

# The transmembrane domain is essential for the microtubular trafficking of membrane type-1 matrix metalloproteinase (MT1-MMP)

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## Summary

Membrane type-1 matrix metalloproteinase (MT1-MMP) degrades the extracellular matrix, initiates the activation pathway of soluble MMPs and regulates the functionality of cell adhesion signaling receptors, thus playing an important role in many cell functions. Intracellular transport mechanisms, currently incompletely understood, regulate the presentation of MT1-MMP at the cell surface. We have focused our efforts on identifying these mechanisms. To understand the transport of MT1-MMP across the cell, we used substitution and deletion mutants, the trafficking of which was examined using antibody uptake and Chariot delivery experiments. Our experiments have demonstrated that the microtubulin cytoskeleton and the centrosomes (the microtubulin cytoskeleton-organizing centers) are essential for the trafficking and the internalization of MT1-MMP. We determined that after reaching the plasma membrane, MT1-MMP is internalized

in the Rab-4-positive recycling endosomes and the Rab-11-positive pericentrosomal recycling endosomes. The microtubular trafficking causes the protease to accumulate in the pericentrosomal region of the cell. We believe that the presence of the transmembrane domain is required for the microtubular vesicular trafficking of MT1-MMP because the soluble mutants are not presented at the cell surface and they are not delivered to the centrosomes. The observed transport mechanisms provide a vehicle for the intracellular targets and, accordingly, for an intracellular cleavage function of MT1-MMP in malignant cells, which routinely overexpress this protease.

Key words: MT1-MMP, Chariot delivery, Antibody uptake, Trafficking, Centrosome, Cytoskeleton, Microtubules, Endocytosis, Exocytosis

## Introduction

MT1-MMP is a prototypic member of a membrane-anchored MMP subfamily (Egeblad and Werb, 2002; Holmbeck et al., 2004). This subfamily consists of the six individual enzymes that share a significant peptide sequence homology. MT1-MMP is associated with the cell membrane by a transmembrane domain followed by a cytoplasmic tail (Hernandez-Barrantes et al., 2002) and is synthesized as a latent zymogen that requires N-terminal proteolytic processing to generate a proteolytically potent, mature, membrane-tethered enzyme.

Once activated, MT1-MMP can be inhibited by tissue inhibitors of metalloproteinases-2, -3 and -4 (TIMP-2, -3 and -4) but not by TIMP-1 (Baker et al., 2002). A potent and multifunctional proteinase, MT1-MMP functions in cancer cells as the main mediator of proteolytic events on the cell surface. MT1-MMP degrades a broad spectrum of the extracellular matrix (ECM) substrata including fibronectin, vitronectin, proteoglycan, collagen and laminin (d'Ortho et al., 1997; Ohuchi et al., 1997; Osenkowski et al., 2004), initiates the activation cascade of soluble MMPs (Cowell et al., 1998; Knauper et al., 1996; Sato et al., 1994; Toth et al., 2003), and is directly involved in the cleavage of cell surface receptors including tissue transglutaminase (Belkin et al., 2001), CD44

(Mori et al., 2002), pro- $\alpha_v$  integrin (Deryugina et al., 2002a), syndecan-1 (Endo et al., 2003), low-density lipoprotein receptor-related protein (Rozanov et al., 2004b) and  $\beta$ -glycan (Velasco-Loyden et al., 2004). The MT1-MMP-mediated pericellular proteolytic events modulate cell attachment and motility, and allow cell functioning to adjust to the continuously changing tissue microenvironment (Katayama et al., 2004; Seiki, 2003; Zucker et al., 2003). Consistent with its role in the migration and invasion of malignant cells, MT1-MMP is frequently overexpressed in aggressive, metastatic neoplasms (Katayama et al., 2004; Seiki, 2003).

Recently, we have demonstrated that MT1-MMP either naturally expressed by the cells or the overexpressed recombinant constructs were trafficked to the pericentrosomal compartment and, as a result, accumulated in the centrosomes (Golubkov et al., 2005). To demonstrate that the microtubular trafficking of MT1-MMP to the pericentrosomal compartment is a general cellular phenomenon, we extended these studies by performing an examination of the trafficking and internalization pathways of MT1-MMP in several cell types. Accordingly, this work was intended to confirm and reinforce the evidence presented in our earlier publication (Golubkov et al., 2005). Here, we report that MT1-MMP employs the

microtubular cytoskeleton for intracellular trafficking in malignant (human glioma U251 and breast carcinoma MCF7 cells) and in non-malignant canine epithelial MDCK cells, and that the transmembrane domain is required both for presentation of MT1-MMP in the centrosomes and for the vesicular transport of the protease across the cell compartment.

## Materials and Methods

### Antibodies

Mouse monoclonal antibodies to  $\alpha$ -tubulin (clone 236-10501) and golgin-97 (clone CDF4), goat anti-rabbit IgG (H+L) conjugated with Alexa Fluor 488 and goat anti-mouse IgG (H+L) conjugated with Alexa Fluor 594 were purchased from Molecular Probes (Portland, OR, USA). Murine monoclonal antibody to  $\gamma$ -tubulin (clone GTU-88) and rabbit polyclonal antibody Ab815 against a hinge domain of MT1-MMP were obtained from Sigma (St Louis, MO, USA) and Chemicon (Temecula, CA, USA), respectively. Murine monoclonal antibodies to Rab-4, Rab-11 and early endosome autoantigen-1 (EEA1) were purchased from Becton Dickinson Biosciences-Transduction Laboratories (San Diego, CA, USA). Murine monoclonal antibody to LAMP-1 (lysosome-associated membrane protein-1) was purchased from the Developmental Studies Hybridoma Bank (Iowa City, IA, USA).

### Cell lines

Madin-Darby canine kidney (MDCK), human breast carcinoma MCF7, human glioma U251 and Chinese hamster ovary (CHO) cells were obtained from ATCC (Manassas, VA, USA). CHO cells were routinely grown in HAM-F12 medium. Other cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum and gentamicin (10  $\mu$ g/ml).

MDCK cells were stably transfected, using Lipofectamine (Invitrogen, Carlsbad, CA), with the empty pcDNA3.1-zeo vector (Invitrogen) to prepare MDCK-zeo control cells or with the pcDNA3.1-zeo bearing the full-length wild-type human MT1-MMP to generate MT1-MMP-expressing MDCK-MT-WT cells. Breast carcinoma MCF7 mock-transfected cells (MCF-mock cells) and MCF7 cells expressing the wild-type MT1-MMP (MCF-MT-WT), the catalytically inert MT1-MMP-E240A mutant (MCF-MT-E240A), and the C-end truncated MT1-MMP lacking the amino acids 563-582 of the cytoplasmic tail (MCF-MT- $\Delta$ CT) as well as glioma U251 mock-transfected cells (U-mock cells) and U251 cells expressing the wild-type MT1-MMP (U-MT-WT) were obtained and characterized earlier (Deryugina et al., 2002a; Deryugina et al., 2004; Deryugina et al., 2002b; Rozanov et al., 2004a; Rozanov et al., 2001). We used MT1-MMP constructs to stably transfect CHO cells and to isolate the cells expressing the wild-type MT1-MMP (CHO-MT-WT), the catalytically inert E240A mutant (CHO-MT-E240A), the inert mutant without the amino acid region 26-284 of the entire catalytic domain (CHO-MT- $\Delta$ CAT), the mutant lacking the peptide sequence 319-508 of the entire hemopexin (PEX) domain (CHO-MT- $\Delta$ PEX), the tailless mutant (CHO-MT- $\Delta$ CT) and the soluble construct lacking the peptide sequence 535-582 that included both the transmembrane domain and the cytoplasmic tail (CHO-MT- $\Delta$ TM/ $\Delta$ CT cells).

### The isolation of the recombinant PEX constructs

We designed and used two constructs in our studies. The first soluble construct was the peptide sequence Leu<sup>282</sup>-Ser<sup>538</sup> of the PEX and hinge regions (PEX- $\Delta$ TM/ $\Delta$ CT) of MT1-MMP lacking both the transmembrane domain and the cytoplasmic tail. In the second construct, the Leu<sup>282</sup>-Ser<sup>538</sup> sequence of the PEX-hinge region was fused directly to the sequence Arg<sup>563</sup>-Val<sup>582</sup> of the cytoplasmic tail. Accordingly, the second construct, PEX- $\Delta$ TM, did not include the

sequence Ala<sup>539</sup>-Val<sup>562</sup> of the transmembrane domain. Both constructs were expressed in the yeast *Pichia pastoris* using the expression system we described earlier (Rozanov et al., 2004b; Rozanov and Strongin, 2003). The properly folded, soluble constructs were secreted in the extracellular milieu and then they were isolated from the medium using FPLC on a Mono S column.

### Antibody uptake by the cells

The antibody uptake procedure was performed as described earlier (Remacle et al., 2003). Cells seeded in DMEM on a 13-mm diameter glass coverslip were washed twice with PBS and placed at 4°C for 15 minutes in a serum-free, CO<sub>2</sub>-independent, L-15 medium Leibovitz (Sigma) containing a 1% insulin-transferrin-selenium (ITS) supplement (Sigma). Chilled cells were then incubated for an additional 60 minutes at 4°C with the MT1-MMP Ab815 antibody (10  $\mu$ g/ml in L15/ITS). After three washes with ice-cold PBS, to remove the unbound antibody, the slides were transferred to 37°C to allow the cells to internalize the cell-bound antibody. After the indicated times, the cells were fixed for 20 minutes at room temperature with 4% paraformaldehyde to inactivate the internalization processes. The fixed cells were permeabilized with 0.1% (v/v) Triton X-100, blocked with 10% BSA for 30 minutes and stained for 1 hour with the Alexa Fluor 488-conjugated secondary antibody. Cells were also stained with DAPI to identify the nucleus and with antibodies to  $\gamma$ -tubulin (a centrosomal marker),  $\alpha$ -tubulin (a cytoskeleton marker), golgin-97 (a Golgi marker), early endosome autoantigen-1 (EEA1, an early endosome marker), lysosome-associated membrane protein-1 (LAMP-1, a late endosome/lysosome marker) and Rab-4 and Rab-11 (both recycling endosomal markers). The cells were then stained with Alexa Fluor 594-conjugated secondary antibodies. Coverslips were then mounted using the VectaShield mounting medium (Vector Lab, Burlingame, CA, USA) and examined on an inverted Nikon TE300 fluorescence microscope equipped with a 60 $\times$  oil objective and a real-time cooled CCD camera SP401-115 (Diagnostic Instruments, Sterling Heights, MI, USA). Where indicated, the cell samples were examined using a laser scanning confocal microscope (MRC-1024; Bio-Rad) (Rozanov et al., 2004a). Acquisition and processing of the images were performed with the Lasersharp (Bio-Rad) and Adobe Photoshop software (Adobe Systems, San Jose, CA, USA).

### Chariot protein transduction

Cells were grown in DMEM on a 13-mm diameter glass coverslip to reach a 75-80% confluence. A Chariot stock suspension (6  $\mu$ l; Active Motif, Carlsbad, CA, USA) was diluted to 150  $\mu$ l with H<sub>2</sub>O and the diluted sample was mixed, to generate the Chariot-protein complex, with an equal volume of PBS containing the MT1-MMP Ab815 antibody (2.5  $\mu$ g/ml) or the soluble MT1-MMP constructs ( $\approx$ 7  $\mu$ g/ml). After incubation for 30 minutes at ambient temperature, the sample was diluted with DMEM to a 1 ml final volume. The diluted sample was added to the cells to initiate the delivery of the Chariot complex inside the cell compartment. After incubation for 1 hour with the Chariot complex at 37°C in a 5% CO<sub>2</sub> humidified incubator (time=0), cells were additionally incubated for 30-120 minutes in DMEM-10% fetal bovine serum (1 ml). Cells were next fixed, permeabilized and stained with the Alexa Fluor 488-conjugated secondary antibody to identify the subcellular compartment that harbors the MT1-MMP antibody. Where indicated, microtubule depolymerizer nocodazole (1  $\mu$ g/ml) and Brefeldin A (5  $\mu$ g/ml) were added and co-incubated with the cells for 1 hour at 37°C to disrupt the microtubulin cytoskeleton and the Golgi apparatus, respectively. The cells were stained with the MT1-MMP antibody Ab815 followed by Alexa Fluor 488-conjugated secondary antibody, if the soluble PEX constructs were used in the Chariot delivery experiments.

### Western blotting and gelatin zymography

Basic protocols for these techniques have been described in detail in our recent publications (Deryugina et al., 2004; Deryugina et al., 2002b; Remacle et al., 2003; Rozanov et al., 2004a). Because CHO cells do not synthesize MMP-2 efficiently, these cells were supplemented with the purified, TIMP-2-free, proMMP-2 (15 ng of proMMP-2 per  $1 \times 10^6$  cells). ProMMP-2 was isolated, by gelatin-Sepharose column chromatography, from the aliquots of the serum-free medium conditioned by the MMP-2-overexpressing, transfected fibrosarcoma HT1080 cells (Strongin et al., 1993).

### Results

#### The tubulin cytoskeleton is essential for MT1-MMP trafficking

To examine the intracellular trafficking of MT1-MMP, we used a newly developed Chariot reagent (Morris et al., 2001). This non-covalent reagent allows the delivery of proteins, including antibodies, to the inside of the cell compartment. Following the penetration through the cell membrane, the delivered Chariot-antibody complex dissociates inside the cell and liberates the antibody. The liberated, functional antibody then diffuses throughout the cell and interacts with the target protein and, thus, allows the identification of the subcellular compartment that harbors the target protein. These findings by several independent groups (Buster et al., 2002; Sebbagh et al., 2005; Zhang et al., 2005) provided a solid rationale for the use of Chariot in our experiments.

We employed Chariot to deliver an MT1-MMP antibody into breast carcinoma MCF-MT-WT cells and into MDCK-MT-WT cells. To facilitate the identification of the subcellular compartments, the cells were co-stained for  $\alpha$ -tubulin, a marker of the microtubulin cytoskeleton. The MT1-MMP immunoreactivity was primarily observed in the vesicles dispersed from the perinuclear region to the cell periphery localized alongside the microtubule cytoskeleton (Fig. 1). A similar transport pattern was also detected in MDCK-MT-WT cells (not shown). Following the Chariot-mediated antibody delivery, there was no significant MT1-MMP immunoreactivity in the control MCF-zeo and MDCK-zeo

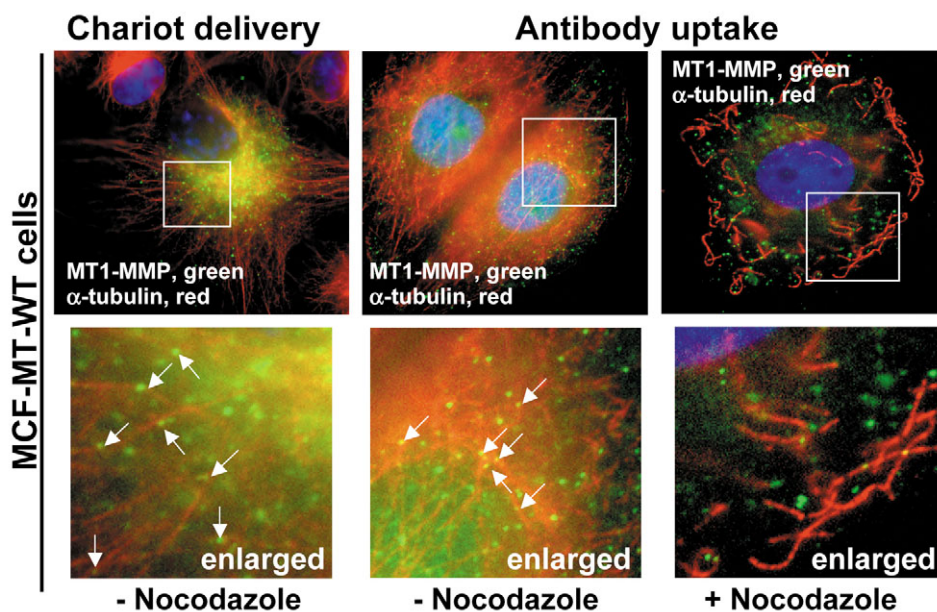
cells, which are both deficient in the protease (not shown). These findings strongly suggest the presence of the vesicular transport of MT1-MMP across the cell compartment.

#### The antibody uptake confirms the endosomal trafficking of MT1-MMP

We next used the antibody uptake to confirm the existence of the vesicular transport of cellular MT1-MMP. For these purposes, MCF-MT-WT cells were allowed to bind the MT1-MMP antibody for 1 hour at 4°C. After washings to remove the unbound antibody, the cells were then moved to 37°C to stimulate the internalization of the cell surface-associated MT1-MMP/antibody complex. The cells were next fixed, permeabilized and stained with a secondary antibody to determine the subcellular localization of the MT1-MMP/antibody complex. The cells were also stained for several cellular markers including  $\alpha$ -tubulin, EEA1, Rab-4 and Rab-11, LAMP-1 and golgin-97.

Consistent with the results of the Chariot-mediated delivery, the antibody uptake experiments in MCF-MT-WT cells identified the multiple MT1-MMP-positive vesicles that were colocalized with  $\alpha$ -tubulin (Fig. 1). To corroborate these results, MCF-MT-WT cells were treated with nocodazole prior to the antibody uptake. As expected, nocodazole disrupted the microtubular cytoskeleton in the cells. As a result, the internalized vesicular MT1-MMP was largely accumulated at the cell periphery rather than being distributed throughout the cell compartment (Fig. 1). Although the most of the internalized MT1-MMP-positive vesicles dissociated from the destroyed tubulin cytoskeleton, a few MT1-MMP-positive vesicles remained associated with the intact fragments of microtubules. This suggests that the  $\alpha$ -tubulin network is essential for the vesicular trafficking of MT1-MMP (Fig. 1).

Our previous data demonstrated that in 1 hour after internalization of the cell surface-associated MT1-MMP in fibrosarcoma HT1080 cells, a significant fraction of the internalized, active enzyme recycles to the plasma membrane and that the recycling occurs through the early endosomes and



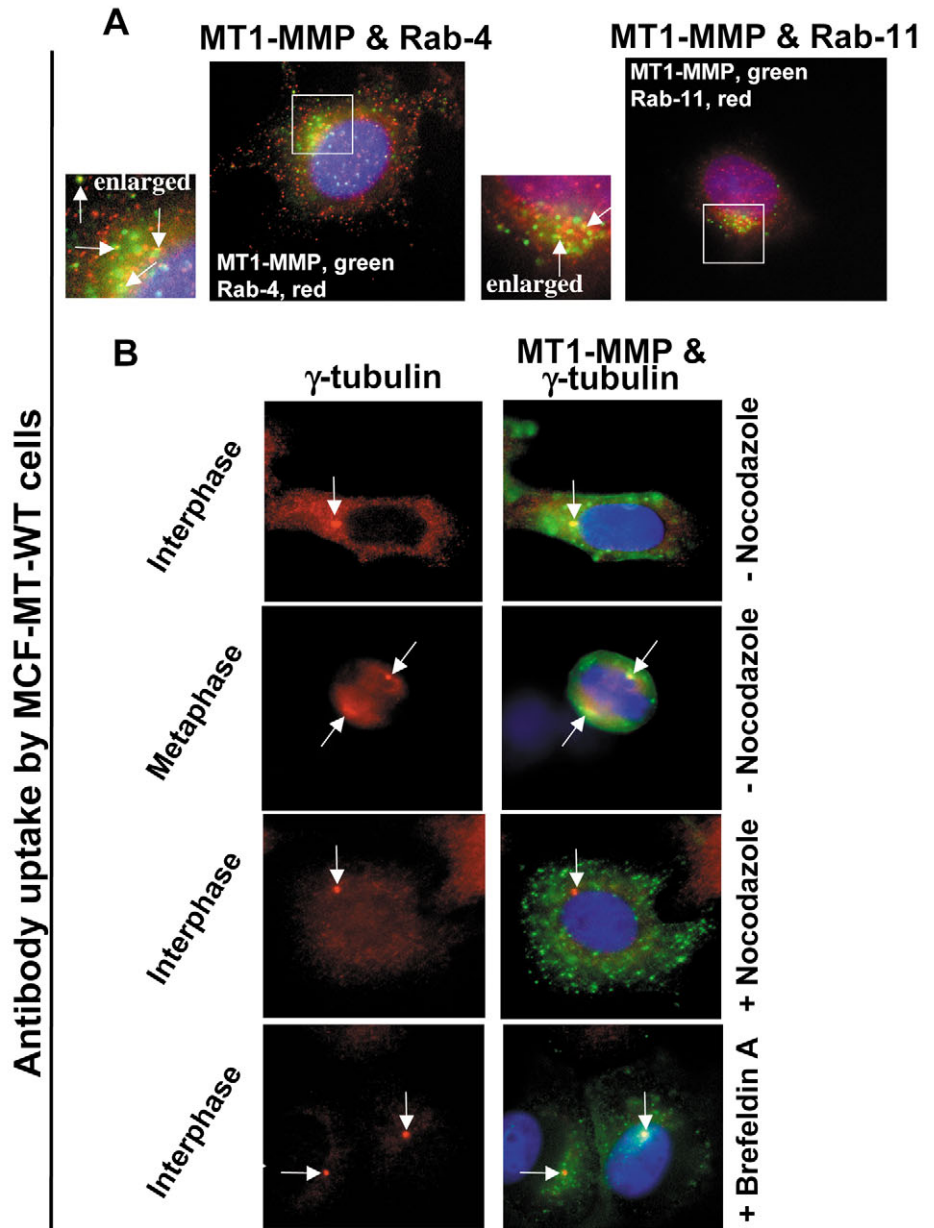
**Fig. 1.** MT1-MMP-positive vesicles in MCF-MT-WT cells. Two hours after Chariot delivery of the MT1-MMP antibody Ab815 (left panels) and 1 hour after the Ab815 uptake (middle and right panels), the cells were fixed, permeabilized and stained for  $\alpha$ -tubulin (red) and Ab815 (green). Where indicated, the cells were pre-treated for 1 hour with nocodazole (1  $\mu$ g/ml). Enlarged areas (boxed in top panels) show the MT1-MMP-positive vesicles (indicated by arrows) localized alongside the microtubule cytoskeleton. DAPI nuclear staining is blue.



then through the Rab-4-positive endocytic vesicles (Remacle et al., 2003). Consistent with these earlier results, the early/recycling endosomes are essential for the trafficking of MT1-MMP in breast carcinoma cells. Thus, by 30 minutes after the initiation of the antibody uptake at 37°C, the MT1-MMP immunoreactivity colocalized with EEA1 (not shown) and Rab-4 (Fig. 2A) in the early/recycling endosomes of MCF-MT-WT cells. By 45 minutes the colocalization of the MT1-MMP cargo was observed with Rab-11 (Fig. 2A) and LAMP-1 (not shown), the markers of the pericentrosomal recycling endosomes and late endosomes/lysosomes, respectively.

Because the colocalization of MT1-MMP was observed with Rab-11 (a known marker of the recycling endosome subset in the microtubule-organizing centrosomes) (Ullrich et al., 1996), we also used antibody uptake to determine the trafficking of MT1-MMP to the centrosomal compartment. By 45 minutes after the initiation of the antibody uptake, cellular MT1-MMP also colocalized with  $\gamma$ -tubulin, thereby suggesting that the protease reached the centrosomal compartment in MCF-MT-WT cells. Nocodazole completely abolished the centrosomal delivery and colocalization of MT1-MMP with  $\gamma$ -tubulin in the MCF-MT-WT cells (Fig. 2B). In turn, Brefeldin A, which is known to block protein trafficking from the endoplasmic reticulum to the Golgi compartment, did not affect the trafficking of MT1-MMP to the centrosomes or the colocalization of the protease with  $\gamma$ -tubulin (Fig. 2B) and the endosomal markers Rab-4 and Rab-11 (not shown). These data suggested that internalized MT1-MMP did not enter the trans-Golgi network (TGN) in its endocytic pathway from the cell surface to the centrosomal compartment. Colocalization of MT1-MMP with  $\gamma$ -tubulin was evident in the centrosomes of the metaphase MCF-MT-WT cells rather than in the interphase cells (Fig. 2B). In turn, there was no significant colocalization of the internalized MT1-MMP with golgin-97, a Golgi marker, either in the presence or the absence of Brefeldin A (Fig. 3).

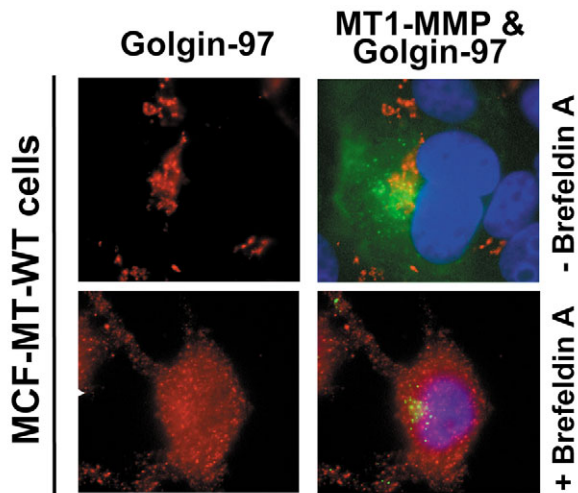
Taken together, our Chariot delivery and antibody uptake studies suggest that both the exocytose and endocytose trafficking of cellular MT1-MMP involves the microtubule cytoskeleton and that the cell surface-associated MT1-MMP is



**Fig. 2.** Internalized MT1-MMP colocalizes with endosomal and centrosomal markers in MCF-MT-WT cells. (A) Following a 30-minute and a 45-minute uptake of the MT1-MMP antibody Ab815, the cells were stained for Rab-4 and Rab-11, respectively (both in red) and for Ab815 (green). Boxed areas are enlarged in the small panels. (B) Following the MT1-MMP antibody Ab815 uptake for 45 minutes, the cells were stained for Ab815 (green) and  $\gamma$ -tubulin (red). Where indicated, cells were pre-treated for 1 hour with nocodazole (1  $\mu$ g/ml) or Brefeldin A (5  $\mu$ g/ml). Arrows indicate the centrosomes. DAPI nuclear staining is blue.

internalized through at least two, distinct pathways, one rapid and one slow, involving the Rab-4-positive, fast-recycling endosomes, which segregate from the early endosomes, and the Rab-11-positive, slow recycling, pericentrosomal endosomes (Sonnichsen et al., 2000; Ullrich et al., 1996). These data correlate well with our earlier findings (Golubkov et al., 2005).

We conclude that the endosomal trafficking of MT1-MMP occurs alongside the microtubulin cytoskeleton. This trafficking pattern governs both the presentation of MT1-MMP



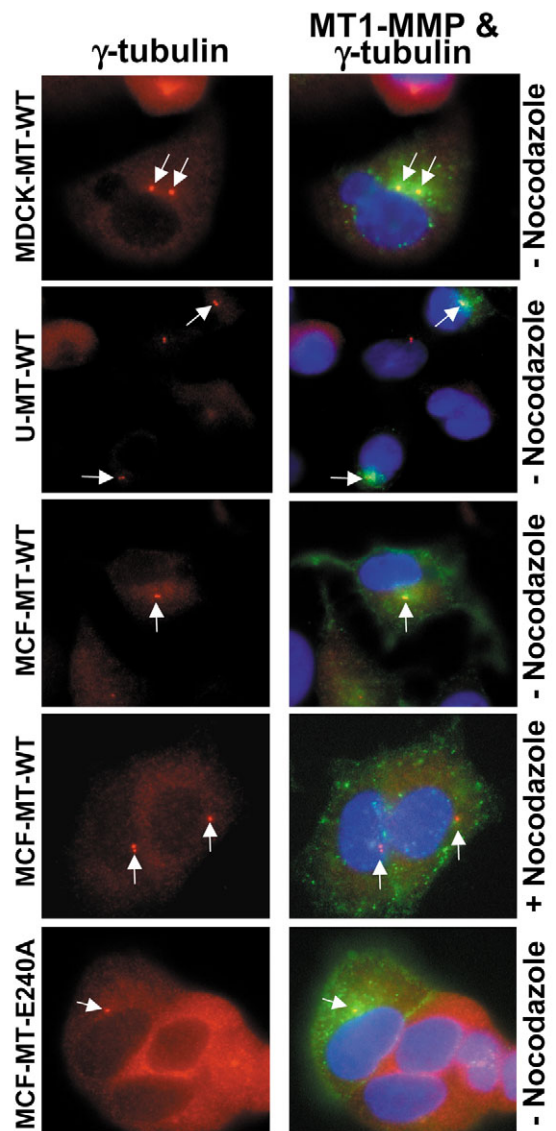
**Fig. 3.** Internalized MT1-MMP does not colocalize with the Golgi marker in MCF-MT-WT cells. Following the MT1-MMP antibody Ab815 uptake for 45 minutes, the cells were stained for Ab815 (green) and golgin-97 (red). Where indicated, cells were pre-treated for 1 hour with Brefeldin A (5  $\mu$ g/ml). DAPI nuclear staining is blue.

at the cell surface and the internalization of the cell surface-associated proteinase inside the cell compartment and the follow-on delivery of the internalized protease to the centrosomal compartment.

#### Trafficking of MT1-MMP to the centrosomes

To examine the trafficking and the centrosomal delivery of MT1-MMP in more detail, we used cells of different tissue origin including canine kidney MDCK-MT-WT cells, and human breast carcinoma MCF-MT-WT and glioma U-MT-WT cells. Chariot was used to deliver the MT1-MMP antibody into these cells. Within 30 minutes of the initiation of the delivery of the Chariot-antibody complex, the MT1-MMP immunoreactivity was found to be primarily localized in the  $\gamma$ -tubulin-positive pericentrosomal region in all cell types (Fig. 4). Nocodazole blocked the transport of MT1-MMP to the centrosomes. In nocodazole-treated cells the MT1-MMP immunoreactivity showed no specific pattern and, in turn, was randomly distributed inside the cells (Fig. 4).

We next examined if the proteolytic activity of MT1-MMP itself is essential for MT1-MMP trafficking to the centrosomes. To answer this question, we used MCF-MT-E240A cells, which express the catalytically inert MT1-MMP bearing the substitution of the essential active site, the Glu-240 residue. Following the Chariot delivery of the MT1-MMP antibody, we determined that there was no difference in the intracellular pattern of the mutant protease in MCF-MT-E240A cells compared to the wild-type MT1-MMP expressed in MCF-MT-WT cells (Fig. 4). We concluded that the proteolytic activity of MT1-MMP itself is not required for the directional delivery of the protease from the cell surface to the centrosomes. In agreement, broad-range hydroxamate inhibitors of MMPs including GM6001 and AG3340 (Levy et al., 1998; Shalinsky et al., 1999), which are both highly potent against MT1-MMP ( $K_i \approx 0.5$  nM), did not affect the trafficking of MT1-MMP to the centrosomes – in the cells co-incubated with 10–25  $\mu$ M



**Fig. 4.** Chariot delivery of the MT1-MMP antibody Ab815 into MDCK-MT-WT, MCF-MT-E240A, U-MT-WT and MCF-MT-WT cells. 30 minutes after delivery, the cells were stained for  $\gamma$ -tubulin (red) and the Ab815 (green). Where indicated, the cells were pre-treated for 1 hour with nocodazole (1  $\mu$ g/ml). Arrows indicate centrosomes. DAPI nuclear staining is blue.

hydroxamates, MT1-MMP was internalized and then trafficked to the pericentrosomal compartment (data not shown).

It has been well established that the inert MT1-MMP-E240 construct is represented by the processed, mature enzyme in breast carcinoma MCF7 and fibrosarcoma HT1080 cells (Hernandez-Barrantes et al., 2000; Rozanov et al., 2001; Toth et al., 2000). Taken together, these observations and our current findings strongly suggest that the processed, mature enzyme form of MT1-MMP can be delivered to the centrosomes.

#### The role of the individual domains in the trafficking of MT1-MMP

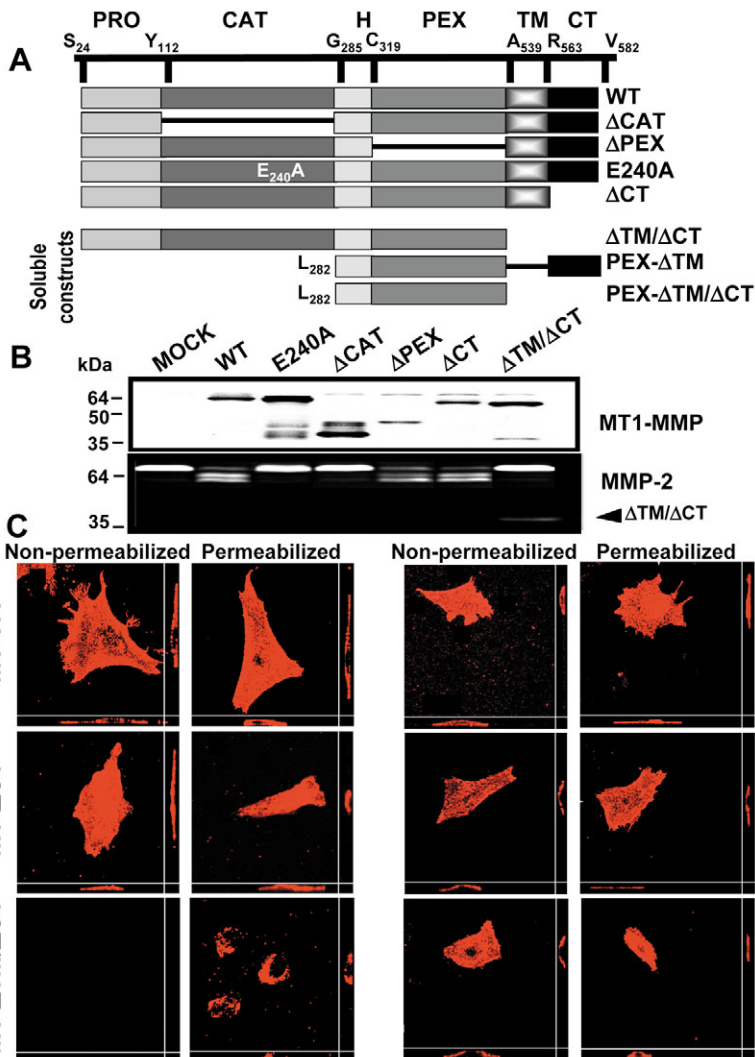
To identify the role of the individual structural domains in the

trafficking mechanisms of MT1-MMP, we used CHO cells. We specifically selected CHO cells because the expression of human MT1-MMP is easily detected against a Chinese hamster background with the available antibodies raised against human MT1-MMP. We transfected CHO cells with the wild-type MT1-MMP (MT-WT) as well as with the mutant MT1-MMP constructs. These constructs represented MT1-MMP with the following sequence regions deleted: the catalytic domain (MT- $\Delta$ CAT), the hemopexin domain (MT- $\Delta$ PEX), the cytoplasmic tail (MT- $\Delta$ CT) and both the transmembrane domain and cytoplasmic tail (MT- $\Delta$ TM/ $\Delta$ CT) (Fig. 5A). For comparison purposes, we also used the catalytically inert full-length MT1-MMP-E240A mutant (MT-E240A) in these experiments. CHO cells expressing MT1-MMP were examined by immunoblotting and immunohistochemistry. The activity of MT1-MMP constructs in initiating the activation pathway of MMP-2 was determined by gelatin zymography of aliquots of conditioned medium (Fig. 5B). Because CHO cells do not synthesize MMP-2, the purified MMP-2 proenzyme was added to the cells to assess the enzymatic activity of MT1-MMP constructs. MT1-MMP was undetectable in CHO-mock cells transfected with the original plasmid without the MT1-MMP insert. Immunoblotting confirmed the existence of similar

expression levels of the MT1-MMP constructs, excluding the MT- $\Delta$ PSEX, in CHO cells (Fig. 5B). Surprisingly, we detected high levels of MT- $\Delta$ TM/ $\Delta$ CT associated with the cell compartment. This finding suggests that though the soluble construct MT- $\Delta$ TM/ $\Delta$ CT was detectable in the extracellular milieu (Fig. 5B), there was a residual fraction of this construct that was incapable of efficiently accomplishing the secretion process and so remained inside the cells.

All MT1-MMP constructs except the catalytically inert MT-E240A and MT- $\Delta$ CAT were functionally active and capable of activating MMP-2 (Fig. 5B). The soluble construct MT- $\Delta$ TM/ $\Delta$ CT was incapable of MMP-2 activation, probably, because it was secreted from the cells into the extracellular milieu and significantly diluted in the medium, and also because of the low efficiency of soluble MT1-MMP in activating MMP-2. However, these data also indicate that the cell-associated portion of the MT- $\Delta$ TM/ $\Delta$ CT construct is not expressed on the cell surface and is inaccessible to MMP-2. In contrast, the cell surface-associated MT- $\Delta$ CT and MT- $\Delta$ PEX mutants were as efficient as the wild-type MT1-MMP in activating MMP-2. These data are consistent with the results published by other groups (Jiang et al., 2001; Jiang and Pei, 2003; Remacle et al., 2003; Rozanov et al., 2004a) and support the non-essential role of the PEX domain of MT1-MMP in the mechanisms of MMP-2 activation (Wang et al., 2004a).

In agreement with the immunoblotting, immunostaining demonstrated the presence of all of the MT1-MMP constructs in CHO cells (Fig. 5C). Cell-surface-associated MT1-MMP expression was especially evident in the non-permeabilized cells. In contrast to other constructs, MT- $\Delta$ TM/ $\Delta$ CT was not detected in the cells unless the cells were permeabilized to provide an antibody access to the intracellular



**Fig. 5.** The transmembrane domain is required for the trafficking of MT1-MMP to the cell surface of CHO cells. (A) Domain structure of mutant MT1-MMP. PRO, prodomain; CAT, catalytic domain; H, hinge region; PEX, hemopexin domain; TM, transmembrane domain; CT, cytoplasmic tail. (B) Immunoblotting and gelatin zymography of CHO cells expressing mutant MT1-MMP. Cells were lysed in a RIPA buffer containing a proteinase inhibitor cocktail-Set III and phenylmethylsulfonyl fluoride (Calbiochem, San Diego, CA, USA). The cell lysates were analyzed by western blotting with the MT1-MMP antibody Ab815 (upper panel). The ability of cells to activate MMP-2 was analyzed by gelatin zymography (bottom panel). An arrowhead indicates the gelatinolytically active, soluble, short MT- $\Delta$ TM/ $\Delta$ CT construct found in the medium. Self-proteolysis of MT1-MMP leads to this short species (Osenkowski et al., 2004; Rozanov and Strongin, 2003). (C) Confocal microscopy of CHO cells expressing mutant MT1-MMP. Permeabilized and non-permeabilized cells were stained with the Ab815 antibody. The XY and ZX projections of cells are shown at the bottom and on the right of each panel, respectively. Notice the absence of staining of the non-permeabilized  $\Delta$ TM/ $\Delta$ CT cells.



compartment. In the permeabilized MT- $\Delta$ TM/ $\Delta$ CT cells, the immunoreactivity was condensed in the perinuclear Golgi-like compartment. Consistent with the results of immunoblotting and MMP-2 activation studies, these observations indicate that the MT- $\Delta$ TM/ $\Delta$ CT construct remains trapped inside the cells. In contrast, the tailless MT- $\Delta$ CT construct and other MT1-MMP constructs, which have the intact transmembrane domain, are efficiently trafficked through the cells and presented on the plasma membrane of CHO cells.

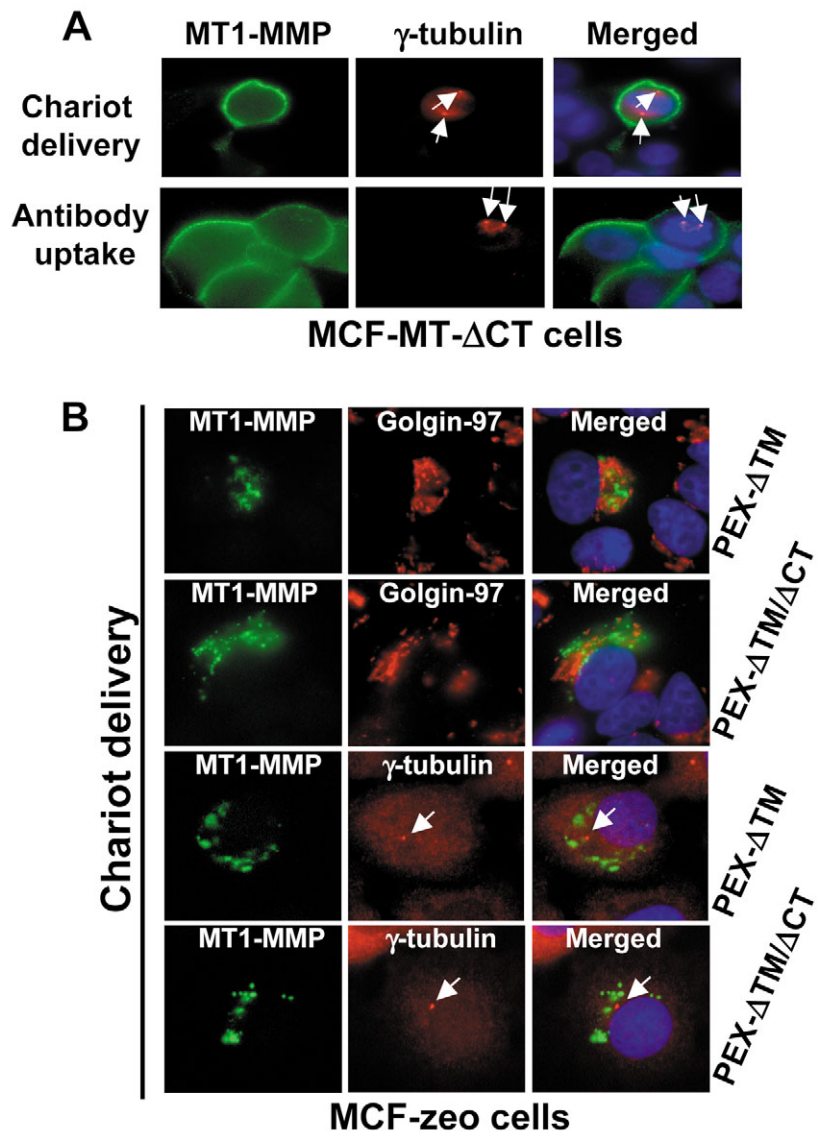
#### The tailless, internalization-deficient mutant MT1-MMP is not delivered to the centrosomes

Our previous and current observations as well as the findings of other authors (Jiang et al., 2001; Lehti et al., 2000; Remacle et al., 2003) indicate that the C-terminal cytoplasmic tail is important for the internalization and the endocytic pathway of MT1-MMP. The tailless MT- $\Delta$ CT construct with the deleted cytoplasmic tail peptide sequence is incapable of efficient internalization and, therefore, the mutant exhibits the significantly enhanced, persistent cell surface-associated expression and a high proteolytic activity (Rozanov et al., 2004a). To confirm and extend these data, we used a Chariot technique to deliver the MT1-MMP antibody into breast carcinoma MCF-MT- $\Delta$ CT cells. In contrast to the wild-type MT1-MMP, the immunoreactivity of the tailless mutant was found only on the plasma membrane 2 hours after the Chariot delivery. It should be noticed that no intracellular immunoreactivity and no colocalization of the mutant with  $\gamma$ -tubulin were observed in MCF-MT- $\Delta$ CT cells (Fig. 6A).

In agreement with the Chariot delivery studies, the antibody uptake experiments also confirmed that the MCF-MT- $\Delta$ CT tailless construct is internalized inefficiently compared to the wild-type protease. The MT- $\Delta$ CT construct was not internalized after 45 minutes at 37°C and, accordingly, no centrosomal immunoreactivity of MT1-MMP was found in MCF-MT- $\Delta$ CT cells (Fig. 6A). We concluded that the presentation of MT1-MMP on the cell surface may precede the trafficking of MT1-MMP to the centrosomes.

#### The role of the transmembrane domain in MT1-MMP trafficking

To examine the role of the PEX and the transmembrane domain in the trafficking of MT1-MMP to the centrosomes, we used a Chariot technique to deliver the soluble PEX- $\Delta$ TM/ $\Delta$ CT and PEX- $\Delta$ TM constructs (Fig. 5A) into MCF-zeo cells. After the delivery of the Chariot-protein complex to the inside of the cells and the required follow-on procedures, the cells were stained for MT1-MMP, and also for golgin-97 and  $\alpha$ -tubulin to



**Fig. 6.** Antibody uptake in MCF-MT- $\Delta$ CT cells and delivery of the soluble MT1-MMP constructs. (A) MCF-MT- $\Delta$ CT cells were treated with the MT1-MMP antibody Ab815. The antibody was then delivered to the cells by Chariot delivery (upper panel) and by antibody uptake (lower panel). The cells were stained for  $\gamma$ -tubulin (red) and Ab815 (green) 2 hours after Chariot delivery and 45 minutes after antibody uptake. Notice that the mutant was transported to the plasma membrane but it was not efficiently internalized and transported to the centrosomes. (B) MCF-zeo cells were treated with the PEX- $\Delta$ TM/ $\Delta$ CT and PEX- $\Delta$ TM constructs. The constructs were then delivered to the cells by Chariot delivery. Two hours after delivery, the cells were stained for MT1-MMP (green), golgin-97 and  $\gamma$ -tubulin (both in red). Notice that the delivered constructs do not colocalize with  $\gamma$ -tubulin and golgin-97, and appear to accumulate in the trans-Golgi network. Arrows indicate centrosomes. DAPI nuclear staining is blue.

identify the TGN and the microtubule cytoskeleton, respectively (Fig. 6B). After 1 hour following delivery, both PEX- $\Delta$ TM/ $\Delta$ CT and PEX- $\Delta$ TM were found in a perinuclear, TGN-like compartment. These findings are consistent with the cell compartmentalization of the MT- $\Delta$ TM/ $\Delta$ CT construct observed in CHO cells (Fig. 5C). After 2 hours following the delivery, both PEX species had accumulated in the same intracellular compartment (Fig. 6B). Both soluble species

failed to be transported to the plasma membrane as shown by the immunostaining of the permeabilized and non-permeabilized cells (not shown). Similarly, both PEX- $\Delta$ TM/ $\Delta$ CT and PEX- $\Delta$ TM constructs were not transported to the centrosomes and did not colocalize with  $\gamma$ -tubulin (Fig. 6B).

## Discussion

Of the 24 known human MMPs, MT1-MMP (also known as MMP-14) is the most closely associated with cancer (Chun et al., 2004; Hotary et al., 2003; Hotary et al., 2002; Sabeih et al., 2004; Seiki, 2003; Yana and Weiss, 2000). In an efficient, but not yet completely understood manner, MT1-MMP acts as a growth factor in malignant cells and assumes tumor growth control (Hotary et al., 2003; Koshikawa et al., 2005). Our previous work has clearly shown that MT1-MMP confers tumorigenicity on non-malignant epithelial cells, thus providing us with substantive evidence of the enormous importance of this protease in cell functioning and, probably, in malignant transformation (Soulie et al., 2004).

MT1-MMP is regulated as a protease via self-proteolysis and inhibition by TIMPs (Baker et al., 2002; Osenkowski et al., 2004), and as a membrane protein via trafficking, internalization and recycling (Jiang et al., 2001; Lehti et al., 2000; Osenkowski et al., 2004; Remacle et al., 2003; Wang et al., 2004c). Previously, there was an impression that MT1-MMP is present only at the cell surface (Sato et al., 1994). Recent observations, however, demonstrate the existence of a major intracellular pool of the protease. This intracellular MT1-MMP pool awaits biological signals before it is rapidly transported to the plasma membrane (Zucker et al., 2002). These joint multidimensional mechanisms regulate MT1-MMP spatially and temporally, and they appear to be essential for the invasion-promoting function of the protease (Itoh and Seiki, 2004; Seiki, 2003). Understanding the transport mechanisms of MT1-MMP is required for their targeting in a clinically advantageous manner.

The transport of the newly synthesized molecules of MT1-MMP to the plasma membrane occurs in only a few minutes, post-synthesis. In contrast, the transport of the newly synthesized integrin  $\alpha_v$  takes 30–60 minutes (Deryugina et al., 2004). The transport of MT1-MMP is resistant to a Golgi inhibitor (Brefeldin A) and is highly sensitive to nocodazole, a reagent that depolymerizes the microtubulin cytoskeleton (Vasquez et al., 1997). We have now demonstrated that the deletion of the individual domains of MT1-MMP including the PEX domain and the cytoplasmic tail peptide sequence did not significantly affect the levels of the constructs delivered to the plasma membrane. The inactivation of the functional activity of MT1-MMP by the substitution of the essential active site Glu-240 or by the deletion of the entire catalytic domain also did not significantly change the levels of MT1-MMP presented on the cell surface. In turn, the deletion of the transmembrane domain, which resulted in the soluble MT1-MMP construct, strongly affected the intracellular localization of MT1-MMP. In agreement, the compartmentalization of the MT- $\Delta$ TM/ $\Delta$ CT construct was highly similar to that of the PEX- $\Delta$ TM/ $\Delta$ CT construct. We believe that the transmembrane domain of MT1-MMP is not directly involved in the interactions with tubulin. However, the presentation on the cell surface and the

subsequent internalization in the endocytic vesicles are both essential for the microtubular trafficking of MT1-MMP. Because the soluble constructs of MT1-MMP are incapable of membrane association and cell surface presentation, they cannot enter into the microtubular trafficking and they cannot be delivered to the centrosomes. The replacement of the transmembrane domain of MT1-MMP with that of MT2-MMP, MT3-MMP and MT5-MMP will be required to ascertain the similar function of the transmembrane domain in the trafficking of other MT1-MMP types (in progress).

Endocytosis is a cellular process of internalizing the membrane proteins (Conner and Schmid, 2003). Evidently, endocytosis plays an important role in regulating the presentation of MT1-MMP on the cell surface. In our previous work, we demonstrated that dynamin-dependent endocytosis of MT1-MMP involves both clathrin-dependent and clathrin-independent pathways. We also showed that, by 60 minutes post-internalization, a significant fraction of MT1-MMP recycles to the plasma membrane (Remacle et al., 2003). Our results were subsequently supported by those of other authors (Wang et al., 2004c). In addition, we determined that MT1-MMP partially accumulates in the centrosomes in its trafficking pathway through the cell. In the centrosomes, enzymatically active MT1-MMP is directly involved in the proteolytic processing of pericentrin (an integral centrosomal protein which is essential to the mitotic spindle formation) (Golubkov et al., 2005). Centrosomes, microtubule-organizing centers, regulate the mitotic spindle during cell division and provide sister chromatid disjunction to support genome inheritance (Dictenberg et al., 1998; Wang et al., 2004b; Zheng et al., 1995). Centrosomes are characterized by  $\gamma$ -tubulin-containing centrioles surrounded by pericentriolar material. Alterations in the centrosome functionality lead to the formation of abnormal mitotic spindles and to premature anaphase and chromosome instability, the latter being routine and frequent features of highly aggressive tumors (Miki et al., 2004; Nakajima et al., 2004; Wang et al., 2004b).

There is a consensus among scientists that cell surface-associated MT1-MMP is a key player in pericellular proteolysis. Our current work supports and extends our earlier findings, which point to the intracellular cleavage function of MT1-MMP (Golubkov et al., 2005). We reasoned, however, that any observations that are counter to dogma need to be definitive. That is why we performed the studies presented here. The purpose of this study was to illustrate the mechanisms of the delivery of MT1-MMP to the centrosomal compartment.

We confirmed, by using several methods and experimental approaches, that the vesicular transport of MT1-MMP occurs alongside the microtubulin cytoskeleton in malignant and non-malignant cells. It appears that tubulin microfilaments are used as railroad tracks for the trafficking of MT1-MMP. Our current data explain the unusually fast trafficking rate of newly synthesized MT1-MMP to the plasma membrane and the high sensitivity of MT1-MMP trafficking to nocodazole (an inhibitor of microtubulin polymerization). Our data obtained with transfected cells correlated well with our earlier results with non-transfected cells (Golubkov et al., 2005) and showed the same intracellular localization and the trafficking pattern of MT1-MMP.

A recent publication confirms the endosomal nature and the



microtubular intracellular trafficking of metalloproteinases such as MMP-2 and MMP-9 (Schnaeker et al., 2004). These results provide indirect support for the data presented here. Our data suggest that following the presentation of MT1-MMP at the cell surface and the internalization of the plasma membrane-associated MT1-MMP, a fraction of the internalized MT1-MMP pool is capable of reaching and accumulating in the centrosomes. Thus, the tailless MT1-MMP mutant with impaired internalization failed centrosomal trafficking. The vesicular trafficking provides a vehicle for MT1-MMP to access the centrosomes and to target the centrosomal proteins.

Overall, our results provide additional evidence for the presence of MT1-MMP in the centrosomal compartment of the cell and suggest that this protease may be involved not only in the pericellular proteolytic events, but it may also target certain intracellular proteins. It is tempting to hypothesize that the intracellular cleavage function of MT1-MMP, which is directly associated with the mechanisms of trafficking of MT1-MMP across the cell, plays an important role in the total tumorigenic effect of this protease.

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