MT1-MMP hemopexin domain exchange with MT4-MMP blocks enzyme maturation and trafficking to the plasma membrane in MCF7 cells

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The hemopexin-like domain of membrane-type matrix metalloproteinase-1 (MT1-MMP) enables MT1-MMP to form oligomers that facilitate the activation of pro-matrix metalloproteinase-2 (pro-MMP-2) at the cell surface. To investigate the role of the MT1-MMP hemopexin domain in the trafficking of MT1-MMP to the cell surface we have examined the activity of two MT1–MT4-MMP chimaeras in which the hemopexin domain of MT1-MMP has been replaced with that of human or mouse MT4-MMP. We show that MT1-MMP bearing the hemopexin domain of MT4-MMP was incapable of activating pro-MMP-2 or degrading gelatin in cell based assays. Furthermore, cell surface biotinylation and indirect immunofluorescence show that transiently expressed MT1–MT4-MMP chimaeras failed to reach the plasma membrane and were retained in the endoplasmic reticulum. Functional activity could be restored by replacing the MT4-MMP hemopexin domain with the wild-type MT1-MMP hemopexin domain. Subsequent analysis with an antibody specifically recognising the propeptide of MT1-MMP revealed that the propeptides of the MT1–MT4-MMP chimaeras failed to undergo proper processing. It has previously been suggested that the hemopexin domain of MT4-MMP could exert a regulatory mechanism that prevents MT4-MMP from activating pro-MMP-2. In this report, we demonstrate unambiguously that MT1–MT4-MMP chimaeras do not undergo normal trafficking and are not correctly processed to their fully active forms and, as a consequence, they are unable to activate pro-MMP-2 at the cell surface.

Key words: gelatin degradation, hemopexin domain, MT1-MMP, MMP-2 activation, MT4-MMP, propeptide.

INTRODUCTION

Matrix metalloproteinase-2 (MMP-2) is a soluble member of the zinc dependent endopeptidases that comprise the matrix metalloproteinase subgroup of the metzincin family of enzymes. It has been identified as a major player in tissue destruction and cell migration associated with a number of pathologies including tumour metastasis [1]. Like other soluble members of this family, pro-MMP-2 is secreted into the extracellular space as a latent enzyme requiring activation by proteolytic removal of the propeptide. In general this is accomplished by other MMPs or by serine proteinases such as plasmin. Pro-MMP-2, however, is not susceptible to plasmin activation and in the last decade a unique cell surface activation mechanism has been identified, which requires the formation of a trimolecular complex composed of MT1-MMP (membrane type 1-matrix metalloproteinase-1, also known as MMP-14), TIMP-2 (tissue inhibitor of metalloproteinases-2) and pro-MMP-2. It has been proposed that activation of pro-MMP-2 involves the formation of homophilic oligomers between the hemopexin-like domains of neighbouring MT1-MMP molecules [2]. This allows clustering of MT1-MMP/TIMP-2 'receptors' in close proximity to unoccupied active MT1-MMP thus facilitating the two-step, MT1-MMP initiated, cleavage of pro-MMP-2 bound to the 'receptors' [3–5]. In agreement with this hypothesis, the MT1-MMP hemopexin domain interactions that are necessary for MMP-2 activation have been demonstrated by two previous studies [6,7]. Controversially, recent studies [8,9] have challenged these findings by showing that MT1-MMP with its hemopexin domain removed could still activate MMP-2, suggesting that the requirement for this domain is critical only for other functions of MT1-MMP, such as collagenolysis and cell invasion.

Two of the former studies [2,8] used domain-exchange MT1-MMP constructs, in which the hemopexin domain was replaced with the hemopexin domain of MT4-MMP. MT4-MMP is a cell-associated member of the membrane bound MMPs that, like MT6-MMP, has a glycophosphatidylinositol anchor instead of the type-1 transmembrane domain of the other members of this group. MT4-MMP is however a poor activator of MMP-2 [10] despite the high degree of similarity between its ectodomain structure and that of all the other membrane associated MMPs. All the aforementioned studies report that MT1-MMP in which its hemopexin domain has been exchanged for that of MT4-MMP is unable to activate pro-MMP-2. This observation has led to speculation that negative regulation exerted by the hemopexin domain of MT4-MMP is a possible explanation for the inability of MT4-MMP to activate pro-MMP-2 [8].

In the course of our research aimed at elucidating the trafficking mechanisms involved in the display of MT1-MMP at the cell surface, we have also made use of domain-exchange MT1-MMP chimaeras, including MT1–hMT4-MMP (MT1-MMP in which the hemopexin domain has been replaced by that of human MT4-MMP). The present study shows that the MT1–hMT4-MMP chimaera is unable to activate pro-MMP-2 or to degrade gelatin when transiently expressed in MCF7 cells. We also demonstrate that MT1–mMT4-MMP (MT1-MMP in which the hemopexin domain has been exchanged for that of mouse MT4-MMP) is similarly unable to activate pro-MMP-2 and degrade gelatin in our cell-based assays. Furthermore, using cell surface biotinylation, immunofluorescence techniques and an antibody that specifically

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; EGFP, enhanced green flourescent protein; ER, endoplasmic reticulum; FCS, foetal calf serum; HRP, horseradish peroxidase; MMP-2, matrix metalloproteinase-2; MT1-MMP, membrane-type matrix metalloproteinase-1; MT1–hMT4-MMP, MT1-MMP substituted with human MT4-MMP hemopexin domain; MT1–mMT4-MMP, MT1-MMP substituted with mouse MT4-MMP hemopexin domain; NHS, *N*-hydroxysuccinimido; SBS, Sorensen buffer; TIMP-2, tissue inhibitor of metalloproteinases-2; wt, wild-type.

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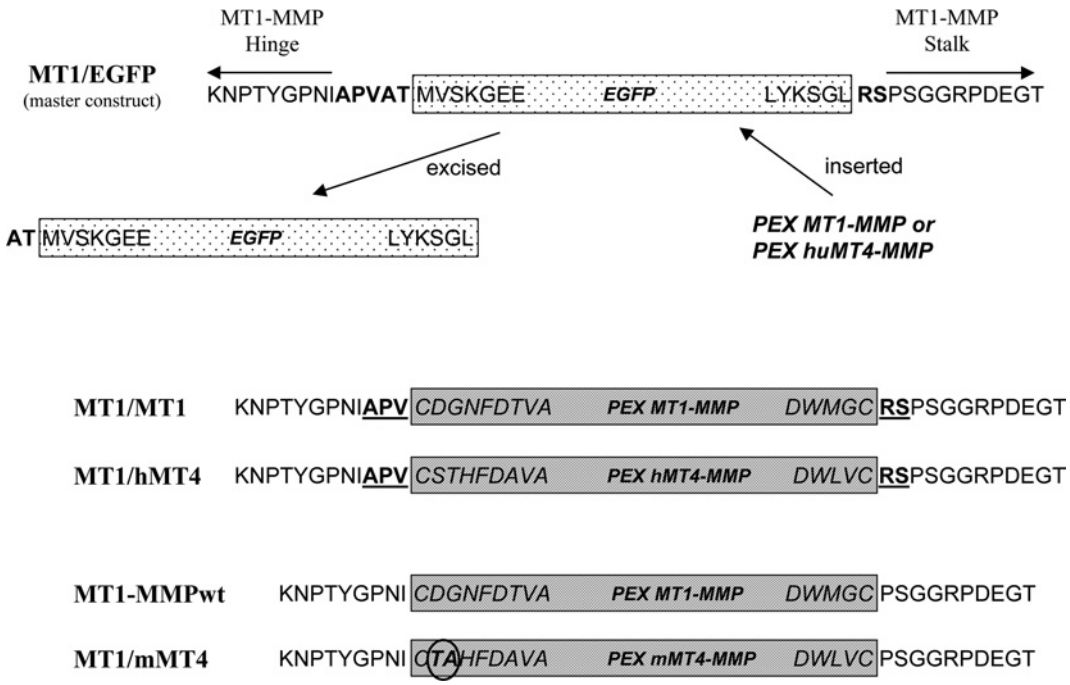

Figure 1 Schematic representation of MT1-MMP chimaeras

MT1-MMP chimaeras were generated as described in the text from the 'master construct', MT1–EGFP, as shown. Shaded areas represent regions replacing MT1-MMP hemopexin domain that were inserted between the MT1-MMP hinge region (separating the catalytic and hemopexin domains) and the stalk region preceding the transmembrane domain. Residues shown in bold type and underlined are additional 'linkers' generated during construction of hemopexin domain substitutes from the master construct, as described in the Experimental section. Circled residues in MT1–mMT4-MMP are those that differ in the mouse MT4-MMP hemopexin domain compared with the human MT1-MMP hemopexin domain. Note that MT1–mMT4 MMP does not have the 'linkers' present in the other constructs.

recognises the propeptide of MT1-MMP, we show that, when expressed in MCF7 cells, the reason for the lack of activity in the MT1–MT4-MMP chimaeras is due to the inability of these mutants to be properly processed and displayed at the cell surface.

EXPERIMENTAL

Cells and cell culture conditions

All cell culture reagents were purchased from Invitrogen Ltd. unless indicated otherwise. MFC7 human breast cancer cells were maintained in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10 % (v/v) FCS (foetal calf serum; Hyclone Laboratories Inc.), 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin at 37 °C in a 5 % CO_2 atmosphere.

DNA constructs

Wild-type MT1-MMP

Full length wt (wild-type) human MT1-MMP cDNA without either of its 5′ or 3′ untranslated regions was amplified and subcloned between the HindIII and EcoRI sites of pcDNA3.1 Zeo$^+$ mammalian expression vector (Invitrogen Ltd.).

MT1–MT1-MMP and MT1–hMT4-MMP

The region of human MT1-MMP cDNA coding for the signal sequence, pro-domain, catalytic domain and hinge region (amino acids 1–318) was amplified by standard PCR and cloned upstream (NheI and AgeI) of EGFP (enhanced green flourescent protein) in the pEGFP-C1 vector (BD Clontech). The recombinant vector was then digested with BglII and EcoRI, and the amplified cDNA coding for human MT1-MMP stalk, transmembrane and cytoplasmic tail regions (amino acids 509–582) were cloned downstream of EGFP in order to generate the 'master construct', MT1–EGFP (originally designed for use in cell trafficking studies that are not described in this study). This led to the creation of two short linkers on both sides of the EGFP (Figure 1). EGFP was then excised from the master construct by digestion with AgeI and BglII restriction enzymes and replaced with human MT4-MMP or human MT1-MMP hemopexin domains to create MT1–hMT4-MMP and MT1–MT1-MMP respectively. Both constructs display the same short linkers as depicted in Figure 1.

MT1–mMT4-MMP

MT1–mMT4-MMP (a gift from Dr Yoshifumi Itoh, Kennedy Institute of Rheumatology, Imperial College, London, U.K.) was generated as described previously [2].

All constructs were checked by sequencing using the Big Dye Terminator kit (Applied Biosystems).

Antibodies

Anti-MT1-MMP pro-domain mouse monoclonal antibody (clone 2A7), was a gift from Professor Marie-Christine Rio, Université Louis Pasteur, Strasbourg, France [11]. Anti-MT1-MMP sheep primary IgGs, directed against the full ectodomain of mature human MT1-MMP (N175) [12], were affinity purified using MT1-MMP ectodomain immobilized on a HiTrap™ NHS (*N*-hydroxysuccinimido)-activated HP (high performace) column (Amersham Pharmacia Biotech UK Ltd) as described previously [13]. All

secondary antibodies were purchased from Jackson ImmunoResearch Laboratories Inc. and used according to the manufacturer's instructions. Anti-giantin mouse monoclonal antibody [14] was a gift from H.-P. Hauri (Biozentrum, University of Basel, Basel, Switzerland).

Pro-MMP-2 activation

MCF7 cells were trypsinized and resuspended in DMEM and 10 % FCS then transfected in suspension (2×10^5 cells/ml) using 0.5 μg of each DNA construct, 1.5 μl of FuGENE™ 6 transfection reagent (Roche Diagnostics Ltd.) according to the manufacturer's instructions. Cells were plated in 24-well culture dishes (2×10^5 cells/well) and incubated at 37 °C.

At 24 h post transfection the medium was removed and replaced with 300 μl/well serum-free DMEM with insulin, transferrin and selenium supplements (Sigma). To each well, 100 ng of purified pro-MMP-2 [15] was added, and the cells were incubated at 37 °C for a further 24 h. Cell supernatants were harvested and analysed by gelatin zymography as described previously [16]. In some instances cell lysates were also prepared as described [17] for analysis by Western blotting. An assessment of TIMP-1 and TIMP-2 in supernatants and cell lysates was made by performing reverse gelatin zymography as described previously [18].

Biotinylation

MCF7 cells were transfected as described above, seeded into 6-well culture dishes (2×10^5 cells/well) and incubated overnight at 37 °C. Biotinylation was performed as described previously [13] with minor modifications. Cells were washed three times with ice-cold Sorensen Buffer (SBS; 14.7 mM KH_2PO_4, 2 mM Na_2HPO_4 and 120 mM Sorbitol, pH 7.8) and then incubated at room temperature (25 °C) with 2.5 ml/well non-permeable EZ-link™ NHS-LC-biotin [*N*-succinimidyl-6-(biotinamido)hexanoate; Perbio Science; 0.5 mg/ml in SBS] for 20 min. The cells were washed carefully three times with SBS and the unreacted biotin was quenched using 100 mM glycine in SBS for 10 min on ice. Following a further three washes with SBS, the cells were lysed using 200 μl/well RIPA lysis buffer [20 mM Tris/HCl, pH 7.4, 150 mM NaCl, 0.1 % SDS, 1 % (v/v) Triton X-100, 1 % (w/v) Deoxycholate and 1 % (v/v) Nonidet P40]. Lysates from replicate wells were pooled and incubated at 4 °C on a rotating rack for 30 min then centrifuged at 9300 ***g*** for 1 min at 4 °C. The lysate supernatants were pre-cleared overnight by incubation on a rotating rack at 4 °C with washed Protein G sepharose (Sigma). At the same time, 30 μg of affinity purified N175 anti-MT1-MMP IgG was bound to pre-washed Protein G–Sepharose.

A proportion (25 μl) of the protein G slurry with the bound antibody was incubated with each of the pre-cleared lysates for 1 h at 4 °C. The immune complexes were separated from the unbound pool by centrifugation at 300 ***g*** for 2 min at room temperature. The beads were then washed four times with RIPA buffer. Immunoprecipitates were eluted from the beads by boiling for 5 min in 2× reducing Laemmli sample buffer (30 μl/sample), and the bound and unbound fractions were analysed by Western blotting.

Western blotting

Samples of transfected MCF7 cell lysates (10 μl) were resolved by reducing SDS/PAGE (10 % gels), and then transferred overnight onto nitrocellulose membranes using a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad Laboratories Ltd). Membranes were blocked at 37 °C using 5 % (w/v) milk powder in Tris-buffered saline with 0.5 % (v/v) Tween 20. Enzymes were detected by first probing with primary antibody (either N175 at 1 μg/ml or 2A7 diluted 1:100), followed by a secondary antibody recognising sheep or mouse IgGs conjugated to HRP (horseradish peroxidase). Bands were revealed using ECL® detection reagents according to the manufacturers' instructions (Amersham). For biotinylated proteins, streptavidin conjugated to HRP (1:1500) was used instead of the primary and secondary antibodies as described.

Gelatin degradation

Texas Red®-labelled gelatin was used to coat 8-well culture slides (Becton Dickinson Labware) using the method described previously [12]. MCF7 cells (10^5 cells/ml) were transfected in suspension with 250 ng of DNA and 0.75 μl of FuGENE™ 6/well, then seeded on top of the gelatin-coated slides (0.5 ml/well) and incubated overnight at 37 °C. The following day the cells were washed in PBS (140 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4 and 8.1 mM Na_2HPO_4), then fixed in 4 % (w/v) paraformaldehyde for 5 min, washed three times in PBS and processed for indirect immunofluorescence microscopy.

Indirect immunofluorescence microscopy

Cells were seeded on 13 mm round glass coverslips or Labtek slides and grown at 37 °C until they reached 70–80 % confluence. Cells were washed with PBS then fixed with 4 % (w/v) paraformaldehyde in PBS for 25 min at room temperature. Cells were left unpermeabilized or permeabilized for 4 min with 0.1 % Triton X-100 (Surfact-Amps X-100 grade, Pierce) in PBS. Staining was carried out as described previously[19]. After extensive washing in PBS the coverslips were briefly rinsed in H_2O and then mounted on glass slides in Mowiol (Calbiochem) containing 25 mg/ml 1,4-diazobicyclo(2,2,2)octane to reduce photobleaching or in Vectashield (Vector Laboratories). Pictures of fluorescently labelled cells were collected using a cooled, slow scan Photometrics Coolsnap HQ CCD camera (Universal Imaging Corporation Ltd) attached to a Zeiss Axioplan 2 imaging microscope fitted with 20×, 63× and 100× plan apochromat objectives (Carl Zeiss Ltd). Image acquisition was performed using Metamorph imaging software (Universal Imaging Corporation). Images were transferred to Photoshop (Adobe Systems) imaging software for digital processing.

RESULTS

Exchanging the hemopexin domain of MT1-MMP for that of MT4-MMP blocks activation of pro-MMP-2

Recent reports on the role of MT1-MMP hemopexin domain in pro-MMP-2 activation have investigated the activity of MT1-MMP chimaeras in which the MT1-MMP hemopexin domain has been substituted with the hemopexin domain of other MT-MMPs [2,8]. A consistent finding from these studies is that hemopexin domain exchanges with MT4-MMP result in a lack of activity of the chimaeras towards pro-MMP-2. To assess the importance of the hemopexin domain in cell surface trafficking and function of MT1-MMP, we generated the chimaera MT1–hMT4-MMP (depicted in Figure 1). In addition we removed the MT1-MMP hemopexin domain and then replaced it in wt MT1-MMP in order to generate MT1–MT1-MMP. Both chimaeras were constructed using the 'master construct' (MT1–EGFP), and as a result the hemopexin domain in each was flanked by the same 'linkers' (described above). Subsequently we compared the activity of our domain-exchange MT1-MMPs with MT1–mMT4-MMP and the wt enzyme in functional assays.

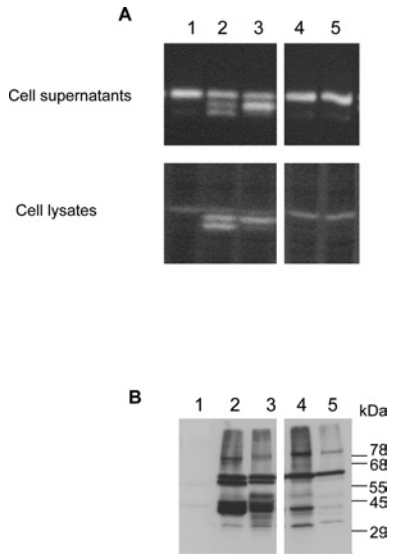

Figure 2 MT1-MMP hemopexin domain chimaeras have impaired pro-MMP-2 activating ability

MCF7 cells were transiently transfected with empty vector pcDNA 3.1 (lane 1), wt MT1-MMP (lane 2), MT1–MT1-MMP (lane 3), MT1–hMT4-MMP (lane 4) or MT1–mMT4-MMP (lane 5). The cells were transfected and incubated for 24 h, the cells were then incubated in the presence of 100 ng of exogenous pro-MMP-2 at 37 °C for a further 18 h. (**A**) The supernatants and lysates were analysed by gelatin zymography. (**B**) Cell lysates were also analysed by Western blotting for MT1-MMP expression levels, as described in the Experimental section.

The constructs were transiently transfected in MCF7 cells and MMP-2 activation was monitored 48 h post transfection by gelatin zymography of the cell culture supernatants and the cell lysates. As expected wt MT1-MMP consistently activated pro-MMP-2 to its intermediate and fully active forms (Figure 2A, lane 2). In agreement with previous reports [2,8], exchanging MT1-MMP hemopexin domain with either human or mouse MT4-MMP hemopexin domain completely blocked MMP-2 activation (Figure 2A, lanes 4 and 5). Interestingly when MT1-MMP hemopexin domain was re-inserted in the presence of the 'linkers' (MT1–MT1-MMP), pro-MMP-2 was cleaved only to the intermediate form and further processing did not occur (Figure 2A, lane 3). To determine whether this result could be explained by differences in TIMP-1 or TIMP-2 levels in the transfected cells we performed reverse gelatin zymography on both cell lysates and cell supernatants. Overall levels of both TIMP-1 and TIMP-2 were low and no significant differences in TIMP-2 were detected in the cell lysates. However there was slightly less secreted TIMP-2 in supernatants from cells transfected with wt MT1-MMP compared with those from cells transfected with the vector control or MT1–MT1-MMP. TIMP-1 was detected at similar levels in all cell supernatants but could not be detected in the cell lysates (results not shown). No MMP-2 activation was seen in supernatants or lysates from cells transfected with pcDNA 3.1 empty vector (Figure 2A, lane 1) or pEGFP-C1 vector alone (results not shown). To establish whether all the constructs were in fact expressed and whether the levels of expression were similar, we analysed the cell lysates by Western blotting. Using the polyclonal antibody to the full ectodomain of MT1-MMP (N175) we detected the presence of MT1-MMP in all cells transfected with the constructs, but the pattern of expression differed from that of wt MT1-MMP (Figure 2B). Western blots of lysates from cells transiently expressing MT1–hMT4-MMP or MT1–mMT4-MMP using the polyclonal antibody (N175) predominately detected a single species of approximately 63 kDa (Figure 2B, lanes 4 and 5). In comparison, lysates from cells expressing wt MT1-MMP and MT1–MT1-MMP (Figure 2B, lanes 2 and 3) revealed the expected pattern of pro- (63 kDa), fully active (60 kDa) and degraded (43–45 kDa) forms of MT1-MMP (the slight variation in molecular mass between wt MT1-MMP and MT1–MT1-MMP can be explained by the presence of the 'linkers' in the latter construct).

Having investigated whether or not the MT1-MMP chimaeras could activate pro-MMP-2 we next examined their capacity to degrade extracellular matrix components directly.

MT1–MT4-MMP does not degrade gelatin and is not detected on the cell surface

We previously demonstrated that Chinese hamster ovary cells can efficiently degrade Texas Red®-labelled gelatin films coated onto culture slides when transiently over-expressing MT1-MMP, but not when transfected with an empty vector control [12]. MCF7 cells do not naturally express either MT1-MMP or MMP-2 proteins at detectable levels (results not shown) and thus provided an excellent model to investigate the ability of our chimaeras to degrade extracellular matrix components. We first assessed the effect of exchanging MT1-MMP hemopexin domain with that of MT4-MMP by transfecting MCF7 cells with wt MT1-MMP or with the MT1–mMT4-MMP chimaera. As reported previously for Chinese hamster ovary cells [12], transient expression of the wt enzyme led to extensive degradation of the labelled gelatin films, resulting in the appearance of 'black holes', which were visible when viewed under the fluorescence microscope using a Rhodamine filter at 568 nm (Figure 3A, panels a, b, e and f). No gelatin degradation was observed for cells transfected with pcDNA 3.1 vector alone (results not shown) or for untransfected cells (just visible in the same field of view when using the FITC filter at 488 nm) (Figure 3A, panels c, d, g and h). Surprisingly, using this assay, transient expression of MT1–mMT4-MMP in MCF7 cells did not induce gelatin degradation and no holes were visible (Figure 3A, panels i, j, m and n). A similar result was obtained with MT1–hMT4-MMP (results not shown), indicating that the result was not due to the species variation of the MT4-MMP hemopexin domain, or to differences in the construction of the chimaeras with respect to the added 'linkers' in MT1–hMT4-MMP (described in the Experimental section). Our results differ from the previous study [2] that showed gelatinolytic activity of Cos-1 cells tranfected with MT1–mMT4-MMP plated on quenched fluorescent DQ gelatin. Differences between the observations most likely result from the nature of the gelatin types used for each assay.

We hypothesized that replacing MT1-MMP hemopexin domain with that of MT4-MMP could affect the intracellular trafficking of the chimaera, and that the absence of gelatin degradation by MT1–mMT4-MMP might be attributable to lack of enzyme presented on the surface of transfected MCF7 cells. We used our polyclonal anti-MT1-MMP IgG (N175) and a FITC-labelled secondary antibody to detect cell surface and intracellular expression of wt MT1-MMP and MT1–mMT4-MMP in transiently transfected MCF7 cells cultured on Texas Red®-labelled gelatin films. Since the polyclonal antibody was raised against the entire MT1-MMP extracellular domain it could be used to detect expression of the chimaeras by both immunofluorescence and Western blotting. When non-permeabilized cells were viewed using the FITC filter at 488 nm it was apparent that there was no surface expression of MT1–mMT4-MMP (Figure 3A, panel k) whereas wt MT1-MMP was clearly detected (Figure 3A, panel c). Likewise no cell-surface expression was observed for MT1–hMT4-MMP (results not shown). Non-permeabilized cells were also stained using a

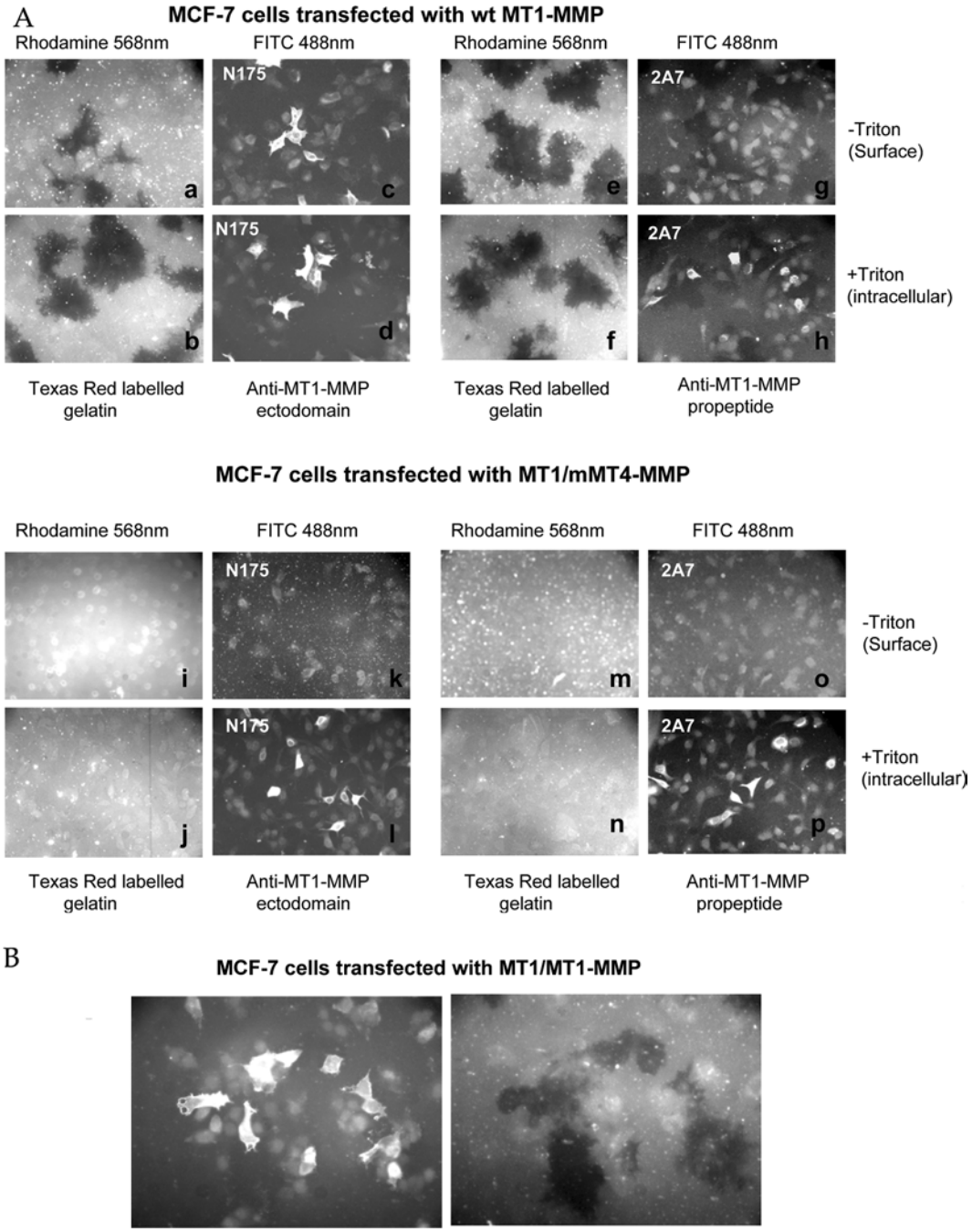

Figure 3 Lack of gelatin degrading activity by MT1/MT4-MMP chimaeras (A), and gelatin degradation by MT1–MT1-MMP chimaera (B)

(**A**) MCF7 cells were transiently transfected with wt MT1-MMP (panels a–h) or with MT1–mMT4-MMP (panels i–p) and cultured for 24 h on gelatin labelled with Texas Red® as described in the Experimental section. MT1-MMP was detected by immunolocalization using antibodies against the full ectodomain (N175; panels c, d, k and l) or the propeptide (2A7; panels g, h, o and p). Cells were permeabilized using Triton X-100 (panels b, d, f, h, j, l, n and p) or left unpermeabilized (panels a, c, e, g, i, k, m and o). Panels a, b, e, f, i, j, m and n show the gelatin films viewed under the Rhodamine filter at 568 nm. Panels c, d, k, l, g, h, o and p show MT1-MMP staining on positively transfected cells in the same fields of view under the FITC filter at 488 nm. Cells not expressing MT1-MMP can also be seen by low level background fluorescence. (**B**) MT1–MT1-MMP was transiently expressed in MCF7 cells cultured on Texas Red®-labelled gelatin films for 24 h as described. Cell surface MT1-MMP was detected by indirect immunofluoresence using the polyclonal antibody to the full ectodomain of MT1-MMP (N175) without permeabilization. Left-hand panel: MT1-MMP detected by FITC-fluorescence. Right-hand panel: Texas Red®-labelled gelatin from the same field of view.

monoclonal antibody that specifically recognizes the MT1-MMP pro-domain (2A7) to assess the presence of this form at the cell surface. As expected neither pro-MT1-MMP (Figure 3A, panel g) nor pro-MT1–mMT4-MMP (Figure 3A, panel o) were detected at the surface of transfected MCF7 cells. To confirm that MT1–mMT4-MMP was indeed expressed in transfected MCF7 cells we assessed the intracellular pool of the protein. Following permeabilization, both wt MT1-MMP (Figure 3A, panels d and h) and MT1–mMT4-MMP (Figure 3A, panels l and p) were detected using either the anti-(extracellular domain) polyclonal or the anti-pro-domain monoclonal MT1-MMP antibodies. These results confirmed that both constructs were expressed in MCF7 cells with

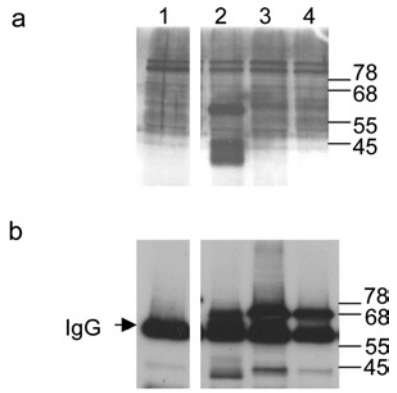

Figure 4 Lack of biotinylation of MT1hMT4-MMP and MT1–mMT4-MMP at the cell surface

(**a** and **b**) MCF7 cells were transiently transfected with empty vector pcDNA 3.1 (lane 1), wt MT1-MMP (lane 2), MT1–mMT4-MMP (lane 3) or MT1–hMT4-MMP (lane 4). Cells were biotinylated 24 h post transfection, and MT1-MMP was immunoprecipitated from the cell lysates using the polyclonal antibody against the full ectodomain of MT1-MMP (N175). Biotinylated (cell surface) MT1-MMP was detected by probing the Western blot with streptavidin peroxidase (**a**) and total MT1-MMP by stripping and reprobing the blot with N175 antibody (**b**).

a similar positive transfection rate of approx. 15–20 %. However neither MT1–mMT4-MMP nor MT1–hMT4-MMP (results not shown) was detected at the cell surface. In contrast, using the same assay, we observed that MT1–MT1-MMP was expressed at the cell surface at similar levels to the wt MT1-MMP, and degraded the gelatin films in a similar manner (Figure 3B), indicating that the presence of the 'linkers' did not affect trafficking of this construct to the plasma membrane.

MT1–hMT4-MMP and MT1–mMT4-MMP are not expressed at the cell surface

Since gelatin degradation by MT1-MMP relies on the cell surface presentation of active enzyme we compared the levels of wt MT1-MMP with the MT1–MT4-MMP chimaeras in transiently transfected MCF7 cells by cell-surface biotinylation using a non-permeable biotinylation reagent. MT1-MMP was immunoprecipitated from cell lysates using the anti-MT1-MMP ectodomain affinity purified IgG. Samples were analysed by Western blotting for MT1-MMP using the polyclonal antibody (N175) to determine the level of immunoprecipitation for each construct (Figure 4b) and for biotinylated (i.e. cell surface) MT1-MMP by probing with streptavidin-HRP (Figure 4a). As expected, we observed the biotinylation of fully active (60 kDa) MT1-MMP and its degraded forms (43–45 kDa), confirming their presence at the surface of cells transiently transfected with wt MT1-MMP (Figure 4a, lane 2). Surprisingly little, if any, biotinylated MT1–mMT4-MMP or MT1–hMT4-MMP was detected at the surface of transfected MCF7 cells (Figure 4a, lanes 3 and 4), suggesting that these two constructs, despite being expressed at similar levels to the wt enzyme (Figure 4b, lanes 3 and 4), do not reach the plasma membrane. To confirm this observation, we carried out immunofluorescence experiments to assess more precisely the intracellular localization of the MT1–hMT4-MMP and MT1–mMT4-MMP chimaeras. MCF7 cells were transiently transfected with wt MT1-MMP or MT1–hMT4-MMP or MT1–mMT4-MMP. After fixation, cells were permeabilized and the chimaeras detected using the anti-MT1-MMP affinity purified IgG. We also used antibodies directed against calnexin and giantin to reveal endoplasmic reticulum (ER) and *cis*-golgi compartments respectively (results not shown). In MCF7 cells transfected with

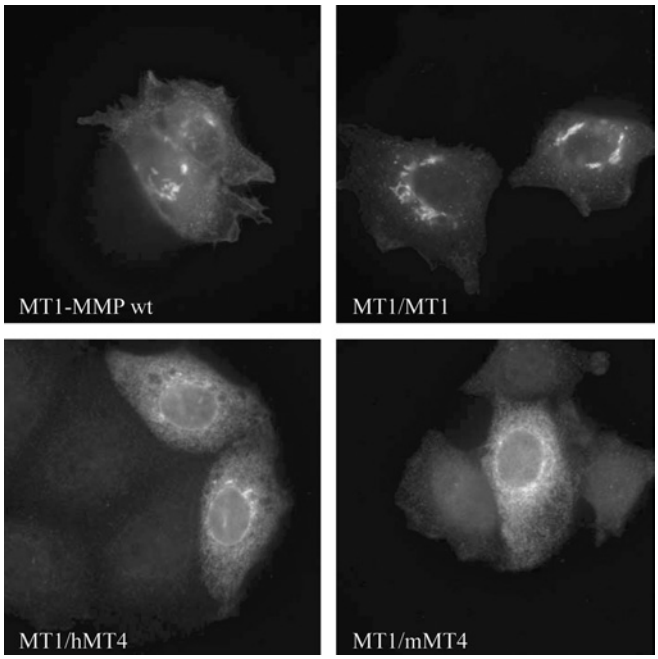

Figure 5 Intracellular localization of wt MT1-MMP and MT1-MMP chimaeras

MCF7 cells transiently transfected with wt MT1-MMP or MT1–MT1-MMP (top panels) or with MT1–hMT4-MMP or MT1–mMT4-MMP (bottom panels) were fixed, permeabilized and stained with affinity purified anti-MT1-MMP IgG (N175).

wt MT1-MMP or with MT1–MT1-MMP we observed that the enzyme is mainly detected in cytoplasmic vesicles as well as in the giantin positive compartment (Figure 5, top panels). Surprisingly both MT1–hMT4-MMP and MT1–mMT4-MMP chimaeras were detected mainly, if not completely, in the ER (Figure 5, bottom panels).

The results confirm that MT1–hMT4-MMP and MT1–mMT4-MMP are both expressed in MCF7 cells, but that these constructs do not reach the cell surface. These results are in contrast to those of Itoh et al. [2], who showed, using detection by biotinylation, that low levels of wt MT1-MMP and MT1–mMT4-MMP were found at the surface of transfected Cos-1 cells.

MT1/MT4-MMP chimaeras are largely unprocessed

The monoclonal antibody (2A7) specifically recognizes the prodomain of MT1-MMP, which allowed us to examine whether the lack of cell surface expression of MT1–hMT4-MMP and MT1–mMT4-MMP compared with wt MT1-MMP could be due to a difference in the processing of these constructs to the mature form.

Western blot profiles of lysates (Figure 2B) from cells transfected with MT1–MT4-MMP chimaeras indicated that fully active forms (60 kDa in wt MT1-MMP) of MT1–hMT4-MMP and MT1–mMT4-MMP were not present. In addition, few degradation products (generated by autocatalysis in wt MT1-MMP) were evident, suggesting a lack of autocatalysis by the MT1–MT4-MMP chimaeras. However it should be noted that wt MT1-MMP auto-catalytically degraded fragments (43–45 kDa) largely comprise the hemopexin domain together with the transmembrane and cytoplasmic domains that remain associated with the cell membrane. Thus if those constructs that had the MT1-MMP hemopexin domain replaced by human or mouse MT4-MMP generated degradation products they may not be fully

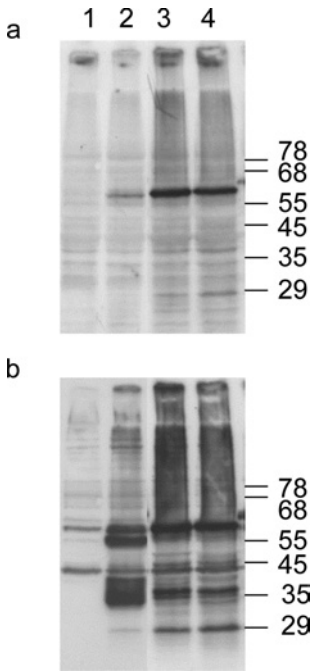

Figure 6 Detection of the pro-peptide domain of MT1-MMP in transfected MCF7 cell lysates

Lysates from MCF7 cells transiently transfected with pcDNA 3.1 (lane 1), wt MT1-MMP (lane 2), MT1–mMT4-MMP (lane 3) or MT1–hMT4-MMP (lane 4) were analysed by Western blotting for pro MT1-MMP using monoclonal antibody 2A7 (**a**) followed by stripping and reprobing with polyclonal antibody N175 for total MT1-MMP (**b**).

recognised by the polyclonal antibody against the ectodomain of human MT1-MMP. To determine whether the lack of activation of pro-MMP 2 by the MT1–MT4-MMP chimaeras, their inability to degrade gelatin and the non-appearance of the enzymes at the cell surface were due to lack of active enzymes, the cell lysates were analysed by Western blotting and probed first with the monoclonal antibody 2A7. The blots were then stripped and re-probed with the polyclonal antibody N175. It is evident from Figure 6 that the major species of the MT1–MT4-MMP constructs have their pro-peptide intact, which can be seen when lanes 2 from Figure 6 (a and b) are compared with lanes 3 and 4 from Figure 6 (a and b).

DISCUSSION

The contributions of the individual domains of the soluble MMPs to their functional activity have been extensively and successfully investigated using chimaeric and domain-exchange proteins. These have predominantly been obtained from mammalian expression systems that provide checkpoints for incorrectly folded proteins. Recently this approach has been used by a number of laboratories to study the role of the individual domains of the membrane-associated MMPs, especially MT1-MMP. In this study we have demonstrated that exchanging the hemopexin domain of human MT1-MMP for that of human or mouse MT4-MMP, followed by transient expression of the chimaeras in MCF7 cells resulted in a failure in trafficking to the cell surface and the retention of the chimaeric proteins in the ER as unprocessed enzymes. In the light of these findings their inability to activate pro-MMP-2 or to degrade gelatin is not surprising. Previously, transiently expressed MT1–mMT4-MMP was detected by surface biotinylation of Cos-1 cells [2], but an assessment of the relative levels of biotinylated enzyme compared to total expression was not made. In our experiments using transfected MCF7 cells we clearly demonstrated that, compared with wt MT1-MMP, levels of surface biotinylated MT1–mMT4-MMP and MT1–hMT4-MMP chimaeras were negligible relative to the total expression levels of the constructs.

By excising EGFP from our master construct (as described in the Experimental section) and then replacing it with the MT1-MMP hemopexin domain instead of the MT4-MMP hemopexin domain we were able to restore MT1-MMP trafficking and function. Nevertheless, although MT1–MT1-MMP was abundantly expressed at the surface and could degrade gelatin, pro-MMP-2 was processed only to the intermediate form generated by MT1-MMP cleavage between residues N^{37} and L^{38} [20]. This result was unexpected: MT1–MT1-MMP with the replaced hemopexin domain should have the ability to form homophilic oligomers and thus concentrate MT1-MMP/TIMP-2 'receptors' for the binding of pro-MMP-2, so why does full activation not occur? While TIMP-1 inhibits only the second autocatalytic step of MT1-MMP-mediated pro-MMP-2 activation, and in excess can cause accumulation of the activation intermediate, there were no significant differences between TIMP-1 levels in transfected MCF7 cell lysates and supernatants. TIMP-2, on the other hand, inhibits both steps in the activation cascade, but the slightly higher level of secreted TIMP-2 in supernatants from MT1–MT1-MMP transfected cells was unlikely to be responsible for complete inhibition of MMP-2 autocatalysis while having no inhibitory effect on the MT1-MMP mediated initial cleavage of pro-MMP-2.

In the absence of TIMP-2, concentration-dependent pro-MMP-2 activation by soluble recombinant MT1-MMP catalytic domain (i.e. without the hemopexin domain) is enhanced by heparin. Furthermore heparin binds the hemopexin domain of MMP-2 at the same site as TIMP-2 [5]. In addition, a catalytically inactive MMP-2 is processed only to the intermediate form by soluble catalytic domain of MT1-MMP, and can be fully processed by the addition of active MMP-2 [21]. Thus the biochemical data support the hypothesis that a localized concentration of pro-MMP-2 via MT1-MMP/TIMP-2 receptors promotes MMP-2 autocatalysis.

We postulated that the presence of the short linker sequences in the reconstructed MT1–MT1-MMP chimaera could conceivably interfere with either MT1-MMP hemopexin domain oligomerization or with TIMP-2/pro-MMP-2 binding and thereby prevent sufficient concentration of pro-MMP-2 to promote autocatalysis. This hypothesis implies that initial pro-MMP-2 cleavage can occur without MT1-MMP hemopexin domain interactions and/or binding of TIMP-2. In agreement, two recent reports [8,9] demonstrated full MMP-2 activation in cells transfected with MT1-MMP expression constructs that lacked the entire hemopexin domain. The results may be explained by evidence that MT1-MMP forms oligomers dependent on interactions between the transmembrane and cytoplasmic domains as well as the hemopexin domain [6,7].

Although TIMP-2 deficiency leads to severely impaired MMP-2 activation [22,23], MT1-MMP processing of pro-MMP-2 to the intermediate form has been demonstrated in TIMP-2 depleted cells [24], leading to speculation that TIMP-2 is required only for the second autocatalytic activation step.

In conclusion, it seems likely that in transfected cells where MT1-MMP is overexpressed, the initial cleavage of pro-MMP-2 can occur in the absence of MT1-MMP hemopexin domain interactions and without TIMP-2. Whether this is a physiological phenomenon is still open to speculation.

Despite the fact that the MT1–MT1-MMP chimaera gave us an unexpected result (with respect to its ability to activate MMP-2),

it may be explained by its method of construction, in that the presence of the 'linkers' did not affect the ability of this chimaera to reach the plasma membrane. We also conclude that in the case of the MT1–MT4-MMP chimaeras the presence or absence of the 'linkers' did not affect the observations.

These experiments highlight the difficulties in interpreting biological effects attributed to individual domains of MT1-MMP by means of this type of domain exchange and transient over-expression. On the basis of the evidence from domain-exchange experiments it has been suggested that negative regulation by their hemopexin domains might account for the inability of MT4-MMP and MT6-MMP to activate pro-MMP-2 [8]. Our data demonstrate that replacing MT1-MMP hemopexin domain with that of either human or mouse MT4-MMP results in a failure of the resulting chimaeras to mature, and that it is this, rather than negative regulation, that is the reason for their lack of activity towards pro-MMP-2.

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REFERENCES

1 Egeblad, M. and Werb, Z. (2002) New functions for the matrix metalloproteinases in cancer progression. Nat. Rev. Cancer. **3**, 161–174
2 Itoh, Y., Takamura, A., Ito, N., Maru, Y., Sato, H., Suenaga, N., Aoki, T. and Seiki, M. (2001) Homophilic complex formation of MT1-MMP facilitates proMMP-2 activation on the cell surface and promotes tumour cell invasion. EMBO J. **20**, 4782–4793
3 Strongin, A. Y., Collier, I., Bannikov, G., Marmer, B. L., Grant, G. A. and Goldberg, G. I. (1995) Mechanism of cell surface activation of 72-kDa type IV collagenase. Isolation of the activated form of the membrane metalloproteinase. J. Biol. Chem. **270**, 5331–5338
4 Atkinson, S. J., Crabbe, T., Cowell, S., Ward, R. V., Butler, M. J., Sato, H., Seiki, M., Reynolds, J. J. and Murphy, G. (1995) Intermolecular autolytic cleavage can contribute to the activation of progelatinase A by cell membranes. J. Biol. Chem. **270**, 30479–30485
5 Butler, G. S., Butler, M. J., Atkinson, S. J., Will, H., Tamura, T., Van Westrum, S. S., Crabbe, T., Clements, J., D'Ortho, M.-P. and Murphy, G. (1998) The TIMP-2 membrane type 1-metalloproteinase "receptor" regulates the concentration and efficient activation of progelatinase A. J. Biol. Chem. **273**, 871–880
6 Rozanov, D., Deryugina, E., Ratnikov, B., Monosov, E., Marchenko, G., Quigley, J. and Strongin, A. (2001) Mutation and analysis of membrane type-1 matrix metalloproteinase (MT1-MMP). The role of the cytoplasmic tail Cys^{574}, the active site Glu^{240}, and furin cleavage motifs in oligomerization, processing, and self-proteolysis of MT1-MMP expressed in breast carcinoma cells. J. Biol. Chem. **276**, 25705–25714
7 Lehti, K., Lohi, J., Juntunen, M. M., Pei, D. and Keski-Oja, J. (2002) Oligomerization through hemopexin and cytoplasmic domains regulates the activity and turnover of membrane-type 1 matrix metalloproteinase. J. Biol. Chem. **277**, 8440–8448
8 Wang, P., Nie, J. and Pei, D. (2004) The hemopexin domain of membrane-type matrix metalloproteinase-1 (MT1-MMP) is not required for its activation of proMMP 2 on cell surface but is essential for MT1-MMP-mediated invasion in three-dimensional type I collagen. J. Biol. Chem. **279**, 51148–51155
9 Remacle, A., Rozanov, D., Baciu, P., Chekanov, A., Golubkov, V. and Strongin, A. (2005) The transmembrane domain is essential for the microtubular trafficking of membrane type-1 matrix metalloproteinase (MT1-MMP). J. Cell Sci. **118**, 4975–4984
10 English, W., Puente, X., Freije, J., Knauper, V., Amour, A., Merryweather, A., Lopez-Otin, C. and Murphy, G. (2000) Membrane type 4 matrix metalloproteinase (MMP17) has tumour necrosis factor-α convertase activity but does not activate pro-MMP-2. J. Biol. Chem. **275**, 14046–14055
11 Chenard, M., Lutz, Y., Mechine-Neuville, A., Stoll, I., Bellocq, J., Rio, M.-C. and Basset, P. (1999) Presence of high levels of MT1-MMP protein in fibroblastic cells of human invasive carcinomas. Int. J. Cancer. **82**, 208–212
12 d'Ortho, M.-P., Stanton, H., Butler, M., Atkinson, S. J., Murphy, G. and Hembry, R. M. (1998) MT1-MMP on the cell surface causes focal degradation of gelatin films. FEBS Lett. **421**, 159–164
13 Remacle, A., Murphy, G. and Roghi, C. (2003) Membrane type 1-matrix metalloproteinase (MT1-MMP) is internalized by two different pathways and is recycled to the cell surface. J. Cell Science **116**, 3905–3916
14 Lindstedt, A. D. and Hauri, H. P. (1993) Giantin, a novel conserved Golgi membrane protein containing a cytoplasmic domain of at least 350 kDa. Mol. Biol. Cell. **4**, 679–693
15 Murphy, G., Willenbrock, F., Ward, R., Cockett, M., Eaton, D. and Docherty, A. J. P. (1992) The C-terminal domain of 72 kDa gelatinase A is not required for catalysis, but is essential for membrane activation and modulates interactions with tissue inhibitors of metalloproteinases. Biochem. J. **283**, 637–641
16 Heussen, C. and Dowdle, E. B. (1980) Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing sodium dodecyl sulfate and copolymerized substrates. Anal. Biochem. **102**, 196–202
17 Stanton, H., Gavrilovic, J., Atkinson, S. J., D'ortho, M. P., Yamada, K. M., Zardi, L. and Murphy, G. (1998) The activation of ProMMP-2 (gelatinase A) by HT1080 fibrosarcoma cells is promoted by culture on a fibronectin substrate and is concomitant with an increase in processing of MT1-MMP (MMP-14) to a 45 kDa form. J. Cell Sci. **111**, 2789–2798
18 Staskus, P. W., Masiarz, F. R., Pallanck, L. J. and Hawkes, S. P. (1991) The 21-kDa protein is a transformation-sensitive metalloproteinase inhibitor of chicken fibroblasts. J. Biol. Chem. **266**, 449–454
19 Roghi, C. and Allan, V. J. (1999) Dynamic association of cytoplasmic dynein heavy chain 1a with the Golgi apparatus and intermediate compartment. J. Cell Sci. **112**, 4673–4685
20 Strongin, A. Y., Marmer, B. L., Grant, G. A. and Goldberg, G. I. (1993) Plasma membrane-dependent activation of the 72-kDa Type IV Collagenase is prevented by complex formation with TIMP-2. J. Biol. Chem. **268**, 14033–14039
21 Will, H., Atkinson, S. J., Butler, G. S., Smith, B. and Murphy, G. (1996) The soluble catalytic domain of membrane type 1 matrix metalloproteinase cleaves the propeptide of progelatinase A and initiates autoproteolytic activation. Regulation by TIMP-2 and TIMP-3. J. Biol. Chem. **271**, 17119–17123
22 Caterina, J., Caterina, N., Yamada, S., Holmbeck, K., Longenecker, G., Shi, J., Netzel-Arnett, S., Engler, J., Yermovski, A., Windsor, J. and Birkedal-Hansen, H. (1999) Murine TIMP-2 gene-targeted mutation. Ann. N.Y. Acad. Sci. **878**, 528–530
23 Wang, Z., Juttermann, R. and Soloway, P. D. (2000) TIMP-2 is required for efficient activation of proMMP-2 *in vivo*. J. Biol. Chem. **275**, 26411–26415
24 Lafleur, M., Tester, A. and Thompson, E. (2003) Selective involvement of TIMP-2 in the second activational cleavage of pro-MMP-2: refinement of the pro-MMP-2 activation mechanism. FEBS Lett. **553**, 457–463

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