lane 2) and we frequently observed fragmentation products of M_r 29 kDa and M_r 27 kDa in these experiments which represent the catalytic and C-terminal domains of MMP-13 respectively (Fig. 2, lane 2, low molecular mass bands). This indicated that proMMP-13 activation in HTC75 cells was more efficient, as MMP-13 fragmentation only occurs at relatively high concentrations of active enzyme [8]. These MT1-MMP expressing cell models allowed us to establish the role of the C-terminal domain and the active site in proMMP-13 activation by cell surface MT1-MMP.

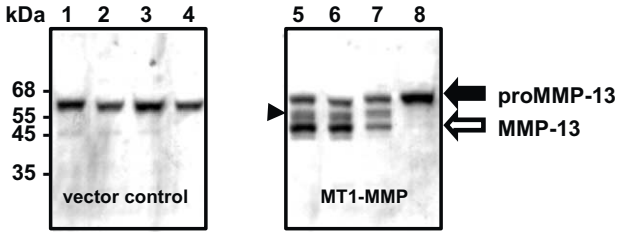

Fig. 1. Activation of proMMP-13 by HT1080 cells transfected with MT1-MMP. Western blot developed with an antibody to proMMP-13. Lane 1, HT1080 cells transfected with vector supplemented with 5.5 nM proMMP-13 were incubated for 24 h at 37°C; lane 2, as lane 1 with 2 μM aprotinin; lane 3, as lane 1 with 11 nM TIMP-1; lane 4, as lane 1 with 15 nM TIMP-2; lane 5, HT1080 cells transfected with MT1-MMP supplemented with 5.5 nM proMMP-13; lane 6, as lane 5 with 2 μM aprotinin; lane 7, as lane 5 with 11 nM TIMP-1; lane 8, as lane 5 with 15 nM TIMP-2. Bands corresponding to proMMP-13 (black arrow), active MMP-13 and the activation intermediate (arrowhead) are indicated.

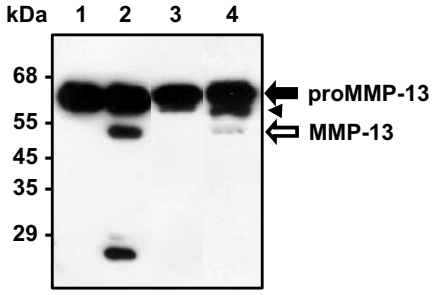

Fig. 2. Processing of E^{205}-A proMMP-13 by MT1-MMP expressing cells. HTC75 cells transfected with pEE12 proMMP-13 or the inactive E^{205}-A proMMP-13 mutant were incubated at 37°C for 24 h following induction of MT1-MMP expression. Lane 1, non-induced proMMP-13 transfected; lane 2, induced proMMP-13 transfected; lane 3, non-induced E^{205}-A proMMP-13 transfected; lane 4, induced E^{205}-A proMMP-13 transfected. Protein bands corresponding to proMMP-13, active MMP-13 and the activation intermediate are indicated on the right. A C-terminal fragmentation product is observed in lane 2, band corresponding to M_r 27 kDa.

3.2. Cellular activation of proMMP-13 is a two step event

In order to assess whether the secondary cleavage event observed during proMMP-13 activation (Fig. 1, lanes 5, 6 and 7) was autoproteolytic we transfected HTC75 cells with pEE12 E^{205}-A proMMP-13 and analysed activation by Western blotting. E^{205}-A proMMP-13 was processed mainly to the 56 kDa intermediate form (Fig. 2, lane 4, arrowhead), with minute amounts of active enzyme detectable, suggesting that efficient conversion to the 48 kDa form required catalytically competent MMP-13. The small amounts of active form may be due to the action of MMP-2, which is less efficient. In contrast, wild type proMMP-13 was efficiently converted to the 48 kDa active form which underwent further fragmentation (Fig. 2, lane 2) [8].

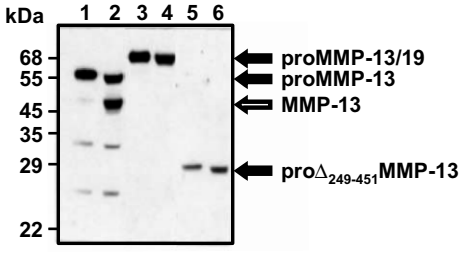

Fig. 3. C-terminal domain dependent activation of proMMP-13 by MT1-MMP membranes. proMMP-13, a chimaeric proMMP-13/19 mutant and $\Delta_{249-451}$ proMMP-13 at concentrations of 5.5 nM were incubated with MT1-MMP containing membranes for 16 h at 37°C. Lane 1, proMMP-13 buffer control; lane 2, proMMP-13 with MT1-MMP membranes; lane 3, proMMP-13/19 buffer control; lane 4, proMMP-13/19 with MT1-MMP membranes; lane 5, $\Delta_{249-451}$ proMMP-13 buffer control; lane 8, $\Delta_{249-451}$ proMMP-13 with MT1-MMP membranes. The protein bands corresponding to proenzymes are indicated with arrows.

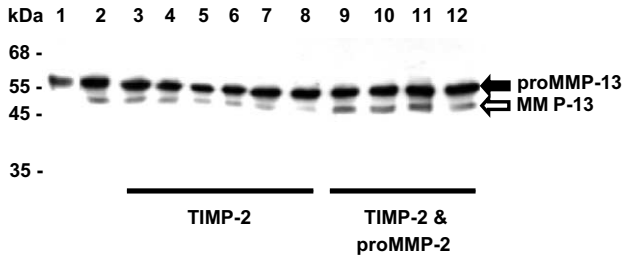

Fig. 4. Activation of proMMP-13 by *TIMP-2−/−* mouse fibroblasts expressing MT1-MMP. *TIMP-2−/−* mouse fibroblasts expressing MT1-MMP were transfected with proMMP-13 and incubated for 24 h with different concentrations of TIMP-2. Lane 1, vector control cells; lane 2, MT1-MMP transfected cells; lanes 3–8, MT1-MMP transfected cells incubated with 1 nM, 5 nM, 10 nM, 20 nM, 40 nM and 80 nM TIMP-2. Lanes 9 and 10, MT1-MMP transfected cells incubated with 1 nM TIMP-2 and 1.7 nM proMMP-2 (lane 9) and 3.5 nM proMMP-2 (lane 10); lanes 11 and 12, MT1-MMP transfected cells incubated with 5 nM TIMP-2 and 1.7 nM proMMP-2 (lane 11) and 3.5 nM proMMP-2 (lane 12). Protein bands corresponding to proMMP-13 and active MMP-13 are indicated on the right.

3.3. *C-terminal domain dependent activation of proMMP-13 by membrane associated MT1-MMP*

To determine whether the C-terminal domain of proMMP-13 regulates MT1-MMP activation, as described for proMMP-2, we investigated whether a chimaeric proenzyme proMMP-13/19 or a C-terminal deletion mutant of proMMP-13 ($\Delta_{249-451}$ proMMP-13) were activated by MT1-MMP containing membranes. Only wild type proMMP-13 was processed to the fully active enzyme (Fig. 3, lanes 2 and 4), while proMMP-13/19 (lane 5) and $\Delta_{249-451}$ proMMP-13 (lane 7) were not activated. Identical results were obtained when cells transfected with MT1-MMP were employed to activate wild type and mutant proMMP-13 preparations (not shown). These results indicate that the C-terminal domain of proMMP-13 is essential for cellular activation by MT1-MMP.

3.4. *TIMP-2 independent processing of proMMP-13 by MT1-MMP expressing cells from the TIMP-2−/− mouse*

To address whether the TIMP-2 component of the MMP-2 activation cascade was required for direct proMMP-13 activation we performed experiments using cells derived from the *TIMP-2−/−* mouse, which express MT1-MMP. proMMP-13 activation proceeded efficiently in the absence of TIMP-2 (Fig. 4, lane 2). Addition of TIMP-2, at concentrations that would potentiate MMP-2 activation, inhibited proMMP-13 activation. Addition of proMMP-2 to the cells in the presence of TIMP-2 potentiated proMMP-13 activation (Fig. 4) [6,5].

4. Discussion

We have previously characterised the activation of proMMP-13 by MT1-MMP in solution and in cell model systems such as concanavalin A stimulated fibroblasts or cytokine and phorbol myristate acetate stimulated SW1353 chondrosarcoma cells [5,6]. Here we have examined the potential parallels between cell based proMMP-13 activation and that elucidated for proMMP-2. Using HT1080 cells overexpressing MT1-MMP we showed that proMMP-13 (60 kDa) was processed via a 56 kDa intermediate to the fully active 48 kDa active enzyme and that production of the intermediate was TIMP-1 insensitive. The initial cleavage to the 56 kDa intermediate (Ile^{36} N-terminus) was mediated by MT1-MMP activity. This was confirmed using catalytically inactive E^{205}-A proMMP-13 which was processed by MT1-MMP to the 56 kDa intermediate. Further processing to the 48 kDa active enzyme required a functional active site, i.e. the secondary cleavage is autoproteolytic. This resembles the activation of proMMP-2 by MT1-MMP or MT2-MMP which also requires an initial cleavage by either MT-MMP within the propeptide domain of proMMP-2 prior to autoproteolytic cleavage generating fully active enzyme [15,16].

The activation of proMMP-13 by cell associated MT1-MMP required the presence of its C-terminal domain, since a C-terminal domain deletion mutant or a form of the enzyme where the C-terminal domain had been replaced with that of MMP-19 were not activated. These findings resemble data on proMMP-2 activation by MT1-MMP and MT2-MMP, which depends on the C-terminal domain of MMP-2 [17,18]. MT1-MMP mediated activation of proMMP-2 requires the establishment of a trimolecular complex between MT1-MMP, TIMP-2 and proMMP-2 where the C-terminal domain of TIMP-2 interacts with the C-terminal domain of proMMP-2 [19]. This is reflected in the strength of the MMP-2 and TIMP-2 inhibitor interactions [10].

We established the role of TIMP-2 in the cell-based MT1-MMP activation of proMMP-13 relative to proMMP-2. Fibroblasts derived from *TIMP-2−/−* mice expressing MT1-MMP activated proMMP-13, in contrast to proMMP-2 which cannot be processed in these cells [13,20]. We can conclude that the C-domain of proMMP-13 is TIMP-2 independent and that proMMP-13 activation by MT1-MMP does not involve the establishment of a trimolecular complex between proMMP-13, MT1-MMP and TIMP-2. Our data reflect the results from the recent X-ray analysis of the proMMP-2/TIMP-2 complex [21]. As the majority of the residues involved in hydrophobic and electrostatic interactions at the complex interphase are not conserved in the C-terminal domain of proMMP-13, the highly positively charged lysine cluster (residues 547–575) at the end of blade 3 of the C-terminal β-propeller is absent in MMP-13, underlining why TIMP-2 fails to potentiate proMMP-13 activation by MT1-MMP. The addition of low doses of TIMP-2, which potentiate proMMP-2 activation by the MT1-MMP transfected fibroblasts from the *TIMP-2−/−* mice, had a small but detectable effect on the amounts of proMMP-13 being activated, indicating that this was probably mediated by MMP-2 activity. Furthermore, we were able to detect some fully processed MMP-13 in experiments where we transfected the cells with the E^{205}-A proMMP-13 mutant. We conclude that MMP-2 plays an auxiliary role in converting the intermediate form (56 kDa) into the fully active form.

Studies by Morrison et al. [18] showed that cellular activation experiments with a C-terminal domain deletion mutant of proMMP-2 showed complete lack of activation and cell surface association and that the MT2-MMP mediated activation of proMMP-2 is only partially inhibited by the C-terminal domain of MMP-2 (500-fold molar excess) [18]. We obtained similar data for proMMP-13 activation by MT1-MMP expressing cells (not shown) and we hypothesise that, although the C-terminal domain is required for activation, the interactions involved are weak and that other domains of proMMP-13 may also contribute to allow processing by MT1-MMP. In cross-linking studies on HTC75 cells overexpressing MT1-

MMP exposed to exogenous MMP-13, we were unable to identify any MMP-13 complexes by either immunoblotting or immunoprecipitation (data not shown). We have recently ruled out that Endo180/urokinase type plasminogen activator receptor associated protein is a MMP-13 'receptor' in MT1-MMP activation of proMMP-13 [22] and thus other binding or interacting partners may be involved in this process. The nature of the binding partner for proMMP-13 binding to the cell surface allowing MT1-MMP mediated processing has yet to be established.

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