Membrane type-1 matrix metalloproteinase activity is regulated by the endocytic collagen receptor Endo180

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Summary

The molecular interactions leading to organised, controlled extracellular matrix degradation are of central importance during growth, development and tissue repair, and when deregulated contribute to disease processes including cancer cell invasion. There are two major pathways for collagen degradation: one dependent on secreted and membrane-bound collagenases, the other on receptor-mediated collagen internalisation and intracellular processing. Despite the established importance of both pathways, the functional interaction between them is largely unknown. We demonstrate here, that the collagen internalisation receptor Endo180 (also known as CD280, uPARAP, MRC2) is a novel regulator of membrane-bound matrix metalloproteinase (MT1-MMP) activity, MT1-MMP-dependent MMP-2 activation and urokinase plasminogen activator (uPA) activity. We show close correlation between Endo180 expression, collagen accumulation and regulation of MT1-MMP cell-surface localisation and activity. We directly demonstrate, using collagen inhibition studies and non-collagen-binding mutants of Endo180, that the molecular mechanism underlying this regulation is the ability of Endo180 to bind and/or internalise collagens, rather than by acting as an interaction partner for pro-uPA and its receptor uPAR. These studies strongly support a functional interaction between two distinct collagen degradation pathways, define a novel mechanism regulating MT1-MMP activity and might have important implications for organised collagen clearance in the pericellular environment.

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Key words: Endo180, Mannose receptor family, MT1-MMP, Membrane type-1 matrix metalloproteinase, Extracellular matrix, ECM

Introduction

Collagens are abundant, ubiquitous protein constituents of the extracellular matrix (ECM). They play essential roles in wound healing and the growth with differentiation and morphogenesis of tissues and in controlling vital cellular functions such as migration, adhesion and signalling (Leitinger and Hohenester, 2007). The coordinated synthesis, deposition and degradation of collagens is essential for physiological tissue homeostasis, and its deregulation underpins inflammatory and fibrotic diseases and tumour growth and metastasis (Perez-Tamayo, 1978).

Collagen remodelling occurs via two distinct pathways. The first pathway is extracellular and/or pericellular collagen degradation by matrix metalloproteinases (MMPs), of which, membrane-anchored membrane-type-1 matrix metalloproteinase (MT1-MMP, also known as MMP-14), is a key collagenolysin (Holmbeck et al., 1999; Sato et al., 1994; Zhou et al., 2000). MT1-MMP directly degrades ECM proteins including collagens I-IV and is also a potent activator of latent proMMP-2, which in the active form degrades gelatin and collagens I and IV (Butler et al., 1998; Ioh and Seiki, 2006; Strongin et al., 1995). In vivo, MT1-MMP collagenolysis is essential for post-natal connective tissue remodelling (Holmbeck et al., 1999; Zhou et al., 2000) and for tumour formation, invasion and metastasis (Hotary et al., 2003; Sabeh et al., 2004; Seiki et al., 2003; Szabova et al., 2008; Wolf et al., 2007). MT1-MMP activity is stringently regulated by both endogenous inhibitors, TIMPs (tissue inhibitors of metalloproteinase) (Brew et al., 2000), and by transcriptional and post-translational mechanisms, including autocatalytic processing (Lehti et al., 1998; Rozanov et al., 2001).

The second collagen degradation pathway requires specific binding and cellular uptake of collagen for delivery to intracellular degradative compartments. This occurs via α2β1-integrin-mediated phagocytic uptake, which is considered the primary pathway in the remodelling of adult tissues (Everts et al., 1996). Recently, a novel collagen-binding and collagen-internalisation receptor, Endo180 (endocytic receptor 180) was identified. Endo180 is a constitutively recycling receptor that binds collagens and internalises them via clathrin-coated pits into early endosomes for lysosomal degradation (Engelholm et al., 2003; Kjoller et al., 2004; Wienke et al., 2003). Moreover, recent in vivo studies identified the importance of Endo180 in ECM remodelling during post-natal bone development (Wagenaar-Miller et al., 2007) and in tumour progression (Curino et al., 2005; Wienke et al., 2007).

Despite the established importance of both these pathways, the relationship between them is virtually unexplored. Studies to date have focused on how MT1-MMP-mediated limited fragmentation of collagen might influence uptake by Endo180 (Madsen et al., 2007; Wagenaar-Miller et al., 2007). By contrast, here we have explored whether Endo180 can regulate protease activity and thus direct pericellular collagen proteolysis.
Results and Discussion
Endo180 regulates protease activity

To investigate whether Endo180 is involved in regulating protease activity, HT1080 human fibrosarcoma cells were treated with Endo180-targeting siRNA oligonucleotides (Fig. 1A). Initially, MMP-2 activity was examined directly by monitoring the activation of proMMP-2 using gelatin zymography.

A striking increase in MMP-2 activity was observed following Endo180 depletion, evidenced by substantially increased conversion of proMMP-2 to the active enzyme in both cell lysates and conditioned media (Fig. 1B). This increased protease activity was not an off-target effect as an Endo180 siRNA SMARTpool and three independent Endo180-targeting oligonucleotides all effectively decreased Endo180 expression (Fig. 1A) and produced an identical phenotype (Fig. 1B). Moreover, this phenotype was rescued by expression of murine wild-type Endo180 (shown below). Additionally, Endo180 depletion did not affect proMMP-9 activation (supplementary material Fig. S1). Finally, proMMP-2 activation cannot be attributed simply to downregulation of an endocytic receptor because siRNA-induced downregulation of transferrin receptor had no effect on proMMP-2 activation (supplementary material Fig. S2).

Endo180 can interact with pro-uromodulin plasminogen activator (pro-uPA) bound to its GPI-anchored receptor, uPAR (Behrendt, 2004; Behrendt et al., 2000). Pro-uPA binds uPA, allowing for conversion to active uPA, and because both uPA and plasmin (generated from plasminogen by uPA) can activate proMMP-2 directly and indirectly via MT1-MMP (Kazes et al., 1998; Keski-Oja et al., 1992; Mazzieri et al., 1997; Monea et al., 2002) we considered the Endo180-uPAR-uPA system to be a potential regulatory component involved in MMP-2 activation. Consistently, a significant increase in uPA activity was observed in the conditioned media of Endo180-depleted cells (Fig. 1C,D). However, treatment with specific uPA inhibitors or uPA siRNA at concentrations that significantly reduced uPA activity (Fig. 1E) failed to inhibit proMMP-2 activation following Endo180 downregulation (Fig. 1F, supplementary material Fig. S3A,B). Likewise, the plasmin inhibitor aprotinin was ineffective (not shown). Thus, regulation of MMP-2 activity by Endo180 is independent of uPA-uPAR and the plasminogen cascade. Conversely, the broad-spectrum MMP...
inhibitor GM6001 completely abrogated proMMP-2 activation (Fig. 1F), indicating a requirement for MMP activity and suggesting that regulation of MMP-2 activity by Endo180 is most probably MT1-MMP-dependent.

To confirm that increased MMP-2 activation occurred as a consequence of the ability of Endo180 to regulate MT1-MMP activity, cells were treated with siRNAs against Endo180 and MT1-MMP. Two separate MT1-MMP siRNAs substantially reduced MT1-MMP expression (Fig. 2A) and prevented the small, basal level of proMMP-2 activation (Fig. 2B) (Ueda et al., 2003). Other MT-MMP family members besides MT1-MMP can activate proMMP-2. Quantitative RT-PCR confirmed that the MT1-MMP siRNAs only targeted MT1-MMP, and not any other MT-MMPs (MT2-MMP to MT6-MMP, not shown). Endo180 siRNA treatment alone or in conjunction with control MT1-MMP siRNA, again resulted in pronounced activation of MMP-2 (Fig. 2B,C). Conversely, activation of MMP-2 was completely abrogated when cells were treated with both Endo180- and MT1-MMP–siRNA-targeting oligonucleotides (Fig. 2B,C), in agreement with data obtained using the MMP inhibitor GM6001 (Fig. 1F). Moreover, as increased uPA activity following Endo180 downregulation is significantly reduced when MT1-MMP is also downregulated (supplementary material Fig. S3C), it is probable that regulation of uPA activity occurs as a secondary consequence of increased MT1-MMP and MMP-2 activity (Prager et al., 2004).

**Endo180 regulates the autolysis and cell-surface level of MT1-MMP**

As reported for other cell types (Galvez et al., 2002; Sheikh et al., 2000), both MT1-MMP and Endo180 are localised in a punctate distribution at the surface of HT1080 cells and in intracellular endosomes, particularly in the perinuclear region (Fig. 3A). Only limited colocalisation of MT1-MMP and Endo180 was observed (Fig. 3A arrows). To address the mechanism by which Endo180 expression influences MT1-MMP activity, we investigated MT1-MMP expression and localisation following Endo180 downregulation. Immunoblot analysis of whole-cell lysates revealed a significant accumulation of the 43-45 kDa autolytic MT1-MMP fragment in Endo180-depleted cells (Fig. 3B,C), directly confirming increased MT1-MMP activity (Lehti et al., 1998). However, the level of mature (~60 kDa) MT1-MMP was unaffected by Endo180 downregulation, even in the presence of GM6001 to prevent increased MT1-MMP autolysis (Fig. 3C). Consistently, no change in MT1-MMP mRNA level was detected (supplementary material Fig. S4; Klingbeil et al., 2009). These data suggest that enhanced MT1-MMP activity and proMMP-2 activation might be mediated by an increased level of cell-surface-active MT1-MMP following Endo180 downregulation. To address this, cell-surface expression of MT1-MMP was analysed by FACS using a mAb directed against the MT1-MMP catalytic domain that recognises active (~60 kDa) MT1-MMP but not the 44 kDa autolysis product (Galvez et al., 2002). These experiments were performed in the presence of GM6001 to facilitate detection of active MT1-MMP, independent of increased autolys. A significantly increased cell-surface level of active MT1-MMP was observed following Endo180 depletion (Fig. 3D), suggesting that Endo180 expression can regulate MT1-MMP membrane localisation and consequent enzyme activity.

**Regulation of MT1-MMP activity requires the collagen binding and/or internalisation function of Endo180**

Endo180 binds and internalises collagens in vitro (Engelholm et al., 2003; Kjoller et al., 2004; Wienke et al., 2003) and native collagens in vivo (Curino et al., 2005; Wagenaar-Miller et al., 2007; Wienke et al., 2007). MT1-MMP activity and MMP-2 activation are induced by collagen in several cell types, including HT1080 cells. Several mechanisms have been proposed to underlie the collagen-mediated increase in MT1-MMP activity involving both transcriptional and non-transcriptional pathways, including increased cell-surface expression of MT1-MMP (Ellerbroek et al., 1999; Lafleur et al., 2006; Maquoi et al., 2000; Ruangpanit et al., 2001; Tam et al., 2002). This raised the possibility that the collagen binding and/or internalisation function of Endo180 directly underpins its ability to control MT1-MMP activity. Consequently, we first confirmed that Endo180 in HT1080 cells, as in other cells, functions to bind and internalise collagens (supplementary material Fig. S5). Next, the turnover of collagen was investigated using a Sircol assay, which detects collagen types I to V (Fineschi et al., 2001; Tam et al., 2002). These experiments were performed in the presence of GM6001 to prevent increased MT1-MMP autolysis (Fig. 3C). Consistently, no change in MT1-MMP mRNA level was detected (supplementary material Fig. S4; Klingbeil et al., 2009). These data suggest that enhanced MT1-MMP activity and proMMP-2 activation might be mediated by an increased level of cell-surface-active MT1-MMP following Endo180 downregulation. To address this, cell-surface expression of MT1-MMP was analysed by FACS using a mAb directed against the MT1-MMP catalytic domain that recognises active (~60 kDa) MT1-MMP but not the 44 kDa autolysis product (Galvez et al., 2002). These experiments were performed in the presence of GM6001 to facilitate detection of active MT1-MMP, independent of increased autolys. A significantly increased cell-surface level of active MT1-MMP was observed following Endo180 depletion (Fig. 3D), suggesting that Endo180 expression can regulate MT1-MMP membrane localisation and consequent enzyme activity.

**Fig. 2.** Loss of MT1-MMP reverses MMP-2 activation following Endo180 downregulation. (A) HT1080 cells were transfected with MT1-MMP or control siRNAs, lysed 48 hours later and immunoblotted for MT1-MMP and α-tubulin. (B,C) HT1080 cells transfected with Endo180 or MT1-MMP control or targeting siRNA, alone or in combination, were cultured as in Fig. 1A-D. (B) Gelatin zymogram of conditioned media. (C) Active MMP-2 levels were quantified as for Fig. 1B; **P<0.001 (n=6).
MT1-MMP activity is regulated by Endo180 (2006). As shown in Fig. 4A, increased collagen levels accumulated in the conditioned media of Endo180-siRNA-treated cells compared with control cells. These data are consistent with in vivo studies where loss of Endo180 (Curino et al., 2005) or expression of an internalisation-defective Endo180 mutant (Wienke et al., 2007) led to increased tumour-associated collagens. To determine whether misregulation of collagen turnover was important for increased MT1-MMP activity, we used the collagen synthesis inhibitor AzC (azetidine-2-carboxylic acid), a proline analogue that incorporates into the collagen polypeptide but cannot be hydroxylated (Maehata et al., 2006). AzC prevented the increased collagen accumulation following Endo180 downregulation (Fig. 4B) and, importantly, inhibited both the increased level of cell-surface active MT1-MMP and MMP-2 activation (Fig. 4C,D). This effect was specific, because MMP-2 activation in response to PMA (phorbol 12-myristate 13-acetate) or concanavalin A (both known inducers of MT1-MMP-dependent MMP-2 activation) was unaffected by AzC (Fig. 4E), as was PMA-induced upregulation of proMMP-9 (Fig. 4E). Additionally, AzC did not affect cell viability (supplementary material Table S2) or the expression levels of non-collagenous proteins (supplementary material Fig. S6).

To directly demonstrate that the mechanism by which Endo180 regulates MT1-MMP activity requires its collagen binding and/or
internalisation function, we performed a rescue experiment. Cells transfected with siRNAs specific for target sequences in human but not murine Endo180 were co-transfected with either murine full-length Endo180, murine N-terminal-truncated Endo180 lacking the collagen-binding domain (East et al., 2003) or empty vector (Fig. 4F). Importantly, expression in Endo180-siRNA-treated cells of wild-type murine Endo180, but not the non-collagen-binding Endo180 mutant, inhibited the collagen

Fig. 4. Regulation of MT1-MMP activity requires the collagen binding-and-uptake function of Endo180. (A) Collagen levels were analysed in conditioned media from control- or Endo180-siRNA-transfected cells and expressed relative to levels from mock-transfected cells; mean + s.d. (n=2). (B) Endo180-siRNA-transfected cells were cultured with or without AzC, or alternatively were co-transfected with murine wild-type Endo180, N-terminal-truncated Endo180 (mutant) or vector. The conditioned media were analysed for collagen; mean + s.d. relative to untreated Endo180-siRNA-transfected cells (n=2). (C) siRNA-transfected cells as indicated were cultured in the presence of GM6001 and with or without AzC for 48 hours and were analysed by FACS for active MT1-MMP (as for Fig. 3D). Data presented are relative to mock-transfected cells for each treatment (n=3). (D) Cells treated as for C in the absence of GM6001. Conditioned media were analysed for MMP-2 activation and quantified as for Fig. 1F (n=4). (E) Cells were cultured with or without AzC for 57 hours and then stimulated with concanavalin A (25 μg/ml) or PMA (100 nM) for 18 hours. The conditioned media were analysed for MMP-2 activation. (F-H) Cells were transfected with control or Endo180 siRNA plus either murine wild-type Endo180 (WT-Endo180), N-terminal-truncated Endo180 (Endo180ΔEx2-6) or vector and cultured with or without GM6001 as above. (F) Lysates were immunoblotted for Endo180, FLAG epitope and α-tubulin. (G) Cells treated with GM6001 were analysed by FACS as for C; mean + s.d. relative to mock-transfected cells (n=2). (H) Conditioned media of cells in the absence of GM6001 were analysed and quantified for MMP-2 activation as for Fig. 1F; mean + s.d. (n=3).
accumulation (Fig. 4B), the increased cell-surface level of active MT1-MMP (Fig. 4G) and the activation of MMP-2 (Fig. 4H).

Conclusion
This study identifies a new role for Endo180 in the control of pericellular proteolysis in HT1080 cells through negative regulation of MT1-MMP activity and MMP-2 activation. The observation that wild-type Endo180, and not a non-collagen-binding mutant, restores the cell-surface-active MT1-MMP and MMP-2 activation to control levels in Endo180-depleted cells directly demonstrates that Endo180 suppresses MT1-MMP activity and MMP-2 activation through a mechanism that is dependent on its collagen binding and/or internalisation function. Although we could robustly immunoprecipitate both Endo180 and MT1-MMP, we were unable to demonstrate a direct physical association between these two receptors using reciprocal co-immunoprecipitation experiments (not shown), suggesting that it is unlikely that Endo180 plays a direct role in sequestering and/or clearance of MT1-MMP.

Together, these data support a model in which Endo180 expression promotes a local pericellular environment favouring reduced collagen-induced MT1-MMP activity, suggesting that Endo180 can act as a functional link between the two major pathways of collagen remodelling. However, it remains possible that uptake of collagen-associated components or Endo180-mediated signalling might impact on the regulation of proteolysis. Although MMP-2 levels in mammary tumours of wild-type and Endo180−/− mice were found not to correlate with Endo180 expression, MMP-2 activation was not addressed (Curino et al., 2005). Furthermore, these data were derived from whole-tumour explants excised at a single time-point, potentially masking any spatio-temporal differences in MT1-MMP activity.

The interplay between extracellular and specific intracellular mechanisms of proteolysis demonstrated here might be important in limiting indiscriminate matrix degradation and in coordinating the balanced level of local proteolysis required for efficient cell migration. Additionally, our data suggest that regulation of Endo180 expression might either promote local pericellular proteolysis and release of substrate degradation products, or act to preserve pericellular collagen as a substrate for cell adhesion and survival. This might be important where one collagen degradation pathway is spatially and/or temporally favoured over the other, for example to control distinct cellular behaviour; note that Endo180 is differentially expressed during early and late stages of wound healing (Honardoust et al., 2006). Moreover, Endo180 and MT1-MMP are required for collagen remodelling during post-natal bone development (Wagenaar-Miller et al., 2007) and in tumour progression (Curino et al., 2005; Wienke et al., 2007), suggesting that functional interactions between these two pathways might be important in physiological and pathological collagen remodelling. Consequently, it will be important in future studies to establish the relevance of these observations in other cell types and in complex in vivo models involving physiological and pathological collagen remodelling.

Materials and Methods
Reagents
Antibodies: anti-human MT1-MMP N175 (a gift of Gillian Murphy, CRUK, Cambridge Research Institute, UK) recognises all forms of the MT1-MMP receptor (d’Ortho et al., 1998); mAb LEM2/15.8 recognises the MT1-MMP catalytic domain (Galvez et al., 2002); anti-transferin receptor (B3/25); anti-Endo180 (A5/158) (Sheikh et al., 2000); anti-TIMP-2 (T2-N IC3, Merck Biosciences); neutralising anti-uPA (Galvez et al., 2002); anti-transferrin receptor (B3/25); anti-Endo180 (A5/158) (Sheikh et al., 2000). MT1-MMP activity is regulated by Endo180

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