# Localization of membrane-type 1 matrix metalloproteinase in caveolae membrane domains

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Membrane-type 1 matrix metalloproteinase (MT1-MMP) is a membrane-associated MMP that has been recently reported to have a central role in tumour cell invasion. Here we report that both the native and overexpressed recombinant forms of MT1-MMP are highly enriched in low-density Triton X-100-insoluble membrane domains that contain the caveolar marker protein caveolin 1. Moreover, the MT1-MMP-dependent activation of proMMP-2 induced by concanavalin A and cytochalasin D was correlated with the processing of MT1-MMP to its proteolytically inactive 43 kDa fragment in U-87 glioblastoma and HT-1080 fibrosarcoma tumour cell lines; this processing was also preferentially observed within the caveolar fraction. Interestingly, whereas the expression of caveolin 1 had no effect on the MT1-MMP-dependent activation of proMMP-2, its co-expression with MT1-MMP antagonized the MT1-MMP-increased migratory potential of COS-7 cells. Taken together, our results provide evidence that MT1-MMP is preferentially compartmentalized and proteolytically processed in caveolae of cancer cells. The inhibition of MT1-MMP-dependent cell migration by caveolin 1 also suggests that the localization of MT1-MMP to caveolin-enriched domains might have an important function in the control of its enzymic activity.

Key words: angiogenesis, cancer, caveolin, cell migration, glioblastoma.

## INTRODUCTION

Matrix metalloproteinases (MMPs) are a broad family of zinc-binding endopeptidases that degrade various components of the extracellular matrix. These enzymes are encoded by different genes and are implicated in several normal and pathological tissue remodelling processes such as wound healing, angiogenesis and tumour invasion [1]. Among these MMPs, a large body of evidence suggests a role for the membrane-type MMPs (MT-MMPs) as physiological activators of latent soluble forms of MMPs. Moreover, the expression of MT-MMPs at the cell surface of invasive cells has also been proposed to represent a key step in tumour-cell invasion and metastasis [2–4]. The pivotal role of MT1-MMP in normal cell function was recently demonstrated in connective-tissue metabolism by gene targeting, and resulted in MT1-MMP-deficient mice developing severe craniofacial dysmorphism, arthritis, osteopenia, dwarfism and fibrosis of soft tissues [5].

Studies of the structure–function relationship of MT1-MMP have been limited by its presence in the hydrophobic domains of membranes. On the basis of immunofluorescence, it was suggested recently that the targeting and degradation/turnover of MT1-MMP at the leading edge of migrating cells was regulated in part by its cytoplasmic domain [6]. Moreover, as subcellular compartmentation has not yet been definitely established for MMP function, it has also been reported that specialized surface protrusions of invasive cells, termed invadopodia and lamellipodia, have differential localizations of MT1-MMP, MMP-2 and tissue inhibitor of MMP 2 (TIMP-2) [7].

The potential invasive character of cancer cells constitutively secreting proMMP-2 is governed by the presence of components essential to the formation of a ternary complex composed of TIMP-2, which binds to the catalytic domain of MT1-MMP, followed by the binding of the C-terminal domain of proMMP-2 to the C-terminal domain of TIMP-2 [8–10]. It has been suggested that this complex enables the processing of proMMP-2 by cleavage and the generation of its fully active form [11]. The intracellular compartmental basis for integrating the transmembrane signalling events leading to the MT1-MMP-dependent proMMP-2 activation thus remains to be established. Compartmentation of receptors and signalling intermediates into specialized membrane domains might therefore represent a mechanism by which cells expressing MT1-MMP could integrate such extracellular information. Among these domains, caveolae have emerged as key plasma membrane specializations involved in transmembrane signalling events [12]. Moreover, recent reports suggest that heritable differences in the interaction between caveolins and their partners lead to the pathogenesis of multiple types of cancer [13]; in parallel, the expression of caveolin 1 was shown to inhibit lamellipod extension and cell migration in a metastatic mammary adrenocarcinoma cell line [14].

In the present study we have therefore attempted to define further the molecular mechanisms underlying the MT1-MMP-dependent activation of proMMP-2 in tumour cell lines. The relatively poor Triton X-100 extractability from membranes, combined with the presence of specific regions within MT1-MMP containing hydrophobic amino acids, a known important mechanism underlying targeting to caveolae, prompted us to investigate MT1-MMP's caveolar function and localization. Our observations suggest that this process is localized in caveolae and might regulate the primary role of the membrane-anchored MMPs, the MT-MMPs, in cellular invasion.

Abbreviations used: Con A, concanavalin A; Cyto D, cytochalasin D; EGCg, epigallocatechin gallate; MMP, matrix metalloproteinase; MT1-MMP, membrane-type 1 MMP; NOG, n-octylglucopyranoside; *ph*MT1-MMP, cDNA encoding human MT1-MMP; rMT1-MMP, recombinant MT1-MMP; RT–PCR, reverse-transcriptase-mediated PCR; TIMP-2, tissue inhibitor of MMP 2.

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## MATERIALS AND METHODS

### Antibodies and chemicals

The following materials were purchased from the indicated sources. The anti-(human MT1-MMP) polyclonal antibody (raised against the hinge region), the anti-(caveolin 1) monoclonal antibody, the anti-(human MMP-2) monoclonal antibody, the anti-(TIMP-2) polyclonal antibody and the enhanced chemiluminescence (ECL)-Western blot kit were from Chemicon. The BCA (bicinchoninic acid) protein assay kit was from Pierce; PVDF membranes were from Boehringer Mannheim; all products for electrophoresis and zymography were bought from Bio-Rad; TriZOL reagent, trypsin, penicillin and streptomycin were from Gibco BRL; fetal bovine serum was from HyClone Laboratories; epigallocatechin gallate (EGCg) was from ICN; the anti-($\beta$-COP) monoclonal antibody (clone no. maD), agarose, CHAPS, concanavalin A (Con A), cytochalasin D (Cyto D), gelatin, n-octylglucopyranoside (NOG), SDS, trichloroacetic acid and Triton X-100 were from Sigma.

### Cloning of cDNA encoding human MT1-MMP (*ph*MT1-MMP)

The cDNA encoding MT1-MMP was isolated by nested PCR. The first amplification was performed for 30 cycles at an annealing temperature of 55 °C with the oligonucleotide primers derived from nt 40–64 (5′-TGGCGGTGCGACCCCAGGGC-GTGGG-3′, sense) and 1937–1913 (5′-ACCCACCACCACCT-GCTGCCACTGG-3′, anti-sense) of the human MT1-MMP cDNA (GenBank accession number D26512) by using total cellular RNA extracted from U-87 glioblastoma cells as template. The resulting 1.9 kb cDNA fragment was reamplified with the oligonucleotides 5′-CAGCTGCAGGAATTCGTGGTCTCGG-ACCATGTCTCCCG-3′ (sense) and 5′-CAGCTGCAGATG-GGCGTCAGACCTTGTCCAGC-3′ (anti-sense) for 40 cycles with annealing at 60 °C, resulting in a 1.4 kb cDNA fragment containing the whole open reading frame of MT1-MMP that was subcloned into pCR-2.1 vector with the TOPO-TA cloning kit (Invitrogen). The *Hin*dIII and *Xba*I restriction sites present in the pCR-2.1 vector were used for subcloning into a pcDNA (3.1+) expression vector (Invitrogen) that uses a cytomegalovirus promoter.

### Cells, media and transfection methods

Except where indicated, all experiments were performed with COS-7 cells. These cells were cultured under an air/$CO_2$ (19:1) atmosphere in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% (v/v) fetal bovine serum, 4 mM glutamine, 100 i.u./ml penicillin and 100 mg/ml streptomycin. The human U-87 glioblastoma cell line and the HT-1080 cell line derived from a metastatic lesion of human fibrosarcoma were purchased from American Tissue Culture Collection and were maintained in modified Eagle's medium containing 10% (v/v) fetal bovine serum (heat-inactivated for the HT-1080 cell line), 2 mM glutamine, 100 i.u./ml penicillin and 100 mg/ml streptomycin. COS-7 cells were transiently transfected with plasmids with the use of the non-liposomal formulation FUGENE-6 transfection reagent (Boehringer Mannheim). All experiments involving these cells were performed 36 h after transfection. Mock transfections of COS-7 cultures with pcDNA (3.1+) expression vector alone were used as controls.

### Detergent-solubility experiments

Crude membranes isolated from MT1-MMP-transfected COS-7 cells were solubilized on ice in 20 mM Tris/HCl, pH 7.4, with 1% Triton X-100, NOG, CHAPS, $Na_2CO_3$ or SDS for 30 min. The homogenates were then centrifuged for 1 h at 200000 ***g*** at 4 °C. The MT1-MMP protein content of the resulting particulate and soluble fractions was then examined by immunoblotting.

### Extraction of caveolae-enriched membrane fractions with alkaline carbonate, and purification

Carbonate extraction was performed as described previously [15]. In brief, cells were grown to confluence in a F-75 dish and were washed twice in cold PBS. After aspiration of the PBS solution, 2 ml of 500 mM $Na_2CO_3$, pH 11.0, was used to scrape the cells off the dish. The sample was transferred to a 5 ml polycarbonate tube and was homogenized with a Polytron instrument followed by sonication. The resulting homogenate was mixed with an equal volume of 90% (w/v) sucrose prepared in 25 mM Mes/0.15 M NaCl (pH 6.5) (MBS). The sample was then transferred to a 12 ml ultracentrifuge tube and overlaid with a discontinuous sucrose gradient [4 ml of 35% (w/v) sucrose, 4 ml of 5% (w/v) sucrose, both prepared in MBS, lacking detergent]. The samples were subjected to centrifugation at 200000 ***g*** (39000 rev./min in a Beckman SWT-1 rotor) for 18 h at 4 °C. A light-scattering band was observed at the 5%/35% sucrose interface. Twelve 1 ml fractions were collected, and 20 $\mu$l aliquots of each fraction were subjected to SDS/PAGE and immunoblot analysis.

### Migration assay

Transwells (8 $\mu$m pore size; Costar) were precoated with 0.5% gelatin/PBS by adding 200 $\mu$l of the solution per transwell and allowing the membranes to dry in air in a laminar-flow hood at room temperature. The transwells were then assembled in a 24-well plate (Falcon 3097); the lower chambers were filled with 600 $\mu$l of DMEM supplemented with 10% (v/v) fetal bovine serum and the upper chamber of each transwell was filled with 200 $\mu$l of COS-7 cells ($7.5 \times 10^4$ cells/ml). The plate was then placed at 37 °C in 5% $CO_2$/95% air for 2 h. Cells that had migrated to the lower surface of the filters were fixed and stained with 0.1% Crystal Violet/20% (v/v) methanol before being counted. Results are presented as average numbers of migrated cells per five fields (magnification × 100).

### Gelatin zymography

Gelatinolytic activity in culture medium from monolayer cultures was detected by gelatin zymography as described previously [16]. In brief, an aliquot (20 $\mu$l) of the culture medium was subjected to SDS/PAGE with a 7.5% (w/v) polyacrylamide gel containing 0.1 mg/ml gelatin. The gels were then incubated for 30 min at room temperature twice in 2.5% (v/v) Triton X-100 to remove SDS, then rinsed five times in doubly distilled water. The gels were incubated at 37 °C for a further 20 h in 20 mM NaCl/5 mM $CaCl_2$/0.02% (v/v) Brij-35/50 mM Tris/HCl buffer (pH 7.6), then stained with 0.1% Coomassie Brilliant Blue R-250, followed by destaining in 10% (v/v) acetic acid/30% (v/v) methanol in water. Gelatinolytic activity was detected as unstained bands on a blue background and was quantified by densitometric measurement. All experiments were performed with cells that had been serum-deprived by incubation overnight.

### Western blot analysis

Cell lysates, crude membrane and sucrose-gradient fractions were subjected to SDS/PAGE under reducing conditions and

transferred to PVDF membranes. Immunoblotting procedures were performed as described previously in detail [16]. PVDF membranes were incubated with primary antibody, washed and incubated with a secondary antibody conjugated with horseradish peroxidase (Jackson). Bound IgGs were detected with a chemiluminescent substrate.

### Isolation of total RNA and reverse-transcriptase-mediated PCR (RT–PCR) analysis

Total RNA was extracted from cultured U-87, HT-1080 and COS-7 cells using the TriZOL Reagent. First-strand cDNA synthesis followed by specific gene-product amplification was performed with the Titan One Tube RT–PCR Kit (Roche Molecular Biochemicals). Primers were all derived from human sequences; PCR conditions were optimized so that the gene products were found to be at the exponential phase of the amplification [17]. PCR products were resolved on 2% (w/v) agarose gels containing 1 $\mu$g/ml ethidium bromide.

## RESULTS

### Expression, function and proteolytic processing of recombinant MT1-MMP in COS-7 cells

To investigate the mechanism underlying proMMP-2 activation by MT1-MMP, we first cloned the full-length MT1-MMP cDNA from human U-87 glioblastoma cells. The recombinant MT1-MMP (rMT1-MMP) protein was overexpressed in COS-7 cells and tested for its ability to promote the activation of an exogenous source of proMMP-2. As shown by gelatin zymography in Figure 1(A), a marked activation of the MMP-2 zymogen was induced in MT1-MMP-transfected COS-7 cells in comparison with mock-transfected cells, in which only the proMMP-2 form was observed. Interestingly, incubation of MT1-MMP-transfected COS-7 cells in the presence of the lectin Con A or the cytoskeleton-disrupting agent Cyto D further increased the activation of proMMP-2 as quantified by densitometric measurements (32% for Con A; 41% for Cyto D), whereas preincubating the transfected COS-7 cells with EGCg, a green-tea polyphenol derivative that we recently identified as a potent inhibitor of MMP-2 gelatinolytic activity [18], decreased the rMT1-MMP-dependent proMMP-2 activation process (Figure 1A). Here we demonstrate for the first time that EGCg might also act at the cell surface of transfected COS-7 cells directly on an MT1-MMP-dependent process involved in the activation of proMMP-2.

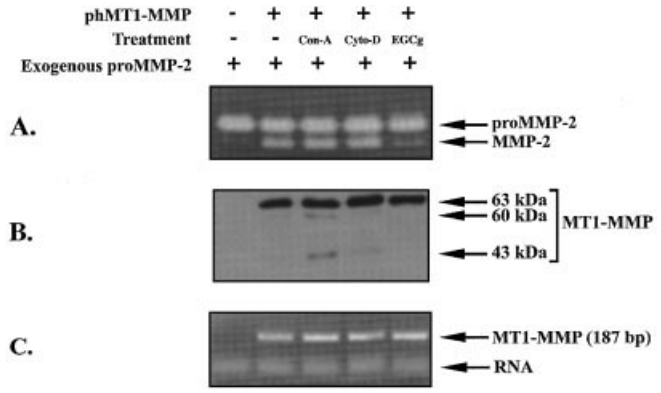


**Figure 1 Recombinant MT1-MMP protein activates proMMP-2 and is processed proteolytically in COS-7 cells**

COS-7 cells were transfected with *ph*MT1-MMP (+) as described in the Materials and methods section and then treated with 10 $\mu$g/ml Con A, 1 $\mu$M Cyto D or 25 $\mu$M EGCg for 18 h. (**A**) The cells were subsequently washed and incubated for 12 h in conditioned medium isolated from U-87 cells and containing high levels of secreted proMMP-2. The activation of proMMP-2 was monitored by gelatin zymography. (**B**) Lysates isolated from mock-transfected (−) and *ph*MT1-MMP-transfected (+) COS-7 cells were resolved (20 $\mu$g per well) by SDS/PAGE [9% (w/v) gel]. MT1-MMP immunoreactivity was analysed; the 63 and 60 kDa bands are respectively the unprocessed and processed active forms of MT1-MMP, whereas the 43 kDa band is the processed inactive form. (**C**) Total RNA was extracted from mock-transfected (−) and *ph*MT1-MMP-transfected (+) COS-7 cells; 1 $\mu$g of this was subjected to RT–PCR analysis of MT1-MMP. The resulting amplified products of 187 bp were resolved on 2% (w/v) agarose gels containing 1 $\mu$g/ml ethidium bromide.

Expression of MT1-MMP at the cell surface of COS-7 cells was explored further with particular emphasis on its own proteolytic processing mechanisms. The MT1-MMP protein was absent from COS-7 cell lysates (Figure 1B); this was further reflected at the gene level by a lack of detectable MT1-MMP transcript (Figure 1C). However, immunoreactive rMT1-MMP bands were detected solely in MT1-MMP-transfected cells and this was correlated by RT–PCR with the amplification of a 187 bp cDNA fragment of MT1-MMP in those transfected cells only (Figure 1C). At the protein level, the rMT1-MMP appeared as its full-length 63 kDa pro-form product in unstimulated COS-7 cells and was shown to be processed first to its 60 kDa form and subsequently to its inactive 43 kDa form after treatment with Con A or Cyto D but not EGCg (Figure 1B). These observations are of particular interest as the Cyto D- and Con A-induced processing of rMT1-MMP expressed in COS-7 cells is not correlated with an increase in either MT1-MMP protein or gene expression levels (Figures 1B and 1C). Taken together, this validation of our rMT1-MMP-encoding gene enabled us to investigate further the subcellular localization of the MT1-MMP-dependent events leading to proMMP-2 activation in U-87 glioblastoma and HT-1080 fibrosarcoma tumour cell lines.

### Localization of MT1-MMP in Triton-insoluble and caveolin-rich domains

The question of whether or not MT1-MMP is located within a detergent-insoluble membrane domain might have important implications with regard to the mechanisms involved in the regulation of its activity. Crude membranes were isolated from MT1-MMP-transfected COS-7 cells and subjected to extraction with Triton X-100, $Na_2CO_3$, NOG, CHAPS or SDS. Figure 2 shows that MT1-MMP is indeed located in detergent-insoluble membranes; only SDS treatment, at increasing temperature, resulted in its complete extraction. Insolubility in Triton X-100 is a characteristic of detergent-insoluble glycolipid-rich domains ('DIGs'), of which caveolae can be considered a subclass.

The MT1-MMP proteolytic processing associated with crude membrane preparations, as well as its poor membrane extractability, prompted us to go one step further and to localize the subcellular compartments that are effectively involved in MT1-MMP processing. Interestingly, sequence alignment analysis of MT1-MMP resulted in close matches to aromatic-rich consensus caveolin-binding motifs (ΦXΦXXXΦ, ΦXXXXΦXXΦ or ΦXΦXXXXΦXXΦ, where Φ represents Trp, Phe or Tyr), reported to interact with the caveolin-scaffolding domain [19]. Four of these potential sequences were found in several members of the MMP family and were all located in their haemopexin-like domain, a region known to have a functional role in substrate binding and/or in interactions with TIMPs [20]. The most conserved caveolin-binding motif found in MT1-MMP was found in its haemopexin-like domain and was located between $Tyr^{323}$ and $Phe^{333}$ (YFFRGNKYYRF), whereas the three other less well conserved sequences were also found in the haemopexin-

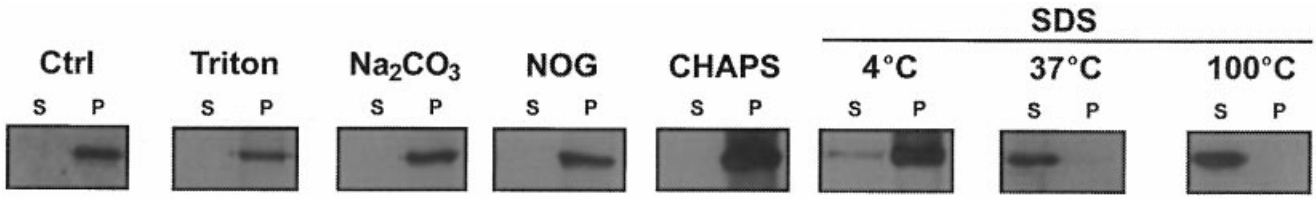


**Figure 2 Solubility of rMT1-MMP in detergents**

Crude membranes were isolated from MT1-MMP-transfected COS-7 cells and solubilized for 30 min on ice in 20 mM Tris/HCl (pH 7.4) containing 1% Triton X-100, NOG, CHAPS, $Na_2CO_3$ or SDS. The homogenates were then centrifuged for 1 h at 200000 ***g*** at 4 °C. The 63 kDa MT1-MMP protein content of the resulting particulate (P) and soluble (S) fractions was examined by immunoblotting.

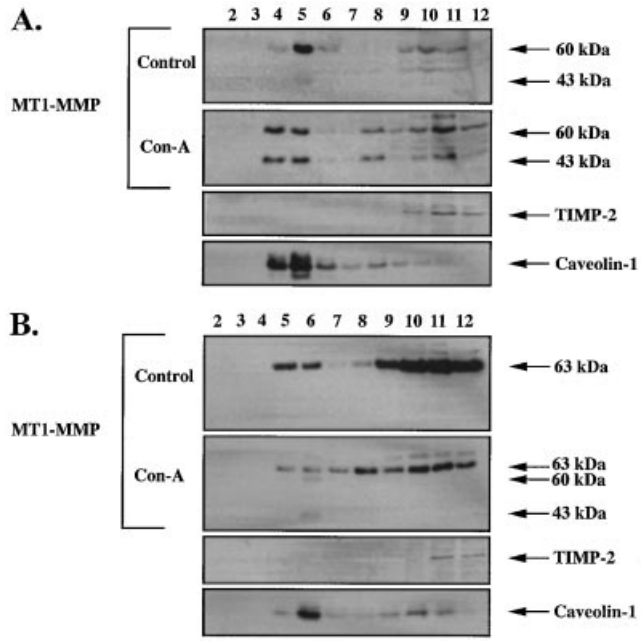


**Figure 3 Localization of MT1-MMP in low-density sucrose-gradient fractions containing caveolin 1 prepared from U-87 glioblastoma and *ph*MT1-MMP-transfected COS-7 cells**

Carbonate extraction was performed as described in the Materials and methods section; lysates were fractionated in a discontinuous sucrose-density gradient. Fractions 2–12 were analysed for MT1-MMP, TIMP-2 and caveolin 1 immunoreactivity in U-87 glioblastoma cells (**A**) and in *ph*MT1-MMP-transfected COS-7 cells (**B**). Treatment with 10 $\mu$g/ml Con A was performed on serum-deprived cells for 18 h.

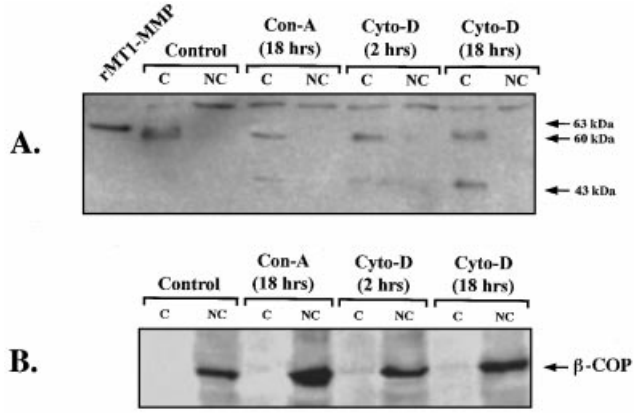


**Figure 4 Preferential localization of the MT1-MMP processing events within the caveolin-rich membrane domains**

Caveolae were isolated from HT-1080 cell lysates by using a discontinuous sucrose-density gradient. Equal amounts (5 $\mu$g) of protein were precipitated with trichloroacetic acid and separated by SDS/PAGE [9% (w/v) gel] for MT1-MMP immunodetection (**A**) or by SDS/PAGE [6.5% (w/v) gel] for $\beta$-COP immunodetection (**B**). The caveolae-enriched fraction (C) is compared with the non-caveolae fraction (NC) isolated for each cell treatment. Cells were treated or not for 18 h with 10 $\mu$g/ml Con A or for 2–18 h with 1 $\mu$M Cyto D. Arrows in (**A**) indicate the processed forms of MT1-MMP as described in the legend to Figure 1(B).

like domain and were located respectively between $Phe^{229}$ and $Phe^{236}$, $Phe^{277}$ and $Phe^{285}$, and $Tyr^{371}$ and $Phe^{381}$. Interestingly, all of the four caveolin-binding motifs were located at each of the putative antiparallel four-stranded $\beta$ sheets within the hydrophobic core of the proposed topology of the haemopexin-like domain of MMPs [21].

On the basis of these observations and on the poor Triton X-100 extractability of MT1-MMP from crude membrane preparations, we investigated the potential localization of MT1-MMP in low-density sucrose gradient fractions by a widely used detergent-free procedure to isolate the specialized membrane domains that represent caveolae [15]. We found that MT1-MMP of U-87 glioblastoma cells (Figure 3A) as well as rMT1-MMP expressed in COS-7 cells (Figure 3B) co-sedimented with caveolae-enriched membrane domains (Figure 3, fractions 4–6 of the sucrose gradient). The absence of MT1-MMP from lysates of untransfected COS-7 cells (Figure 1B) was confirmed in caveolae isolated from untransfected COS-7 cells (results not shown). The function of the MT1-MMP protein and its caveolar localization were also investigated with Con A and Cyto D. We show that incubation of the U-87 glioblastoma and MT1-MMP-transfected COS-7 cells with Con A induced the processing of MT1-MMP, which was reflected by the appearance of the 43 kDa MT1-MMP-immunoreactive bands associated with the caveolae-enriched fractions (Figures 3A and 3B). The caveolin 1 profile distribution was unaffected by the treatment with Con A; only a representative 22 kDa caveolin 1 immunodetection is therefore shown for each cell line for the sake of clarity. Similar co-sedimentation profiles of caveolin 1 and of MT1-MMP on sucrose-density gradients were observed in the human HT-1080 fibrosarcoma cell line. We also examined whether COS-7 and U-

87 glioblastoma cells produce endogenous TIMP-2, a soluble and secreted protein involved in the MT1-MMP-dependent activation of proMMP-2. Caveolae were isolated and intracellular TIMP-2, which has no potential caveolin-binding motif and no myristoylation site, sedimented exclusively as a 25 kDa protein in non-caveolar sucrose-gradient fractions isolated from both cell lines (Figure 3, fractions 10–12 of the sucrose gradient).

### Proteolytic processing of MT1-MMP takes place preferentially in caveolae

To assess further the proportion of MT1-MMP associated with caveolae fractions, caveolae were isolated from these cells and equal amounts of protein were separated by SDS/PAGE. Immunodetection of MT1-MMP in the caveolae-enriched fraction (fraction 5 from the sucrose gradient) was compared with that in the non-caveolae fraction (fraction 12 from the sucrose gradient). As shown in Figure 4(A), immunoreactive MT1-MMP forms were preferentially enriched in the caveolar fraction. Furthermore, treatment with Con A or Cyto D clearly resulted in the processing of the 60 kDa MT1-MMP protein to its 43 kDa form in comparison with the MT1-MMP located in the caveolae fraction isolated from control untreated cells. Finally, some evidence suggesting that caveolae prepared in the absence of detergent might have been contaminated by non-caveolar fragments prompted us to examine the presence of $\beta$-COP, a marker for Golgi membranes [22]. In contrast with MT1-MMP, Figure 4(B) clearly shows that $\beta$-COP was enriched in non-caveolar fractions, confirming that MT1-MMP was indeed preferentially localized in caveolae-enriched domains.

### Recombinant expression of caveolin 1 blocks MT1-MMP-induced migration in COS-7 cells

Caveolin 1 has been proposed to function as a putative tumour suppressor by inhibiting signalling molecules involved in cell growth and mitogenesis [12,13,19]. We next examined whether the expression of caveolin 1 interfered with any of the MT1-MMP-dependent intracellular events leading to the activation of proMMP-2. We also examined the role of caveolin 1 and MT1-MMP in cell movement. We therefore transfected U-87 glioblastoma and COS-7 cells with caveolin 1 cDNA (Cav-1) and assessed their MT1-MMP-dependent proMMP-2 activation ability. As shown in Figure 5 (upper panels), the expression of recombinant caveolin 1 did not affect the potential of U-87 cells to activate proMMP-2; neither did it potentiate proMMP-2 activation induced by Con A or Cyto D. Similarly, MT1-MMP-transfected COS-7 cells did not show any significant change in rMT1-MMP-induced proMMP-2 activation, whether Cav-1 was transfected alone or in combination with MT1-MMP cDNA (Figure 5, upper panels). However, when cell migration was assayed in gelatin-coated transwells, MT1-MMP-transfected COS-7 cells showed a marked increase in their migratory potential, and the expression of recombinant caveolin 1 completely antagonized this increase (Figure 5, lower panels). In contrast, Cav-1-transfected U-87 glioblastoma cells did not show any significant change in their MT1-MMP-induced migratory potential (results not shown), possibly owing to the rapid MT1-MMP protein turnover in these cells [23], which would diminish the potential interaction of the protein with recombinant caveolin 1 or with intracellular partners through caveolin-1-mediated events. As caveolin 1 has an inhibitory role in many intracellular signalling events by interacting with membrane-bound components, it is tempting to suggest that caveolin 1 might indeed either interact physically with MT1-MMP or modulate MT1-MMP-dependent transduction events involved in cell migration.

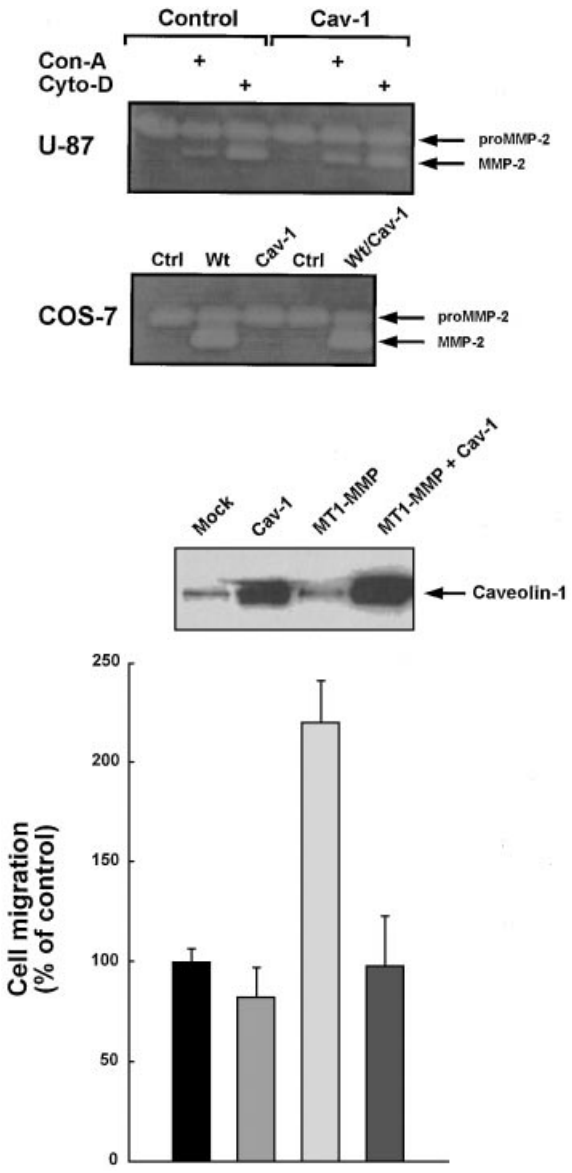


**Figure 5 Recombinant expression of caveolin 1 does not antagonize MT1-MMP-dependent proMMP-2 activation but blocks MT1-MMP-induced cell migration**

Upper panel: U-87 glioblastoma cells were transfected or not with Cav-1 cDNA and subsequently treated with 10 $\mu$g/ml Con A or 1 $\mu$M Cyto D for 18 h. Similarly, COS-7 cells were transfected with pcDNA(3.1 +) vector alone (Ctrl) or with Cav-1 cDNA and incubated for 12 h in conditioned medium containing high levels of proMMP-2. The activation of proMMP-2 was monitored by gelatin zymography. Lower panels: COS-7 cells (Mock) were transfected with cDNA encoding caveolin 1 or MT1-MMP or were co-transfected with MT1-MMP–caveolin 1. Evaluation of cellular migration was performed for 2 h at 37 °C by using transwells with gelatin-coated membranes. The numbers of cells that crossed the membrane were normalized to those of mock-transfected cells. The values presented are averages for five fields from three separate experiments.

## DISCUSSION

Here we provide evidence that MT1-MMP, present in two highly invasive glioblastoma (U-87) and fibrosarcoma (HT-1080) tumour cell lines, is preferentially associated with low-density caveolae-enriched membrane domains and that the cellular events leading to proMMP-2 activation, that are correlated with the

MT1-MMP proteolytic processing, are also associated with these domains. Moreover, the expression of a recombinant form of the protein in a heterologous system (COS-7 cells) also resulted in its preferential localization to these domains. This strongly suggests that some intrinsic features of the MT1-MMP protein enable its targeting and association to caveolae. In this respect it is noteworthy that MT1-MMP, like some other members of the MMP family, possesses highly conserved consensus sequences reported to be involved in the interaction with caveolin 1, a caveolae marker protein thought to be differentially regulated by angiogenesis activators and inhibitors [24]. However, whether the caveolin-binding motifs located in MT1-MMP are recognized by caveolin 1 will most probably depend on the conformation specificity of the MT1-MMP protein because these motifs are localized in its extracellular haemopexin-like domain. These criteria for interaction with caveolin 1 might therefore strictly regulate the expression and intracellular vesicular trafficking leading to secretion of the other soluble members of the MMP family. Alternatively, until the three-dimensional structure of MT1-MMP has been elucidated, we can speculate that the four caveolin-binding motifs might oligomerize to some extent and regulate the formation of the four-bladed $\beta$-propeller haemopexin-like structure that is common to all MMPs except matrilysin [25]. It is also tempting to suggest that the expression of MT1-MMP at specialized cell plasma-membrane domains such as caveolae might similarly be controlled by the caveolin-scaffolding domain, which could also serve ultimately as a natural regulator of the invasive behaviour of cancer cells. Point mutations of the caveolin-1-binding domain found in MT1-MMP will be needed to address this question and are currently under investigation. Given that integrins are linked both to extracellular matrix components and to the cytoskeleton, our observation of the preferential localization of MT1-MMP into caveolae of cancer cells might also provide new insight and have a potential effect in terms of cell adhesion and proteolytic mechanisms involved in angiogenesis and tumour cell invasion.

MT1-MMP has been implicated as a physiological activator of proMMP-2 that in various cancers has an important role as a cell-surface receptor for the TIMP-2–MMP-2 complex [2]. MT1-MMP expression (along with MMP-2 and MMP-9) is correlated with the malignancy of gliomas [26] and also with that of lung, cervical and breast cancer and melanomas [3]. Interestingly, the invasion of malignant melanoma cells occurs only when MT1-MMP localizes in plasma membrane invadopodia, which are cell-membrane protrusions contacting or invading the surrounding matrix [27]. More recently, a role for caveolin 1 in the inhibition of lamellipod extension and cell migration in metastatic mammary adrenocarcinoma cells has also been demonstrated [14]. Thus the presence of MT1-MMP in specialized plasma membrane domains such as caveolae not only emphasizes its role in its recognized function in recruiting the MT1-MMP–MMP-2 complex to sites of invasion but also implies a potential role for caveolae-associated MT1-MMP, which might somehow be crucial in cancer cell lines, similarly to the role of caveolae in regulating several pathophysiological states such as in insulin signalling in diabetes and in the processing of $\beta$-amyloid peptide in brain in Alzheimer disease [13].

MT1-MMP enables the invasive migration of glioma cells in white matter in the central nervous system [3]; regulation of this cell invasion activity is thought to involve MT1-MMP's cytoplasmic and transmembrane domains [6]. This observation is interesting because the cytoplasmic tail of MT1-MMP contains potential phosphorylation sites that might facilitate the deposition of MT1-MMP in integrin-rich invadopodia. Moreover, a role for caveolin in the stimulation of MMP in the extracellular matrix, as well as implications for angiogenesis *in vivo* and tumour invasion, is under investigation. Caveolin and the urokinase receptor (uPAR), for example, are necessary for normal $\beta$1-integrin function in 293 cells; the depletion of caveolin disrupted both uPAR–integrin complexes and uPAR-dependent adhesion [28]. The results presented here provide for the first time evidence not only that MT1-MMP is preferentially located in caveolae but that its proteolytic processing, which is correlated with a rapid proMMP-2 activation, also takes place in such specialized domains. Although the 43 kDa form of MT1-MMP is predicted to be catalytically inactive, it might still have important biological properties because it retains both its haemopexin-like domain and its cytoplasmic tail. Moreover, our observations also indicate a caveolae-regulated function of MT1-MMP in the invasive behaviour of malignant cells. The specific localization of key members of the enzymic machinery leading to the activation of proMMP-2 within caveolae might finally afford the cell a tighter control of the activation and repression of signalling than would be possible if all players diffused freely throughout the cytoplasm. This implies that these domains might represent novel targets for manipulating the invasive potential of tumour cells.

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