Calmodulin inhibitors trigger the proteolytic processing of membrane type-1 matrix metalloproteinase, but not its shedding in glioblastoma cells

Borhane ANNABI*†, Anthony PILORGET*†, Nathalie BOUSQUET-GAGNON*†, Denis GINGRAS*† and Richard BÉLIVEAU*†¹

*Centre de Cancérologie Charles-Bruneau, Hôpital Sainte-Justine, 3175 Côte Ste-Catherine, Montréal, Québec, Canada H3T 1C5, and †Université du Québec à Montréal, C.P. 8888, Succ. Centre-ville, Montréal, Québec, Canada H3C 3P8

Most transmembrane proteins are subjected to limited proteolysis by cellular proteases, and stimulation of cleavage of membrane proteins by calmodulin (CaM) inhibitors was recently shown. The present study investigated the ability of several CaM inhibitors to induce the proteolytic cleavage of the membrane type-1 matrix metalloproteinase (MT1-MMP) from the cell surface of highly invasive U-87 glioblastoma cells. Although no shedding of a soluble MT1-MMP form was induced by CaM inhibitors in the conditioned media, we showed that these inhibitors induced MT1-MMP proteolytic processing to the 43 kDa membrane-bound inactive form that was not correlated with an increase in proMMP-2 activation but rather with an increase in tissue inhibitor of MMPs (TIMP)-2 expression levels. Moreover, this proteolytic processing was sensitive to marimastat

suggesting the involvement of MMPs. Interestingly, CaM inhibitors antagonized concanavalin A- and cytochalasin D-induced proMMP-2 activation, and affected the cytoskeletal actin organization resulting in the loss of migratory potential of U-87 glioblastoma cells. Cytoplasmic tail-truncated MT1-MMP constructs expressed in COS-7 cells were also affected by CaM inhibitors suggesting that these inhibitors stimulated MT1-MMP proteolytic processing by mechanisms independent of the CaM–substrate interaction. We also propose that TIMP-2 acts as a negative regulator of MT1-MMP-dependent activities promoted by the action of CaM inhibitors in U-87 glioblastoma cells.

Key words: actin cytoskeleton, cell migration, glioma, MT1-MMP, TIMP-2.

INTRODUCTION

Matrix metalloproteinases (MMPs) are a family of zinc-dependent enzymes that contribute to the degradation of the extracellular matrix (ECM). Most MMPs produced by tumour cells are originally implicated in the uncontrolled destruction of the ECM, eventually leading to tumour invasion and metastasis. However, other roles for MMPs during multiple stages of tumour progression include functions such as angiogenesis, cell growth and migration [1]. Since increased expression of proMMP-2 is particularly correlated with tumour invasion and metastasis *in vivo* and *in vitro* [2], it is of interest to clarify the mechanism of proMMP-2 activation. It is now well established that membrane type-1 (MT1)-MMP is an endogenous cell-surface receptor and activator of proMMP-2 and is expressed as an active form in most normal and neoplastic cells [3]. MT1-MMP is synthesized as a zymogen that can be processed to its mature, catalytically active form after the removal of its regulatory propeptide domain [4]. Moreover, the existence of a proprotein convertase-MT1-MMP axis that can regulate ECM remodelling through both furin-dependent and furin-independent MT1-MMP processing pathways was also demonstrated, suggesting that an auto-proteolytic cleavage may be programmed in eliciting MT1-MMP functions [5]. In addition to propeptide cleavage, MT1-MMP is also proteolytically processed at the cell surface to a 43 kDa membrane-bound inactive form, a process that is closely associated with proMMP-2 activation [6].

Interestingly, although the potential transmembrane domain of MT1-MMP deduced from the amino acid sequence functions as a membrane linker, the specific sequence of that trans-

membrane domain does not seem to be essential for functional activity [7]. More recently, expression and purification of active soluble forms of MT1-MMP lacking the transmembrane and cytoplasmic domains [8] has provided support for the existence of a soluble latent form of MT1-MMP secreted by primary human cells in culture [9,10], and to the concept of proteolytic cleavage and release of the membrane-anchored MT1-MMP protein from the outside of the cell surface. Many cellular membrane proteins are subjected to limited proteolysis giving rise to soluble forms consisting of the entire extracellular domains of the proteins [11]. Recent reports have suggested that shedding from the membranes may be an important regulatory mechanism of MT1-MMP activity [10,12,13].

The ectodomain of a number of transmembrane proteins can be shed by proteolytic cleavage and released as soluble fragments by the action of cell surface proteases. The activity of the proteases involved in shedding is highly regulated by several intracellular second messenger pathways, such as protein kinase C and intracellular Ca^{2+} [14]. Interestingly, Ca^{2+} influx has been reported to inhibit MT1-MMP processing and to block MT1-MMP-dependent proMMP-2 activation [15,16]. An important intracellular mediator in the actions of Ca^{2+} is calmodulin (CaM), which was recently shown to bind to the endodomain of the adhesion molecule L-selectin and to regulate its shedding [17]. CaM was also required for the interleukin-1-enhanced synthesis of tissue inhibitor of MMP (TIMP) and was found to modulate the synthesis of proMMP-1 and -3 [18], as well as the expression of MT1-MMP [19]. Moreover, it is noteworthy that CaM inhibitors were recently reported to trigger the shedding of a number of transmembrane proteins [20]. Whether CaM inhibitors

Abbreviations used: CaM, calmodulin; CLM, calmidazolium; Con-A, concanavalin A; Cyto-D, cytochalasin D; ECM, extracellular matrix; MMP, matrix metalloproteinase; MT1-MMP, membrane type-1 MMP; TIMP, tissue inhibitor of MMPs; TFP, trifluoperazine; W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide; MEM, modified Eagle's medium; RT, reverse transcriptase.

¹ To whom correspondence should be addressed at Laboratoire de Médecine Moléculaire, Université du Québec à Montréal C.P. 8888, Succ. Centre-ville, Montréal, Québec, Canada, H3C 3P8 (e-mail molmed@justine.umontreal.ca).

affected any MT1-MMP-mediated events, or induced shedding of the membrane-anchored MT1-MMP protein is not known.

In the present study, we examined the intracellular events regulating MT1-MMP activation through proteolytic processing at the cell surface. Moreover, the potential cell-surface cleavage and release mechanisms of MT1-MMP from U-87 glioblastoma cells after treatment with CaM inhibitors and cytoskeleton-disrupting agents were also investigated. Our data suggest that CaM inhibitors trigger intracellular signalling events resulting in MT1-MMP proteolytic processing to its inactive 43 kDa membrane-bound form, and that this occurred through the action of metalloproteinase(s). However, CaM inhibitors did not trigger MT1-MMP shedding from the cell surface into the conditioned media, although potential CaM binding sites were found to be located in the cytoplasmic domain of MT1-MMP. In addition, a role for TIMP-2 is suggested in regulating the activated MT1-MMP levels that, in turn, may regulate the invasive capacity of U-87 glioblastoma cells.

MATERIALS AND METHODS

Antibodies and chemicals

Anti-(human MT1-MMP) polyclonal antibody (raised against the hinge region), anti-(human MMP-2) monoclonal antibody, anti-TIMP-2 polyclonal antibody, and the enhanced chemiluminescence-Western blot kit were from Chemicon International (Temecula, CA, U.S.A.). The bicinchoninic acid protein assay kit was from Pierce and the PVDF membranes were from Boehringer Mannheim (Laval, QC, Canada). All products for electrophoresis and zymography were bought from Bio-Rad Laboratories (Mississauga, ON, Canada), TriZOL reagent, trypsin, penicillin and streptomycin were from GIBCO BRL (Burlington, ON, Canada), and fetal bovine serum was from HyClone Laboratories (Logan, UT, U.S.A.). Agarose, calmidazolium (CLM), concanavalin A (Con-A), cytochalasin D (Cyto-D), gelatin, SDS, trifluoperazine (TFP), Triton X-100, and *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide (W-7) were from Sigma Chemical Co. (Oakville, ON, Canada). The inhibitor compound marimastat (BB-2516) was generously provided by British Biotech (Oxford, U.K.).

cDNA constructs

The cloning of MT1-MMP cDNA from U-87 glioblastoma cells as an *HindIII/XbaI* fragment into a pcDNA(3.1+) (Invitrogen, Burlington, ON, Canada) eukaryotic expression vector has been described previously [21]. Briefly, the cDNA encoding MT1-MMP was isolated by nested PCR. The first round of amplification was performed for 30 cycles at an annealing temperature of 55 °C using oligonucleotide primers derived from the human MT1-MMP cDNA (GenBank® accession number D26512) and total cellular RNA extracted from U-87 glioblastoma cells as the template. The resulting 1.9 kb cDNA fragment was re-amplified with the oligonucleotides [5'-CAGCTGCAGGAATTCGTGGTCTCGGACCATGTCTCCCG-3' (sense) and 5'-CAGCTGCAGATGGGCGTCAGACCTTGTCAGC-3' (antisense)] for 40 cycles with an annealing temperature of 60 °C, resulting in a 1.4 kb cDNA fragment containing the whole open-reading frame of MT1-MMP, which was then subcloned into a pCR-2.1 vector using the TOPO-TA cloning kit (Invitrogen). The *HindIII* and *XbaI* restriction sites present in the pCR-2.1 vector were used for subcloning into a pcDNA (3.1+) expression vector (Invitrogen) employing a cytomegalovirus promoter. Cytoplasmic-domain-truncated forms of MT1-MMP were generated by PCR, subcloned into pcDNA(3.1+), and 3'-ends verified by automated

DNA sequencing (Sheldon Biotechnology Center, McGill, Canada). The respective MT1-MMP constructs are as follows: Wt encodes the full length MT1-MMP protein (Met¹-Val⁵⁸²); Δ1 encodes a protein which lacks the entire C-terminal 20 amino acid cytoplasmic domain (Met¹-Phe⁵⁶²); Δ2 lacks the terminal 16 amino acids (Met¹-Gly⁵⁶⁶); Δ3 lacks the terminal 10 amino acids (Met¹-Leu⁵⁷²); ΔTM encodes a soluble form of MT1-MMP that lacks the entire transmembrane and cytoplasmic domain (Met¹-Cys⁵⁰⁸).

Cells, media and transfection methods

The human U-87 glioblastoma cell line was purchased from A.T.C.C. and was maintained in modified Eagle's medium (MEM) containing 10% (v/v) fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin and 100 mg/ml streptomycin. Except where indicated, all transfection experiments were performed using COS-7 cells. These cells were cultured under a 5% CO₂ atmosphere in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 4 mM glutamine, 100 units/ml penicillin and 100 mg/ml streptomycin. COS-7 cells were transiently transfected with plasmids using the non-liposomal formulation FUGENE-6 transfection reagent (Boehringer Mannheim). All experiments involving these cells were performed 36 h post-transfection. Mock transfections of COS-7 cultures with pcDNA (3.1+) expression vector alone were used as controls.

Migration assays

To assess whether the proteolytic state of MT1-MMP affected U-87 glioblastoma cells migratory potential, transwells (8-μm pore size; Costar, Acton, MA, U.S.A.) were precoated with 0.5% gelatin/PBS by adding 200 μl of the solution per transwell and allowing the membranes to air dry in a laminar flow hood at room temperature (25 °C). The transwells were then assembled in a 24-well plate (Falcon 3097), the lower chambers were filled with 600 μl of MEM supplemented with 10% fetal bovine serum, and 200 μl of U-87 glioblastoma cells (7.5 × 10⁴ cells/ml) was inoculated into the upper chamber of each transwell. The plate was then placed at 37 °C in 5% CO₂/95% air for 2 h. Cells that had migrated to the lower surface of the filters were fixed, stained with 0.1% crystal violet/20% (v/v) MeOH and counted. Data are presented as the average number of migrated cells per 5 fields (× 100).

Immunofluorescent staining

U-87 glioblastoma cells grown in chamber slides (Nalge Nunc International, Naperville, IL, U.S.A.) for 24 h at 37 °C were washed with PBS, fixed by the addition of 3% (v/v) para-formaldehyde, and permeabilized with 0.2% Triton X-100 for 5 min. TRITC-phalloidin (200 nM in PBS) was applied for 30 min to permeabilize the cells for the staining of F-actin.

Gelatin zymography

Gelatinolytic activity in culture media from monolayer cultures was detected by gelatin zymography as described previously [21]. Briefly, an aliquot (20 μl) of the culture medium was subjected to SDS/PAGE using a 7.5% (w/v) acrylamide gel containing 0.1 mg/ml gelatin. The gels were then incubated for 30 min at room temperature (25 °C) twice in 2.5% (v/v) Triton X-100 to remove SDS and rinsed five times in double-distilled water. The gels were further incubated at 37 °C for 20 h in 20 mM NaCl, 5 mM CaCl₂, 0.02% Brij-35, 50 mM Tris/HCl buffer, pH 7.6,

then stained with 0.1 % Coomassie Brilliant Blue R-250, and destained in 10 % (v/v) acetic acid, 30 % (v/v) methanol in H₂O. Gelatinolytic activity was detected as unstained bands on a blue background and was quantified by densitometric measurement. All experiments were carried out with cells that had been serum-deprived by an overnight incubation.

Western blot analysis

Cell lysates were subjected to SDS/PAGE under reducing conditions and transferred to PVDF membranes. Immunoblotting procedures were performed as described in detail previously [6]. PVDF membranes were incubated with primary antibody, washed, and incubated with a secondary antibody conjugated with horseradish peroxidase (Jackson Immuno-research Laboratories, Mississauga, ON, Canada). Bound IgG was detected using a chemiluminescent substrate.

Total RNA isolation and reverse transcriptase (RT)-PCR analysis

In order to amplify DNA fragments specific to MMP-2, MT1-MMP and TIMP-2, total RNA was extracted from cultured U-87 glioblastoma 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, Laval, Quebec, Canada). RT-PCR was performed with specific oligonucleotide primers derived from human sequences, and PCR conditions had been optimized so that the gene products were found to be at the exponential phase of the amplification [21]. β -Actin was used as an internal control and was found to be constant in all test conditions. PCR products were resolved on 2 % (w/v) agarose gels containing 1 μ g/ml ethidium bromide.

RESULTS

CaM inhibitors trigger the proteolytic processing of MT1-MMP but antagonize proMMP-2 activation

We have reported previously that proMMP-2 activation could be triggered through a post-translational MT1-MMP-dependent mechanism [6], and that this process may be regulated into specialized plasma-membrane domains such as caveolae [21]. U-87 glioblastoma cells were thus treated with either cytoskeleton-disrupting agents such as Con-A and Cyto-D, or CaM inhibitors such as TFP, CLM and W-7. These CaM antagonists have been reported to increase expression of MT1-MMP in human uterine cervical fibroblasts [19]. Interestingly, while intracellular levels of CaM remained constant (results not shown), CaM inhibitors triggered MT1-MMP proteolytic processing in U-87 glioblastoma cell lysates similar to that occurring with Con-A or Cyto-D (Figure 1). This was reflected by the appearance of a 43 kDa MT1-MMP immunoreactive form that was thought to be catalytically inactive, since amino acid sequencing was previously correlated with the generation of an N-terminal Ala²⁵⁵ MT1-MMP fragment deleted of most of its catalytic domain [22]. Interestingly, Con-A and Cyto-D treatment caused the appearance of an additional MT1-MMP immunoreactive form at 45 kDa which was absent from CaM-inhibitor-treated cells. Although speculative, this may be attributed to the putative state of phosphorylation of MT1-MMP cytoplasmic residues located at Ser⁵⁷⁷, Tyr⁵⁷³ and Thr⁵⁶⁷ [23]. These residues may well be phosphorylated in response to Con-A, as reported recently [24], but not to CaM inhibitors.

Con-A and Cyto-D are known to activate proMMP-2 through an MT1-MMP-dependent process, and proteolytic processing of

Figure 1 CaM inhibitors trigger the proteolytic processing of MT1-MMP in U-87 glioblastoma cells

U-87 glioblastoma cells were cultured in serum-free media and treated or not treated with 10 μ g/ml Con-A, 1 μ M Cyto-D, 25 μ M TFP, 25 μ M W-7 or 25 μ M CLM for 18 h at 37 °C. The conditioned media was saved and the cells were subsequently washed once in PBS. Lysates were resolved (20 μ g/well) on an SDS/9% (w/v) polyacrylamide gel. MT1-MMP immunoreactivity was analysed and the 55 kDa band represents the processed active form of MT1-MMP, whereas the 43 kDa band represents the processed inactive form.

MT1-MMP is closely associated with this event [6,22]. Since we had shown that CaM inhibitors induced intracellular events leading to MT1-MMP proteolytic processing, we next examined by gelatin zymography the extent of CaM inhibitor-induced proMMP-2 activation in U-87 glioblastoma cells. Surprisingly, not only did TFP, W-7 and CLM not activate proMMP-2, but they further antagonized Con-A- and Cyto-D-induced proMMP-2 activation (Figure 2A). This effect was dose-dependent since Con-A was able to activate proMMP-2 in the presence of up to 10 μ M TFP, while higher TFP concentrations antagonized this activation (Figure 2B). Moreover, Con-A- and TFP-induced MT1-MMP proteolytic processing appeared to act synergistically, as the intensity of both the 55 and 43 kDa MT1-MMP immunoreactive forms increased during co-treatment (Figure 2C). The inhibition of the autocatalytic turnover of MT1-MMP on the cell surface thus results in a concomitant build-up of both the 43 kDa and 55 kDa forms as a consequence of *de novo* synthesis and membrane incorporation of new MT1-MMP molecules. This was later shown, in the current study, to be mediated through increased TIMP-2 protein levels, which was shown recently to regulate the nature of MT1-MMP forms at the cell surface [25]. Cell viability, based on the cleavage of the tetrazolium salt WST-1 {4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulphonate} by mitochondrial dehydrogenases (Roche Diagnostics, Laval, Quebec, Canada), was not significantly affected, and treatment of U-87 glioblastoma cells for up to 18 h combined with higher concentrations (100 μ M) of CaM inhibitors did not result in further activation of proMMP-2 (results not shown).

Metalloproteinase activities mediate TFP-induced proteolytic processing of MT1-MMP

To analyse whether Con-A- and TFP-induced MT1-MMP proteolytic processing had a similar inhibitory action, U-87 glioblastoma cells were incubated in the absence or in the presence of an MMP inhibitor, marimastat, and treated with either Con-A or TFP. As shown in Figure 3(A), Con-A-induced proMMP-2 activation was antagonized by marimastat, and this is correlated with the concomitant inhibition of MT1-MMP processing to its 43 kDa form (Figure 3B). TFP, which does not activate proMMP-2, induced MT1-MMP proteolytic processing, and this processing was also inhibited by marimastat (Figure 3B). This suggests that generation of the 43 kDa fragment by either Con-A or TFP occurred via the action of a marimastat-sensitive metalloproteinase.

Figure 2 ProMMP-2 activation is antagonized by CaM inhibitors in U-87 glioblastoma cells

(A) U-87 glioblastoma cells were cultured in serum-free media and (co-) treated or not with 10 $\mu\text{g/ml}$ Con-A, 1 μM Cyto-D, 25 μM TFP, 25 μM W-7 or 25 μM CLM for 18 h at 37 $^{\circ}\text{C}$. (B) U-87 glioblastoma cells were treated with increasing amounts of TFP in the presence or absence of 10 $\mu\text{g/ml}$ Con-A. In both approaches, the conditioned media was saved and the activation state of proMMP-2 was monitored by gelatin zymography. Arrows indicate the pro- and active forms of MMP-2. (C) Cell lysates from (B) were resolved, as described in Figure 1, and immunoblotted for MT1-MMP. The arrows indicate the 55 kDa active and 43 kDa inactive forms of MT1-MMP.

Figure 3 Metalloprotease activities mediate TFP-induced proteolytic processing of MT1-MMP

U-87 glioblastoma cells were (co-) treated or not with 5 μM marimastat in the presence or absence of 10 $\mu\text{g/ml}$ Con-A or 25 μM TFP for 18 h at 37 $^{\circ}\text{C}$. (A) Conditioned media was used to monitor proMMP-2 activation levels by gelatin zymography, whereas in (B) cell lysates were used to assess the proteolytic states of MT1-MMP by immunoblotting.

MT1-MMP-induced proMMP-2 activation is antagonized by TFP, but is independent of the cytoplasmic domain of the membrane-bound metalloproteinase

Association of CaM with the cytoplasmic tail of L-selectin has been proposed to mediate a regulatory role in L-selectin shedding [17]. CaM binding to proteins depends on small interaction modules in target proteins consisting of basic and hydrophobic motifs. Such a binding site was located in the juxtamembrane region of L-selectin and was composed of the RRLK sequence [17,26]. Analysis of the short 20 amino acid cytoplasmic domain of MT1-MMP indicated the presence of the sequence RRLK

Figure 4 MT1-MMP constructs

Only the intracellular cytoplasmic domain of MT1-MMP is depicted. The putative intracellular CaM-binding sites are underlined, the most conserved one with dotted lines. TM, transmembrane domain.

(Figure 4), which is analogous to the one involved in the L-selectin–CaM interaction. Since two other less conserved potential CaM-binding sites were also found in MT1-MMP, RRHG at the juxtamembrane region and LLDK at the C-terminal end, three MT1-MMP-intracellular-domain deletions ($\Delta 1$, $\Delta 2$ and $\Delta 3$) were constructed together with a transmembrane- and cytoplasmic-domain truncated (ΔTM) MT1-MMP soluble form (Figure 4). These constructs were transiently transfected into COS-7 cells to evaluate their proMMP-2 activating potential. As shown in the zymogram in Figure 5(A), overexpression of recombinant Wt-MT1-MMP in untreated COS-7 cells was reflected by an increase in proMMP-2 activation. This increase in MT1-MMP-dependent proMMP-2 activation was slightly potentiated by the addition of Con-A and Cyto-D, but strongly antagonized by all the CaM inhibitors tested (Figure 5A). Interestingly, similar proMMP-2 activation was observed when COS-7 cells over-expressed either the recombinant Wt- or MT1-

Figure 5 CaM inhibitors antagonize the recombinant MT1-MMP-induced activation of proMMP-2 in COS-7 cells independently of the cytoplasmic domain of MT1-MMP

(A) COS-7 cells were transiently transfected with MT1-MMP cDNA (Wt) or vector alone (—), as described in the Materials and methods section, and then treated with 10 $\mu\text{g}/\text{ml}$ Con-A, 1 μM Cyto-D, 25 μM TFP, 25 μM W-7 or 25 μM CLM for 18 h. The cells were subsequently washed and incubated for 12 h in conditioned media isolated from U-87 glioblastoma cells and containing high levels of secreted proMMP-2. (B) MT1-MMP cytoplasmic-deleted domains were similarly over-expressed in COS-7 cells and treated or not treated with 25 μM TFP for 18 h. proMMP-2 activation was assessed by gelatin zymography.

MMP-deleted cytoplasmic domain ($\Delta 1$, $\Delta 2$ and $\Delta 3$) proteins (Figure 5B, control). No proMMP-2 activation was observed when expression vector alone or a recombinant soluble form of MT1-MMP (ΔTM) was overexpressed in COS-7 cells, confirming the need for MT1-MMP to be expressed and anchored at the plasma membrane in order to induce proMMP-2 activation. However, TFP, which antagonized rMT1-MMP-dependent proMMP-2 activation (Figure 5A), induced a marked decrease ($56 \pm 6\%$) in proMMP-2 activation that was independent of the length of the MT1-MMP cytoplasmic-domain deletion (Figure 5B, +TFP). While protein overexpression in cell lysates was equivalent between all MT1-MMP constructs (results not shown), these results suggest that the MT1-MMP intracellular cytoplasmic domain does not directly participate in the CaM-cytoplasmic domain-mediated interaction.

CaM inhibitors induce TIMP-2 expression levels and antagonize *in vitro* migration of U-87 glioblastoma cells

Cell migration is an essential process in physiological and pathological conditions such as wound healing and tumour invasion. This phenomenon involves cell adhesion on the ECM mediated by integrins, and cell detachment promoted in part by MMPs. Recently, it was shown that *in vitro* cell invasion assays through matrigel were strongly enhanced when cells expressed recombinant Wt-MT1-MMP [23]. On the other hand, Kurschat et al. [27] have also shown that TIMP-2 expression levels regulated MT1-MMP-mediated activation of proMMP-2 and, most importantly, inhibited *in vitro* invasiveness of melanoma cells. Thus we assessed at the gene and protein levels whether TIMP-2 expression was modulated upon treatment of U-87 glioblastoma cells with CaM inhibitors. Furthermore, whether proteolytically processed MT1-MMP contributed to the invasive behaviour of glioblastoma cells was further analysed by the

Figure 6 CaM inhibitors induce TIMP-2 gene and protein expression levels but not MT1-MMP shedding

(A) Total RNA was isolated from U-87 glioblastoma cells treated or not with 10 $\mu\text{g}/\text{ml}$ Con-A, 1 μM Cyto-D, 25 μM TFP, or 25 μM W-7, as described in Figure 1. RT-PCR was performed to amplify a 224 bp DNA fragment for MMP-2, 187 bp for MT1-MMP and 387 bp for TIMP-2. (B) The corresponding cell lysates and conditioned media were resolved on an SDS/15% (w/v) polyacrylamide gel, and immunoblotted for TIMP-2. (C) Soluble MT1-MMP was monitored in the conditioned media of treated U-87 glioblastoma cells. The membrane-bound recombinant MT1-MMP (Wt) and the secreted recombinant soluble form of MT1-MMP (ΔTM) were expressed in COS-7 cells. The latter was isolated from the conditioned media, and both recombinant proteins were loaded as controls. U-87 glioblastoma cells were treated as described in Figure 1.

ability of the cells to penetrate a barrier composed of gelatin in Boyden chamber assays.

As shown in Figure 6(A), MT1-MMP and MMP-2 transcript levels were, as expected, increased in Con-A- and Cyto-D-treated U-87 glioblastoma cells, while TFP and W-7 treatment did not significantly change MMP-2 and MT1-MMP gene expression. However, TIMP-2 transcript levels were markedly increased when cells were treated with those CaM inhibitors. The latter observation was correlated with an increase in intracellular TIMP-2 protein levels in cell lysates, together with an increase in extracellular secreted TIMP-2 levels (Figure 6B). Interestingly, there was no release of a soluble form of MT1-MMP in the conditioned media of U-87-treated cells (Figure 6C). This observation is of importance, since the membrane-bound Wt-rMT1-MMP is shown to migrate as its full-length 63 kDa form, while a soluble secreted rMT1-MMP (ΔTM) form, isolated from the conditioned media of transfected COS-7 cells, is shown to migrate as its unprocessed (57 kDa) and processed (42 kDa) forms, and that neither of these forms is immunodetectable in concentrated conditioned media isolated from CaM inhibitor-

Figure 7 Migration of U-87 glioblastoma cells *in vitro* is antagonized by CaM inhibitors through cytoskeleton reorganization

(A) Migration was assayed in a modified Boyden chamber as described in the Materials and methods section. U-87 glioblastoma cells were treated as described in the legend of Figure 1. The cells were then trypsinized and resuspended in MEM supplemented with serum. U-87 glioblastoma cells (7.5×10^4 cells/ml) were inoculated into the upper chamber of each transwell. Migration was performed for 2 h at 37 °C and the number of cells that had migrated was determined by visually counting the cells on the lower compartment of the filter. Data represent the mean of cells per microscope field for triplicate experiments. (B) U-87 glioblastoma cells were plated (5×10^3 cells/well) in chamber slides and treated with Cyto-D, CLM or TFP. The cells were fixed with paraformaldehyde, permeabilized with Triton X-100 and stained with TRITC-phalloidin to display the actin cytoskeleton.

treated U-87 glioblastoma cells (Figure 6C), suggesting the absence of a potential cell-surface shedding process.

Finally, we show in Figure 7(A) that cell migration through gelatin-coated filters was significantly inhibited in CaM inhibitor-treated U-87 glioblastoma cells. The invasive capacity of these cells was reduced by 47 % in CLM-treated cells, 75 % in W-7-treated cells, and by 79 % in TFP-treated cells. Moreover, this reduced migratory potential may be attributed, at least in part, to cytoskeletal actin re-organization induced by CaM inhibitors (Figure 7B). Interestingly, immunofluorescent detection shows significant condensation of the actin into the cells treated with Cyto-D and TFP, whereas CLM, which resulted only in partial inhibition of migration, was not characterized by dramatic

cytoskeletal perturbation. These observations emphasize the relationship that MT1-MMP proteolytic states may have on cytoskeletal reorganization, and on the different intracellular transduction pathways that may be involved in its proteolytic processing.

DISCUSSION

Several factors and intracellular second messengers have been reported to regulate MT1-MMP expression and activity. Increased intracellular Ca^{2+} levels have been shown to inhibit the conversion of MT1-MMP protein to its active form [15,16,22], while elevated cAMP levels were reported to down-regulate the

Scheme 1 Proposed events in CaM-inhibitor-mediated inhibition of proMMP-2 activation and induction of MT1-MMP proteolytic processing in U-87 glioblastoma cells

CaM inhibitors regulate MT1-MMP proteolytic processing at the cell surface through an MMP-mediated event sensitive to marimastat (A). The nature of that MMP is unknown, and may also be a member of the ADAM family. This results in the conversion of an active 55 kDa MT1-MMP membrane-bound form to a 43 kDa MT1-MMP inactive form [5]. CaM-inhibitor-treatment of U-87 glioblastoma cells also results in an increase in the TIMP-2 transcript and protein levels (B). Low or normal secreted TIMP-2 protein levels promote the formation of the ternary complex composed of the active 55 kDa MT1-MMP, TIMP-2 and the latent proMMP-2. This promotes ECM hydrolysis through the generation of an active MMP-2 [6]. TIMP-2 and MMP-2 dissociate from MT1-MMP presumably by cleaving MT1-MMP to the observed inactive 43 kDa form. Alternatively, high secreted TIMP-2 levels, resulting from CaM-inhibitor-treatment of the cells, will eventually both bind to the activated MT1-MMP form and complex the latent proMMP-2 enzyme [36]. Cytoskeletal actin organization is also profoundly affected as CaM inhibitors induce actin condensation and lower cell migration (C). Whether this is an MMP-mediated event is unknown. This model reflects the dynamic and unique interactions between MT1-MMP and TIMP-2 in living cells that tightly regulate MT1-MMP pericellular activity.

constitutive production of MT1-MMP mRNA and protein in MDA-MB-231 human breast cancer cells [28]. In an effort to identify signal-transduction pathways that may either contribute to or modulate MT1-MMP-dependent proMMP-2 activation in U-87 glioblastoma cells, we have decided to investigate the potential role of CaM in the MT1-MMP overall proteolytic processing. CaM, a ubiquitous intracellular Ca^{2+} receptor that acts as a cellular intermediate of multiple Ca^{2+} actions, is also suggested to act as a bifunctional regulator for the interleukin-1-induced production of MMPs and TIMP in connective tissue cells [18]. Moreover, CaM also mediates changes in intracellular Ca^{2+} levels that in turn regulate the cleavage of several membrane proteins [14]. Finally, a more recent study provided evidence that a novel pathway, triggered by CaM inhibitors, may control the shedding of the ectodomain of several membrane proteins [20]. Whether MT1-MMP, which possesses potential CaM-binding sites in its cytosolic domain, is similarly shed through such an intracellular regulatory mechanism may provide a new insight into the recently reported concept and function of soluble forms of MT1-MMP [7–10,29].

In the present study, we provide evidence that CaM inhibitors triggered intracellular events that led to cell-surface proteolytic processing of MT1-MMP, but that did not induce its shedding as a soluble form into the conditioned media of treated U-87 glioblastoma cells. In these cells, treatment with structurally different CaM inhibitors triggered the processing of MT1-MMP to its inactive 43 kDa form. Such proteolysis was similarly triggered by two cytoskeletal-disruptive agents, namely Con-A and Cyto-D. Intriguingly, whereas the latter induced a concomitant proMMP-2 activation, CaM inhibitors were unable to

generate the active form of proMMP-2, although MT1-MMP was proteolytically processed. It is thus tempting to suggest that CaM inhibitors preferentially generate a proteolytically processed form of MT1-MMP that may have other biological functions in addition to its role in proMMP-2 activation. Among these functions, it is noteworthy that gene disruption of MT1-MMP resulted in the development of severe cranial dysmorphism, dwarfism, osteopaenia, arthritis, and fibrosis of soft tissues [30,31]. How Ca^{2+} /CaM is related to these different functions involving MT1-MMP has yet to be elucidated. Interestingly, the MT1-MMP proteolytic processing triggered by Con-A, Cyto-D or the CaM inhibitor TFP was highly sensitive to the metalloprotease inhibitor marimastat, indicating that the final components of the processing machinery may in fact be under the control of a member of the MMPs family, or from recently described proteins containing a disintegrin and MMP domains known as ADAMs.

MT1-MMP is thought to enable and regulate cell-invasion activity through its cytoplasmic and transmembrane domains [32]. This observation is interesting, as the short 20 amino acid cytoplasmic tail of MT1-MMP contains potential phosphorylation sites that may indeed facilitate deposition of MT1-MMP to integrin-rich invadopodia [33]. However, the intriguing observation that overexpressed MT1-MMP cytoplasmic-domain-deleted mutants ($\Delta 1$, $\Delta 2$ and $\Delta 3$) were similarly affected compared with the Wt-MT1-MMP protein, suggests that alternative mechanisms are involved in CaM inhibitor-mediated proteolytic processing of MT1-MMP and regulation of proMMP-2 activation in U-87 glioblastoma cells. ProMMP-2 activation is dependent on the balance of many components at the cell surface

of invasive cells. In this respect, a large body of evidence suggests that the equilibrium existing between MMPs and TIMPs determines whether cells will invade the ECM or not [34]. Several recent studies pointed out the importance of TIMP-2 expression levels in the regulation of MT1-MMP-mediated activation of proMMP-2 in invasive melanoma cells [27,35]. Moreover, the dynamic and unique interactions between MT1-MMP and TIMP-2, that tightly regulate MT1-MMP pericellular activity, were recently investigated [36] and prompted us to determine the importance of TIMP-2 in the CaM inhibitors actions.

The stringent temporal and spatial co-expression of the *MT1-MMP* and *TIMP-2* genes during development suggests common regulatory pathways that may have important functional implications for the activation of proMMP-2 in health and disease [37]. Perhaps the one aspect of MT1-MMP that deserves our attention is the nature of its interaction with TIMP-2 and the role they play in proMMP-2 activation. In this study, we provide evidence that the MT1-MMP proteolytic processing triggered by CaM inhibitors in a highly invasive glioblastoma cell line is also accompanied by increased expression levels of TIMP-2. Increased gene expression levels were also reflected by increased secreted TIMP-2 protein, whereas TIMP-2 protein levels remained constant when cells were treated with Con-A or Cyto-D. Interestingly, all tested CaM inhibitors significantly inhibited U-87 glioblastoma cell migratory potential. Accordingly, it is thus tempting to hypothesize that the high TIMP-2 protein levels may interact and be involved in MT1-MMP processing through the action of a distinct metalloproteinase, generating an inactive MT1-MMP form at the cell surface or limiting the availability of active MT1-MMP to transduce cell migration. As the activation of proMMP-2 at the cell surface is regulated by the balance between MT1-MMP complexed by TIMP-2 and TIMP-2-free MT1-MMP [38], high concentrations of TIMP-2 will thus bind and inhibit any active MT1-MMP by potentially increasing MT1-MMP proteolytic processing, hence completely preventing any proMMP-2 activation by CaM inhibitors (Scheme 1). Accordingly, surface-associated MT1-MMP-bound TIMP-2 was recently proposed to be internalized and then degraded in human tumour cell lines, controlling the binding and hence the subsequent activation of proMMP-2 [39].

It is of interest to note that, independently of its binding to and regulation of MT1-MMP proteolytic processing [38–40], TIMP-2 also functions as a regulator of cell proliferation where Ca^{2+} -dependent cytoskeletal rearrangements are tightly regulated [41,42]. In this light, it is known that proMMP-2 and -9 activation is regulated by the organization of the polymerized actin cytoskeleton [43,44]. Whether the actin cytoskeleton is affected by CaM inhibitors and whether this cytoskeletal modulation is dependent on the proteolytic state of MT1-MMP deserves attention and remains to be further investigated with regard to the migratory potential of U-87 glioblastoma cells. In a recent study, overexpression of MT1-, MT2-, and MT3-MMP in epithelial cells promoted invasion of basement membrane, whereas a wide range of soluble MMPs did not [45]. In agreement with this observation, we have also shown that overexpression of rMT1-MMP in COS-7 cells increased cell migration [21].

Although MT1-MMP soluble forms have been reported to be secreted by human mesangial cells [9] or a breast carcinoma cell line [10,29], their function in wound healing, cancer progression and metastasis remains unexplained. Recombinant MT1-MMP soluble forms were able to activate proMMP-2 and to readily degrade known ECM substrates of MT1-MMP including gelatin, fibronectin, vitronectin, laminin and tenascin [7,8]. Here, we show that stimulation and regulation of MT1-MMP shedding is not triggered by CaM inhibitors, and that cytoplasmic-domain

deletion analysis indicated that CaM inhibitors induced MT1-MMP proteolytic processing by mechanisms independent of the association of CaM with its intracellular cytosolic domain. Moreover, in light of our observations, identification of the molecular components implicated in MT1-MMP proteolytic processing may allow us to further link MT1-MMP-dependent cell migration and cytoskeletal re-organization.

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