Membrane Type-1 Matrix Metalloproteinase (MT1-MMP) Exhibits an Important Intracellular Cleavage Function and Causes Chromosome Instability*

Elevated expression of membrane type-1 matrix metalloproteinase (MT1-MMP) is closely associated with malignancies. There is a consensus among scientists that cell surface-associated MT1-MMP is a key player in pericellular proteolytic events. Now we have identified an intracellular, hitherto unknown, function of MT1-MMP. We demonstrated that MT1-MMP is trafficked along the tubulin cytoskeleton. A fraction of cellular MT1-MMP accumulates in the centrosomal compartment. MT1-MMP targets an integral centrosomal protein, pericentrin. Pericentrin is known to be essential to the normal functioning of centrosomes and to mitotic spindle formation. Expression of MT1-MMP stimulates mitotic spindle aberrations and aneuploidy in non-malignant cells. Volumes of data indicate that chromosome instability is an early event of carcinogenesis. In agreement, the presence of MT1-MMP activity correlates with degraded pericentrin in tumor biopsies, whereas normal tissues exhibit intact pericentrin. We believe that our data show a novel proteolytic pathway to chromatin instability and elucidate the close association of MT1-MMP with malignant transformation.

Matrix metalloproteinases (MMP(s))1 are a comprehensive family of zinc-enzymes that degrade the extracellular matrix and cell surface molecules (1). Understanding the function of these enzymes in carcinogenesis is critical for the design of anti-cancer pharmaceuticals (2). MT1-MMP is a prototypic member of the membrane-tethered MMP subfamily (3). A transmembrane domain and a cytoplasmic tail (CT) of MT1-MMP associate this abundant membrane-tethered protease with discrete regions of the plasma membrane and the intracellular milieu, respectively. Although MT1-MMP is present in normal tissues, its enhanced expression, unlike of any other of the 23 known human MMPs, is closely associated with aggressive, invasive malignancies (1, 3–5). MT1-MMP transgenic mice displayed mammary gland abnormalities and tumor promotion in mammary gland (6).

MT1-MMP functions as one of the main mediators of proteolytic events on the cell surface, and it is directly involved in the pericellular proteolysis of the extracellular matrix, cell surface adhesion, and signaling receptors and in the activation pathway of soluble secretory MMPs (5, 7–9) Cell surface-associated MT1-MMP acts as a growth factor in malignant cells and assumes tumor growth control (4). The conditional expression of MT1-MMP can, by itself, confer tumorigenicity on non-malignant epithelial cells and cause the formation of invasive tumors (10). MT1-MMP also plays an important role in normal development; MT1-MMP knock-out mice are dwarfs, and they die prematurely (8, 11). A loss of the structurally similar primordial At2-MMP induces dwarfism in Arabidopsis plants (12). There is no extracellular matrix in plants, however, that is similar to the collagenous extracellular matrix of mammals. This datum alone is enough to suggest that the protease plays a role in certain functionally relevant intracellular events in addition to its role in pericellular proteolysis.

MT1-MMP is tightly regulated at the transcriptional and posttranscriptional levels both as a protease (through activation and inhibition) and as a membrane protein (via trafficking, internalization, and recycling) (13–15) The trafficking and the internalization, via clathrin-coated pits and caveolae, have emerged as the essential mechanisms that regulate the biological function of MT1-MMP (16–23). These new data, combined together, provided a compelling argument to investigate the trafficking and the intracellular compartmentalization of MT1-MMP in greater detail. These data also argue that there is a role for the protease in intracellular events in addition to its role in pericellular proteolysis.

Here, we have discovered compelling evidence that MT1-MMP is trafficked along the tubulin cytoskeleton. A fraction of cellular MT1-MMP accumulates in the centrosomal compartment. In the pericentrosomal compartment, active, functionally potent MT1-MMP degrades an integral centrosomal protein, pericentrin. Pericentrin is essential to the normal functioning of centrosomes in the mitotic spindle formation. MT1-MMP proteolysis of pericentrin causes chromosome instability, which is an early predictor of carcinogenesis. Overall, our results suggest an intracellular function for the membrane-tethered protease and an important role of MT1-MMP in the transition of cells from normalcy to malignancy.
**Intracellular Cleavage Function of MT1-MMP**

**MATERIALS AND METHODS**

**Antibodies and Cells**—Rabbit polyclonal antibodies against the catalytic domain and against the hinge region of MT1-MMP were from Chemicon (Temecula, CA), Sigma, and Triple Point Biologics (Portland, OR). Rabbit polyclonal antibodies 4b and 6s to the C-terminal and N-terminal of MT1-MMP were also used for study. The 4b antibodies were characterised earlier (24, 25). A murine monoclonal antibody against α-tubulin was from Sigma. Monoclonal antibodies against α-tubulin, RAB-4 and RAB-11, were from BD Biosciences.

Human U251 glioma, human MCF7 breast carcinoma, and Madin-Darby canine kidney (MDCK) cells were from ATCC (Manassas, VA). All cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS). For MT1-MMP overexpression, MDCK cells were transfected with the pcDNA3.1-zeo vector (mock cells) and with the plasmid bearing human MT1-MMP to overexpress the protease. Control and MT1-MMP-expressing breast carcinoma MCF7 and glioma U251 cells were obtained earlier (18, 26). In this work, U251 cells were also transfected with α1-antitrypsin (PDX). MCF7 cells were also transfected with the catalytically inert MT1-MMP-E240A construct and the internalization-deficient, tailless MT1-MMP-ΔCt construct. MCF7 cells were also transfected with MT1-MMP tagged with a flag tag. To avoid interference with the trafficking of MT1-MMP, the flag tag was inserted into the hinge region of the protease. Peptide cleavage and the mass spectrometry analysis of the digest were performed as described earlier (27). All of the buffer solutions used for the preparation of cell lysates and for the isolation of centrosomes were supplemented with a protease inhibitor mixture (pepsin, trypsin, antipain, aprotinin, and leupeptin) and additionally with phenylmethylsulfonyl fluoride and EDTA (1 mM each).

**MT1-MMP Small Interfering (si)RNA Constructs**—The MT1-MMP siRNA target sequence was designed by using the siRNA Designer software (Promega). From six tested sequences, the sequence 5′-GAAGCCUGGCUACAGCAAAAU-3′ repressed the expression of MT1-MMP most efficiently. The 5′-GUCCAGGUCCAGAAACUU-3′ scrambled RNA sequence was used as a control in our studies. Both sequences were cloned into the psi-LentGene vector (Promega) and used to transfect U251 cells. Transfected cells were selected and cloned in the medium supplemented with 2 μg/ml puromycin. The level of expression of MT1-MMP in the clones was determined by Western blotting.

**Isolation of Centrosomes**—Centrosomes were isolated from ncodazole-synchronized metaphase U251 cells (25). Mitotic cells were harvested by mitotic shake off and lysed in 1 mM Tris-HCl, pH 8.0, containing 0.5% Igepal. Cell lysates were spun at 1500 rpm for 15 min and then fixed with Carnoy's fixative. The fixed cells were spread on slides and centrifuged on a 20% w/w sucrose gradient at 30,000 rpm for 30 min. The supernatant fractions were filtered through a nylon mesh (70-m pore size) and centrifuged on a 40–80% sucrose gradient at 30,000 rpm for 2 h.

**Immunofluorescence**—Cells were fixed in 4% paraformaldehyde for 10 min, permeabilized with 0.1% Triton X-100 for 5 min, and blocked with 1% bovine serum albumin. Cells were incubated with primary antibodies (1:400) for 4 h and then with secondary antibodies (1:200) for 2 h. DNA was stained with 4′,6-diamidino-2-phenylindole. Images were acquired with a real time, cooled CCD camera SP402-115 (Diagnostic Instruments, Sterling Heights, MI).

**MMP-2 Activation Assays**—The ability of cellular MT1-MMP to activate proMMP-2 was demonstrated by gelatin zymography. For the analysis of centrosomal MT1-MMP, the isolated centrosomes were diluted 1:100 in 25 mM HEPES, pH 7.5. Diluted aliquots were co-incubated for 14 h at 37 °C with the purified proMMP-2 (10 ng). The samples were then analyzed by gelatin zymography.

**Fluorescence-activated Cell Sorter Analysis**—Cells were detached in trypsin-EDTA, fixed in 70% ethanol, washed in phosphate-buffered saline, and resuspended in 1% bovine serum albumin, phosphate-buffered saline solution supplemented with 50 μg/ml propidium iodide. The DNA content of cells was analyzed on a FACScan flow cytometer.

**Metaphase Spreads and Chromosome Count**—Cells were incubated for 15 min with 0.005% colchicine and then in 0.005% colchicine and 0.005% sodium borate and rinsed with medium. Cells were then fixed with Carnoy's fixative. The fixed cells were mounted on glass slides. After 72 h, chromosomes were stained with Giemsa stain and examined on a microscope. Digital images of chromosome spreads were analyzed, and chromosomes were counted in >100 spreads of each cell line.

**The Design of the MT1-MMP Chimeras**—Using a QuikChange mutagenesis system (Stratagene), the Asp-Tyr-Lys-Asp-Asp sequence was inserted immediately prior to the Asp-Lys-Thr sequence of MT1-MMP. As a result, the final construct exhibited the Asp-Lys-Thr sequence of the hinge domain of MT1-MMP. To construct MT1-MMP-GFP, the Thr-Ser-Thr sequence of the hinge domain of MT1-MMP was modified to insert PacI and BpiI restriction sites. The enhanced GFP sequence (Clontech) flankd at both ends with (Gly)5 was then inserted into the PacI/BpiI sites of MT1-MMP to generate the MT1-MMP-GFP chimera. MCF7 and U251 cells were stably transfected with the pcDNA3.1-zeo plasmids bearing MT1-MMP-GFP and MT1-MMP-GFP, respectively. To avoid the aberrant trafficking of the recombinant constructs, the clones expressing low levels of the chimeras were specifically selected and analyzed further.

The Analysis of Tumor Biopsies—Frozen samples of colon adenocarcinomas and invasive mammary grade II-III carcinomas and the matched normal tissues were obtained from the NCI Cooperative Human Tissue Network. The homogenized samples were extracted on ice with a radioimmune precipitation assay buffer containing the protease inhibitors. The extract aliquots (60 μg each) were analyzed by immunoblotting with the MT1-MMP Ab815 and pericentrin 4b antibodies.

**RESULTS AND DISCUSSION**

**Centrosomal MT1-MMP**—We examined the subcellular localization of endogenously expressed MT1-MMP in breast carcinoma MCF7 and glioma U251 cells, both of which synthesize MT1-MMP naturally. The level of MT1-MMP in MCF7 cells was, however, very low. U251 cells (Fig. 1a) and MCF7 cells (not shown) demonstrated specific centrosomal MT1-MMP immunoreactivity. The centrosomal association of MT1-MMP was confirmed by using α- and α-tubulin as centrosomal and mitotic spindle markers, respectively. Excess antigen blocked the centrosomal MT1-MMP immunoreactivity (Fig. 1d).

Several individual antibodies to MT1-MMP, which were raised against the hinge region and against the catalytic domain, generated similar MT1-MMP immunostaining. The staining of cells with the isotype control was negative. The centrosomal MT1-MMP immunoreactivity was strongly enhanced in the dividing metaphase cells. Overall, only a fraction of MT1-MMP accumulates in centrosomes, whereas the bulk of cellular MT1-MMP is associated with the plasma membrane and the multiple intracellular vesicles (Fig. 1b). Nocodazole abrogated the association of MT1-MMP with centrosomes in the interphase cells. Nocodazole had no effect on the association of MT1-MMP with centrosomes in the metaphase cells (Fig. 1a).

To corroborate further the presence of endogenous MT1-MMP in centrosomes, U251 cells were stably transfected with the siRNA construct (GAAGCCUGGCUACAGCAAAAU). MT1-MMP silencing by siRNA repressed both the expression of cellular MT1-MMP and its centrosomal immunoreactivity (Figs. 1a and 2c).

To demonstrate the existence of centrosomal MT1-MMP in transfected cells, we used MT1-MMP chimeras. The use of chimeras allowed us to avoid using MT1-MMP antibodies to confirm the centrosomal localization of the protease. The MT1-MMP-GFP construct was detected via the GFP moiety fluorescence without using antibody staining. The FLAG and the GFP protein sequences were both inserted into the hinge region of MT1-MMP. Following transfection of the cells with the chimeric constructs, MT1-MMP-FLAG and MT1-MMP-GFP were each detected in the centrosomes and co-localized with γ-tubulin in breast carcinoma MCF7 and glioma U251 cells, respectively (Fig. 1c). The accumulation of the MT1-MMP chimeras in the pericentrosomal space and the partial co-localization with the centrosomes is a result of MT1-MMP overexpression. Evidently, excess MT1-MMP is incapable of fitting into the tight centrosomal compartment.

To further corroborate the presence of MT1-MMP in the centrosomes, we isolated centrosomes from the synchronized...
metaphase U251 cells and determined that MT1-MMP co-fractionates with γ-tubulin (Fig. 2a). The concentration of MT1-MMP in the cytoplasm fraction was significantly lower than that in the centrosomes and that is why the cytoplasm fractions did not demonstrate observable amounts of the protease. In contrast, the centrosome samples were free of MT1-MMP. Our experiments have led us to the discovery that the microtubule cytoskeleton is essential for the nocodazole-sensitive trafficking of MT1-MMP (28, 29). Centrosomes are the microtubule-organizing centers, which play a key role in rapid protein trafficking. Proteins, e.g. caveolin, have been shown to travel from the perinuclear space to the plasma membrane and back using the tubulin cytoskeleton as “railroad tracks” (29, 30).

To demonstrate the functional activity of centrosomal MT1-MMP, purified proMMP-2 was co-incubated with the centrosomal samples. Centrosomal MT1-MMP activated proMMP-2 and converted the latent zymogen proenzyme into the active MMP-2 enzyme (Fig. 2b). Hydroxamate inhibitors GM6001 and AG3340, which are potent against MT1-MMP (Kᵢ ≈ 0.5 nM for both inhibitors), blocked MMP-2 activation (not shown). Consistent with the ability of centrosomal MT1-MMP to activate MMP-2, immunoblotting of the purified centrosomes using an MT1-MMP antibody confirmed that centrosomal MT1-MMP is represented by the active enzyme species (Fig. 2b, upper panel).

It is not surprising that MT1-MMP traverses and partially accumulates in the pericentrosomal area, because the microtubule cytoskeleton is essential for the nocodazole-sensitive trafficking of MT1-MMP (28, 29). Centrosomes are the microtubule-organizing centers, which play a key role in rapid protein trafficking. Proteins, e.g. caveolin, have been shown to travel from the perinuclear space to the plasma membrane and back using the tubulin cytoskeleton as “railroad tracks” (29, 30).

Our experiments have led us to the discovery that the microtubulin cytoskeleton and the centrosomes (the microtubulin cytoskeleton-organizing centers) are essential for the trafficking and the internalization of MT1-MMP and that MT1-MMP is trafficked to the pericentrosomal space most probably in the endosome-like vehicles. An analysis of the cells showed the existence of MT1-MMP-positive vesicles localized alongside the tubulin cytoskeleton (Fig. 2d). Rab-4 and Rab-11 (the markers of late/recycling endosomes and pericentrosomal/recycling endosomes, respectively) (31) co-localize with MT1-MMP, suggesting its endosomal nature (29, 32) (Fig. 2, e and f). To examine the intracellular trafficking of MT1-MMP, we

Fig. 1. Centrosomal MT1-MMP. a, immunostaining of the metaphase and the interphase glioma U251 and breast carcinoma MCF7 cells. Where indicated, cells were pretreated with nocodazole to destroy the cytoskeleton. Silencing by siRNA abrogates MT1-MMP immunoreactivity (in U251 cells, bottom panel). An antibody to the catalytic domain of MT1-MMP was used in immunostaining, b, immunostaining of endogenously expressed MT1-MMP in U251 cells. Arrows point to the plasma membrane, c, the MT1-MMP-GFP fluorescent chimera and the MT1-MMP-FLAG chimera in the centrosomes of U251 cells and MCF7 cells, respectively. Anti-FLAG antibody M2 antibody (Sigma) was used to detect the MT1-MMP-FLAG construct. d, excess antigen blocks centrosomal MT1-MMP immunoreactivity. The GM6001-inactivated catalytic domain of MT1-MMP (a 10-fold molar excess) was co-incubated with the centrosomal MT1-MMP immunoreactivity. The GM6001-inactivated catalytic domain of MT1-MMP was used in immunostaining. The GM6001-inactivated catalytic domain of MT1-MMP was used in immunostaining. Immunoblotting confirms co-fractionation of MT1-MMP with centrosomal γ-tubulin in U251 cells. Equal amounts of total protein from the cytoplasm and the centrosomal fractions were analyzed by Western blotting (upper panel) and Western blotting (lower panel) demonstrate that centrosomal MT1-MMP is largely represented by the active 60-kDa enzyme and that centrosomal MT1-MMP activates external proMMP-2 and converts the 68-kDa proMMP-2 into the mature 62-kDa MMP-2 enzyme. U251 cells co-expressing MT1-MMP with PDX (a potent inhibitor of furin that is an activator of MT1-MMP) were used as a side-by-side control. PDXMT1-MMP cells express the proenzyme, the activation intermediate, and the mature enzyme, and the 38–45-kDa degraded forms of MT1-MMP. c, Western blotting shows that siRNA silencing blocks the expression of cellular MT1-MMP in U251 cells. d, MT1-MMP (red) is localized alongside the α- tubulin microtubules (green) in the interphase cells. e and f, MT1-MMP (red) co-localizes (arrowheads) with endosomal markers RAB-4 and RAB-11 (green). DAPI, 4′,6-diamidino-2-phenylindole.

Fig. 2. Endosomal origin of functionally active centrosomal MT1-MMP. a, immunoblotting confirms co-fractionation of MT1-MMP with centrosomal γ-tubulin in U251 cells. Equal amounts of total protein from the cytoplasm and the centrosomal fractions were analyzed by Western blotting (upper panel) and Western blotting (bottom panel) demonstrate that centrosomal MT1-MMP is largely represented by the active 60-kDa enzyme and that centrosomal MT1-MMP activates external proMMP-2 and converts the 68-kDa proMMP-2 into the mature 62-kDa MMP-2 enzyme. U251 cells co-expressing MT1-MMP with PDX (a potent inhibitor of furin that is an activator of MT1-MMP) were used as a side-by-side control. PDXMT1-MMP cells express the proenzyme, the activation intermediate, and the mature enzyme, and the 38–45-kDa degraded forms of MT1-MMP. c, Western blotting shows that siRNA silencing blocks the expression of cellular MT1-MMP in U251 cells. d, MT1-MMP (red) is localized alongside the α-tubulin microtubules (green) in the interphase cells. e and f, MT1-MMP (red) co-localizes (arrowheads) with endosomal markers RAB-4 and RAB-11 (green). DAPI, 4′,6-diamidino-2-phenylindole.
used a newly developed non-covalent protein delivery Chariot reagent (33). 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 dissociated inside the cell compartment and liberated the antibody. The liberated, functional antibody then diffused throughout the cell and interacted with the target protein and, thus, allowed the identification of the subcellular compartment that harbors the target protein. The transduction of cells with the antibodies to MT1-MMP, by using a Chariot reagent, as well as the uptake of the MT1-MMP antibody by cells (29) also confirmed the microtubular transport of vesicular MT1-MMP to the centrosomes (not shown). The most recent publication (34) confirms the endosomal nature and the microtubular intracellular trafficking of metalloproteinases such as MMP-2 and MMP-9. These results provide indirect support for the data presented in our manuscript. Taken together, our data suggest that the tubulin cytoskeleton is involved in the rapid, vesicular MT1-MMP trafficking.

**MT1-MMP Targets the Centrosome Proteome**—Centrosomes play a central role in the organization of the tubulin cytoskeleton and microtubule nucleation by the γ-tubulin ring complex (24, 35, 36). They regulate the mitotic spindle during cell division and provide sister chromatid disjunction (37). Centrosomal MT1-MMP is proteolytically potent, and therefore, it may attack the centrosomal targets. Knowing the identity of these targets is of great importance to a more complete understanding of the tumorigenic function of MT1-MMP. In our earlier work, we identified the cleavage preferences of MT1-MMP through the proteolysis of protein substrates and the substrate phage libraries (27). We determined that the Pro-X-X ↓ X_{P4} hydrophobic Collagen-like cleavage motif is not ideally selective for MT1-MMP because this motif is recognized by several other individual MMPs. Highly selective MT1-MMP substrates lack the characteristic Pro at the P3 position; they contain, instead, an Arg at the P4 position (27). This P4 Arg is essential for efficient hydrolysis and for selectivity for MT1-MMP (38). MT1-MMP appears to recognize cleavage substrates in two distinct modes, using contacts at the P3 and the P1’ to recognize less selective substrates and using contacts at the P4 and the P1’ to recognize highly selective substrates (27).

We used these data to construct a probabilistic cleavage profile of MT1-MMP using a system for the prediction of protease specificity (PoPS) (39). Using a conventional set of parameters such as charge, polarity, and size, the phage library data for the P4–P1’ positions were used to produce a position specific scoring matrix on a scale of −5.0 to +5.0, as required by PoPS. The matrix contained a strong preference for Arg at P4 and excluded non-hydrophobic residues from the P1’ position. The matrix was also biased against collagen-like cleavage sites by excluding Pro from the P4 position. Lastly, the matrix was weighted in favor of the P4 and P1’ positions. To filter these predictions further, the programs PSIPRED (40) and NCOILS (41) (integrated in the PoPS system) were used to predict secondary structure and to search for sites that were localized in regions of low structure. PoPS was then used to search for the presence of this profile in the human proteome (∼25,000 proteins) and in the centrosomal proteome consisting of 114 proteins (42).

This analysis returned a score for each identified site, based on the weighted matrix. The analysis revealed 111 top scoring sites in the human proteome. A significant fraction of known MT1-MMP cleavage targets, including tissue transglutaminase, fibronectin, vitronectin, the low density lipoprotein receptor-related protein LRP, and the complement component C3 (43–48) were in this group. The subset of centrosomal proteins was significantly enriched in the high scoring, MT1-MMP-sensitive hits compared with the whole human proteome; −14% (total of 16) centrosomal proteins have the highest scores of 56–58 (60 is the highest possible score in PoPS), compared with −2.4% in the same score group of the entire proteome. Of the 111 human top scoring proteins, three proteins are of centrosomal origin.

**Fig. 3** shows the number of the known centrosomal proteins that were assigned the “MT1-MMP cleavage score” according to PoPS. One of the three top-scoring targets was the integral centrosomal protein, pericentrin (PoPS score = 58). Two other top-scoring targets were centrosomal Nek-2-associated protein 1 and a protein with an unknown function, KIAA1731. Overall, our in silico analyses suggest that centrosomes, relative to the total human proteome, are strongly enriched in the MT1-MMP cleavage targets and that the cleavage of the centrosomal proteins is an important proteolytic function of MT1-MMP.

**Pericentrin Is an MT1-MMP Cleavage Target**—Pericentrins 1 and 2, which are the splice variants of the same chromosomal gene (GenBank PCN2_HUMAN), are integral and essential centrosomal proteins (49). Pericentrin directly binds γ-tubulin and anchors the γ-tubulin-containing ring complexes to the centrosomes (50). Pericentrin silencing and mutations interfere with normal spindle formation and γ-tubulin localization in the centrosomes and result in G2 cell-cycle arrest, chromosome instability, and mitotic spindle aberrations (25, 36). The proteolyzed pericentrin was routinely observed in tumor cell lines (24, 25, 36). No individual proteases capable of cleaving pericentrin, however, have been identified so far. Inhibitors of serine and aspartic proteases as well as the specific inhibitors of calpain and caspases and proteasome inhibitors failed to inhibit the proteolysis of cellular pericentrin.

To assess whether pericentrin is susceptible to cleavage by MT1-MMP and to confirm our computer predictions, we synthesized the 10-mer peptides derived from the putative cleavage sites of pericentrin. The peptides were subjected to cleavage by the individual catalytic domain of MT1-MMP at a 1:100 enzyme:substrate ratio. Mass spectrometry was used to determine the mass of the cleavage products and the localization of the scissile bond (Fig. 4e). The A42A peptide (SGAIGF ↓ LRTA) that is highly sensitive to MT1-MMP (27) was used as a control. GM6001 fully blocked the cleavage of the A42A peptide, thus confirming the absence of contaminating proteases in the MT1-MMP samples. From 12 tested peptides, only the pericentrin peptides bearing the predicted ALRLLG1136 ↓ L1157FG and RAARVLG672 ↓ L673ET cleavage sites were susceptible to MT1-MMP.

We examined further the ability of MT1-MMP to cleave pericentrin in the purified centrosome sample in vitro. To avoid
the degradation of pericentrin by endogenous MT1-MMP, we purified the centrosomes from U251 cells transfected with H9251 and 1-anti-trypsin Portland (PDX) (51, 52). In these cells, MT1-MMP is present in the latent proenzyme form, because furin (an activator of MT1-MMP) is repressed by PDX. Co-incubation of the purified centrosomal samples with the recombinant catalytic domain of MT1-MMP followed by the Western blotting of the digest demonstrated the sensitivity of pericentrin to MT1-MMP. GM6001 rescued pericentrin from MT1-MMP cleavage. Because the antibody M8 to the N-terminal portion of pericentrin was used, the C-terminal cleavage fragments were not observable in this experiment. In turn, -tubulin was unaffected by this treatment (Fig. 4b). These data argue that centrosomal pericentrin is a likely target of MT1-MMP proteolysis in vivo.

To confirm the MT1-MMP cleavage of pericentrin in the cell...
system, we analyzed MT1-MMP-transfected and mock-transfected breast carcinoma MCF7 cells (Fig. 4d). To the contrary, the expression of the internalization-deficient, tailless MT1-MMP-ΔCT mutant (Fig. 4e), which is not delivered to the centrosomes, or the catalytically inert MT1-MMP-E240A construct (the Ala substitutes for an essential Glu240) rescued pericentrin from the proteolysis. Overall, our data suggest that pericentrin is the cleavage target of MT1-MMP in vivo. MT1-MMP proteolysis of pericentrin, however, is limited and results in the generation of the 150-kDa degradation fragment, which is associated, as well as intact pericentrin, with the centrosomes. Additional studies are required to identify the function of the pericentrin fragment in malignancy. Consistent with our data, pericentrin also interacts with the cation channel polycystin-2 membrane protein (55), thereby providing evidence of the interactions between membrane and centrosomal proteins. Conversely, interactions of pericentrin with polycystin-2 provide a rationale for the similar interactions of pericentrin with MT1-MMP. The most recent data suggest that a vesicular form of pericentrin also exists in the cells and that vesicular pericentrin could be, in fact, the target of MT1-MMP proteolysis.2 On the other hand, MT1-MMP is known to autolytically shed its highly potent ectodomain, which could be the major soluble form of intracellular MT1-MMP (56) following the release of the endosomal cargo.

Intracellular Cleavage Function of MT1-MMP

We also identified the number of chromosomes in the cells. There was a direct correlation between the MT1-MMP expression and the DNA content/aneuploidy (Fig. 5, a and b). As a control we used MDCK cells transfected with the empty vector (mock). The MT#6 clone demonstrated the centrosomal MT1-MMP immunoreactivity (Fig. 5c). Similar immunoreactivity of MT1-MMP was determined in the MT#5 clone. As expected, pericentrin was strongly degraded in both the MT#5 and MT#6 clones (not shown).

Intracellular Cleavage Function of MT1-MMP

In agreement with the MT1-MMP proteolysis of pericentrin observed in glioma cells, intact pericentrin was not found in MT1-MMP-overexpressing breast carcinoma MCF7 cells (Fig. 4d). To the contrary, the expression of the internalization-deficient, tailless MT1-MMP-ΔCT mutant (Fig. 4e), which is not delivered to the centrosomes, or the catalytically inert MT1-MMP-E240A construct (the Ala substitutes for an essential active site Glu240) rescued pericentrin from the proteolysis in MCF7 cells (Fig. 4d). Similar to PDX, the MT1-MMP siRNA-silencing rescued pericentrin from MT1-MMP cleavage in U251 cells (Fig. 4f).

To confirm our hypothesis that MT1-MMP causes proteolysis of pericentrin, we examined invasive mammary carcinoma, colon adenocarcinoma biopsies, and matching normal tissues. The samples were extracted with a radioimmune precipitation assay buffer containing the protease inhibitor mixture, phenylmethylsulfonyl fluoride and EDTA. MT1-MMP and pericentrin were each assessed by immunoblotting of the extracts. The intact ~220-kDa pericentrin was found in the normal tissues. In contrast, the 150-kDa degradation fragment of pericentrin was found in mammary carcinoma and colon carcinoma biopsies. In colon carcinoma samples (not shown) the pattern of pericentrin cleavage is similar to that observed in breast cancer biopsies (Fig. 4g). The presence of proteolyzed pericentrin in tumor biopsies correlated with the presence of the 45-kDa form of MT1-MMP, which is indicative of MT1-MMP self-proteolysis and, consequently, the protease activity. The pattern of pericentrin cleavage and the positions of the pericentrin antibody binding sites are summarized in Fig. 4h.

Intracellular Cleavage Function of MT1-MMP

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It is highly likely that pericentrin is not a singular intracellular target of MT1-MMP. Our additional proteomics study of the centrosome proteome (~400 individual proteins in glioma U251 cells) demonstrated that ~30 centrosomal proteins represent potential targets of MT1-MMP because they distinguish the cells in which MT1-MMP was silenced by siRNA from the cells in which MT1-MMP was overexpressed. The identification of these putative centrosomal targets of MT1-MMP by mass spectrometry analyses of the tryptic digest fragments is currently in progress.

MT1-MMP Induces Chromosome Instability—To test the hypothesis of whether MT1-MMP causes aberrations in genome inheritance, MDCK epithelial cells were transfected with human MT1-MMP. Tumor cell lines, including U251 and MCF7, demonstrate preexisting chromosome instability and multiple spindle aberrations and, therefore, cannot be used for the identification of MT1-MMP-induced chromatin aberrations. We selected MDCK cells because the conditional expression of human MT1-MMP is, by itself, sufficient to confer tumorigenicity on these non-malignant epithelial cells and to cause the formation of invasive tumors (10). From numerous stably transfected MDCK clones, we selected clones number 5 (MT#5) and number 6 (MT#6) with the high and the low expression of MT1-MMP, respectively, for the analysis (Fig. 5, a and b). As a control we used MDCK cells transfected with the empty vector (mock). The MT#6 clone demonstrated the centrosomal MT1-MMP immunoreactivity (Fig. 5c). Similar immunoreactivity of MT1-MMP was determined in the MT#5 clone. As expected, pericentrin was strongly degraded in both the MT#5 and MT#6 clones (not shown).

As detected by fluorescence-activated cell sorting, the total DNA content was increased in MT#6 and markedly so in MT#5 cells at 2 months following transfection (Fig. 5d). In contrast, the total DNA content in MDCK cells expressing the tailless, internalization-deficient MT1-MMP-ΔCT construct was close to that in mock cells.

We also identified the number of chromosomes in the cells. There was a direct correlation between the MT1-MMP expression and the DNA content/aneuploidy (Fig. 5, a, 6, and d). Mock cells contained 80.2 ± 0.87 chromosomes with a 10% aneuploidy frequency. In the MT1-MMP-transfected cells both of these figures were significantly higher (89.1 ± 2.1 chromosomes/27% aneuploidy in MT#6 cells, and 100.3 ± 2.9 chromosomes/48% aneuploidy in MT#5 cells). We inferred that MT1-MMP induced aneuploidy in MDCK cells in a dose-dependent manner.

2 S. Doxsey, unpublished observations.
Immunofluorescent staining revealed numerous aberrations of the mitotic spindle in metaphase MT#5 cells (Fig. 5c). We concluded, therefore, that MT1-MMP enhances chromosome instability in MDCK cells. These data are consistent with the enhanced tumorgenesis observed in the MT1-MMP-expressing MDCK xenografts in immunodeficient mice (10).

The aberrant functionality of centrosomes correlates with chromosome instability, a predictor of carcinogenesis (57–61). Cells with multiple centrosomes tend to form multipolar spindles, which result in abnormal chromosome segregation during mitosis (57, 62–65). It has been postulated that centrosome aberration may compromise the fidelity of cell division and cause chromosome instability. The acquisition of genomic instability is a crucial step in the development of human cancer (66). The ubiquity of aneuploidy in human cancers, particularly in solid tumors, suggests a fundamental link between errors in chromosome segregation and tumorigenesis. The observed aneuploidy in MT1-MMP-expressing cells suggests the presence of a novel, previously uncharacterized proteolytic pathway to chromatin instability.

It is also highly likely that cellular proteases exhibit the additional, previously unexpected, functions in mitosis. Thus, activation of calpain during mitosis is required for cells to establish the chromosome alignment, suggesting that this protease is also involved in the cleavage of certain centrosomal proteins (67). Consistent with our hypothesis, MMP-2 is present and functions in the nucleus of cardiac myocytes (68). It is premature to extrapolate our data to other members of the MT1-MMP family. We suspect, however, that MT2-MMP and MT3-MMP, similar to MT1-MMP, are likely to be found in the centrosomes and to function in the pericentrosomal compartment. It appears also that pericentrin is not a single intracellular target of MT1-MMP. Additional targets of MT1-MMP proteolysis have already been detected, and an effort to determine their identity is currently in progress.

Overall, we suggest that there is a causal link between MT1-MMP, pericentrin proteolysis, and chromosome instability. We also suggest that an intracellular proteolytic function of MT1-MMP is an important element in the transition of cells from normalcy to malignancy and that this novel function elucidates the close association of MT1-MMP with malignant transformation and cancer.

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Membrane Type-1 Matrix Metalloproteinase (MT1-MMP) Exhibits an Important Intracellular Cleavage Function and Causes Chromosome Instability
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