Centrosomal pericentrin is a direct cleavage target of membrane type-1 matrix metalloproteinase in humans but not in mice: potential implications for tumorigenesis

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Running title: MT1-MMP cleaves centrosomal pericentrin

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Membrane type-1 matrix metalloproteinase (MT1-MMP) exhibits distinctive and important pericellular cleavage functions. Recently, we determined that MT1-MMP was trafficked to the centrosomes in the course of endocytosis. Our data suggested that the functionally important, integral, centrosomal protein, pericentrin-2, was a cleavage target of MT1-MMP in human and in canine cells and that the sequence of the cleavage sites were ALRRLLG¹¹⁵⁶↓L¹¹⁵⁷FG and ALRRLLS²⁰⁶⁸↓L²⁰⁶⁹FG, respectively. The presence of Asp-948 at the P₁ position inactivated the corresponding site (ALRRLLD⁹⁴⁸↓L⁹⁴⁹FGD) in murine pericentrin. To confirm that MT1-MMP itself cleaves pericentrin directly, rather than indirectly, we analyzed the cleavage of the peptides which span the MT1-MMP cleavage site. In addition, we analyzed glioma U251 cells, which co-expressed MT1-MMP with the wild type murine pericentrin and the D948G mutant. We determined that the D948G mutant that exhibited the cleavage sequence of human pericentrin was sensitive to MT1-MMP, while unmodified murine pericentrin was resistant to proteolysis. Taken together, our results confirm that MT1-MMP cleaves pericentrin-2 in humans, but not in mice, and that mouse models of cancer probably cannot be used to critically examine MT1-MMP functionality.

MT1-MMP/MMP-14 is a prototypic member of the membrane-tethered matrix metalloproteinases (1). Although MT1-MMP is present in normal tissues, its enhanced expression is directly linked to tumor progression and metastasis (2-6). Cell surface-associated MT1-MMP is a multifunctional enzyme (7) and it is involved in the pericellular proteolysis of the extracellular matrix, the activation of soluble MMPs and the cleavage of adhesion and signaling cell receptors (8-10). The functional activity of MT1-MMP is regulated by its activation by furin-like proprotein convertases, by its inhibition by TIMPs and by its self-proteolysis and shedding (11-13). Evidence is also emerging that exocytosis, endocytosis and re-cycling also regulate the presentation of MT1-MMP on the cell surface and, consequently, its cell surface-associated proteolytic activity (14-17).

Recently, we determined that functionally active MT1-MMP, which was presented on the cell surface, was internalized, trafficked alongside the microtubular cytoskeleton and delivered to the centrosomal compartment (16,18). The presence of MT1-MMP in the pericentrosomal space correlated with the cleavage of human pericentrin-2 (kendrin), an integral and functionally important centrosomal, 3336-amino acid residue long, protein (19-22), and chromosome instability in non-malignant epithelial Madin-Darby canine kidney (MDCK) cells (18,23). Centrosomes, spindle pole bodies, are cellular organelles that exhibit an ability to organize microtubules and to nucleate (24). The normal functionality of centrosomes is essential to the organization of the cytoskeleton and the mitotic spindle, self-duplication and cell cycle progression (25,26).
Conversely, centrosomal abnormalities, early predictors of carcinogenesis, promote mitotic spindle aberrations and chromosome instability, events which are frequently observed in neoplastic cells (27-31). It is well established that pericentrin supports the normal functioning of the centrosomes and the cytoskeleton and that its function is important to cell-cycle progression (27,32,33). Despite the evident functional link of MT1-MMP activity with the cleavage of pericentrin observed in both human and canine cells (18), there were suspicions that MT1-MMP is indirectly, rather than directly, involved in these unorthodox, intracellular, proteolytic events. To demonstrate that MT1-MMP cleaves pericentrin directly, we used mutagenesis of murine pericentrin. The peptide sequence of murine, canine and human pericentrin-2 is homologous. There is, however, a single amino acid substitution at the P1 position of the MT1-MMP cleavage site in murine pericentrin when compared to that of human and canine proteins. Consistent with the cleavage preferences of MT1-MMP (34), we hypothesized that Asp-948 inactivates the cleavage site in murine pericentrin.

Here, we reconstructed the MT1-MMP human cleavage site in the murine pericentrin-2 sequence. Consistent with the proteolysis of human pericentrin-2 at the ALRRLLGLFG site, a single D948G mutation transformed murine pericentrin into the cleavage target of MT1-MMP. We suggest that these results confirm that MT1-MMP cleaves pericentrin in humans, but not in mice, and that the intracellular function of centrosomal MT1-MMP in humans cannot be fully recapitulated in the cellular and animal models in mice.

**Materials and Methods**

**Reagents** - Rabbit polyclonal antibody AB815 to the hinge region of MT1-MMP was from Chemicon (Temecula, CA). Murine monoclonal antibody 5D1 to the MT1-MMP catalytic domain was generated jointly by our laboratory and Chemicon. Rabbit polyclonal antibodies 4b and M8 to the C-terminal and N-terminal parts of pericentrin, respectively, were characterized earlier (22,35).

The recombinant catalytic domain of human and murine MT1-MMP was each expressed in *E.coli*, and then purified from the inclusion bodies and refolded to restore the catalytic activity (36). The peptides ALRRLLGLFG, ALRRLLSLFG and ALRRLLGLFG which span the MT1-MMP putative cleavage site in murine, canine and human pericentrin, respectively, were synthesized by GenScript (Piscataway, NJ). The peptides were cleaved for 2 h by the catalytic domain of MT1-MMP at the enzyme-substrate ratio of 1:1000 and the digest samples were analyzed by MALDI-TOF mass-spectrometry (18,34).

**Mutagenesis and cell transfection** – The cDNA construct of murine pericentrin was inserted in the pLPX7-blasticidin vector using routine manipulations. The oligonucleotide direct and reverse primers (5'-CTCCGAGATGCCCTGAGGAGACTTAGCCGTTTGGGGACACACTGAAAGCAGC-3' and GCTGCCTTCAGTGTCCCAAAACAGGCCTAGAAGTCCTCTCAGGGCATCTCAGGAG-3’, respectively; mutant positions are underlined) were used in PCR mutagenesis to insert the D948G mutation in the sequence of murine pericentrin. The presence of the mutation in the mutant construct was confirmed by DNA sequencing.

Mock-transfected human U251 glioma cells (mock), the cells stably overexpressing MT1-MMP (MT cells) and the cells in which MT1-MMP was stably silenced by the 5'--GAAGCCUGGCUACAGCAAUAU-3' siRNA construct (siMT cells) were constructed and partially characterized earlier (18,37,38). The 5'-GGUCCAUGCUGCAGAAAAACU-3' scrambled siRNA construct was used as a control. Both siRNA constructs were cloned in the psiLentGene-puromycin vector (Promega, Madison, WI). There was no effect of the scrambled siRNA construct on the expression of MT1-MMP in U251 cells (not shown). Cells were routinely grown in DMEM supplemented with 10% fetal bovine serum.

In this work, mock, MT and siMT cells were additionally transiently transfected with the pLPX7 plasmid coding for the wild type and the D948G mutant murine pericentrin-2. Blasticidin-resistant cells were selected in 7 days. The expression of murine pericentrin was analyzed by Western blotting of the cell lysates.
which were prepared from the total pool of blasticidin-resistant cells. In order to identify both the N-terminal and the C-terminal fragments of pericentrin, a mixture of 4b and M8 antibodies was used in these experiments. The expression of the murine pericentrin constructs was also analyzed by immunostaining the transfected cells.

**Immunofluorescence** - Cells were fixed in 4% paraformaldehyde for 10 min, permeabilized with 0.1% Triton X-100 for 5 min and blocked with 1% BSA. Cells were then incubated for 4 h with the primary antibody followed by incubation for 2 h with the species-specific secondary antibody conjugated with green Alexa Fluor 488 or red Alexa Fluor 594 (Molecular Probes, Eugene, OR). DNA was stained with DAPI. Images were acquired at a x600 original magnification on a Olympus BX51 fluorescent microscope equipped with a cooled MagnaFire camera (Olympus, San Diego, CA).

**General methods** – Gelatin zymography of MMP-2 from medium aliquots, cell surface biotinylation, cell lysate preparation, immunocapture of biotin-labeled MT1-MMP and Western blotting analysis of MT1-MMP and pericentrin were performed as described in our earlier publications (18,39). The buffers used for the preparation of cell lysates were supplemented with a protease inhibitor cocktail (pepstatin, leupeptin, bestatin, aprotinin, and E-64) and in addition, with PMSF and EDTA (1 mM each) to prevent additional, artificial proteolysis of the lysate samples.

**Results**

**Analysis of the peptides which span the MT1-MMP cleavage site in human, canine and murine pericentrin** - Our earlier data suggested that cellular MT1-MMP cleaved human centrosomal pericentrin at the ALRRLLG<sup>1156</sup>L<sup>1157</sup>FG cleavage site (18). Sequence alignment of human, murine and canine pericentrin shows that the ALRRLLG<sup>1156</sup>L<sup>1157</sup>FG and ALRRLLS<sup>2068</sup>L<sup>2069</sup>FG sequences are present in humans and canines, respectively, while mice exhibit the ALRRLLD<sup>948</sup>L<sup>949</sup>FG sequence (Fig. 1). In agreement, in the cleavage tests in vitro, MT1-MMP cleaved the synthetic peptides that span the human and canine cleavage site sequences. In contrast, the peptide ALRRLLD<sup>948</sup>L<sup>949</sup>FG that corresponded to the sequence of murine pericentrin was resistant to proteolysis by human MT1-MMP. Murine MT1-MMP also did not cleave this peptide (not shown). These data are consistent with the cleavage preferences of MT1-MMP and they suggest that the presence of a negatively charged Asp residue at the P1 position is likely to inactivate the site and to protect the peptide substrate from MT1-MMP proteolysis (34).

**MT1-MMP cleaves the murine pericentrin D948G mutant that exhibits the MT1-MMP cleavage site** – To validate our in vitro cleavage data in a cell setting, we first mutated the sequence of murine pericentrin ALRRLLD<sup>948</sup>L<sup>949</sup>FG and then isolated the D948G mutant. This mutant exhibited the human MT1-MMP cleavage site ALRRLLG<sup>948</sup>L<sup>949</sup>FG. We specifically selected robust U251 cells for these studies, because they, as in other aggressive malignant cells, exhibit the required compensatory mechanisms to overcome the overexpression of pericentrin. Other cell types usually do not survive the overexpression of this multifunctional, integral, centrosomal protein, the levels of which control cell cycle progression and genetic stability (29).

We next transfected mock, MT and siMT cells with the wild type and D948G mutant murine pericentrin. Finally, we determined if the pericentrin constructs were cleaved in the transfected cells. Fig. 2 shows that the expression of MT1-MMP was silenced in siMT cells. Low levels of MT1-MMP were observed in mock cells, which synthesized MT1-MMP naturally. The expression of MT1-MMP was up-regulated in MT cells, in which a characteristic 42-45 kDa self-degradation form of MT1-MMP was observed in addition to the 60-64 kDa full-length forms of the protease. According to our earlier data and the observations of the others, the presence of the 42-45 self-proteolytic form in the cell samples indicates the presence of high levels of the catalytically potent MT1-MMP (11,12).

Both the wild type and the D948G mutant murine pericentrins were stable in mock and siMT cells. The cleavage of the wild-type murine pericentrin was not observed in MT cells, which overexpress the protease. In contrast, there was an extensive proteolysis of
the D948G mutant in MT cells. The combined size of the observed N-terminal and C-terminal cleavage fragments of pericentrin (105 kDa and 140 kDa, respectively) correlated well with the size of intact murine pericentrin (240-250 kDa). Because these experiments were performed with the individual total cell pools obtained via transiently transfecting cells with the pericentrin constructs, the levels of pericentrin differ insignificantly among the samples.

As shown by gelatin zymography of the medium samples, mock and siMT cells did not activate secretory MMP-2, which was produced naturally by glioma cells. Consistent with many other reports (12), transfection of the cells with MT1-MMP stimulated extensive activation of the MMP-2 zymogen and its conversion into the mature enzyme. The efficiency of MMP-2 activation in MT cells co-transfected with either unmodified murine pericentrin or the D948G mutant was similar when compared to the cells expressing MT1-MMP alone (Fig. 2). These results indicate that the intracellular, pericentrin-cleaving, function of MT1-MMP and the status of pericentrin do not affect the proteolytic pericellular function of MT1-MMP. Overall, these results confirmed our in vitro peptide cleavage data and suggested that the reconstruction of the MT1-MMP human cleavage site ALRRLLG948↓L949FG transformed murine pericentrin into the cleavage target of MT1-MMP. In agreement with the proteolysis of mutant pericentrin by MT1-MMP, there was an evident centrosomal co-localization of these two proteins in the transfected cells (Fig. 3).

Discussion

From the twenty four known human MMPs, an elevated expression of MT1-MMP is most closely associated with malignancies (5,40). There is extensive evidence that cell surface-associated MT1-MMP functions as one of the key players of pericellular proteolysis in humans and mice (3,7,8). Knockout mice models generated volumes of highly valuable information about the proteolytic function of cellular MT1-MMP (41-45). There is evidence, however, that after being delivered to the plasma membrane, MT1-MMP, along with many other membrane-tethered proteins (46,47), is internalized. The clathrin-dependent and caveolin-dependent internalization pathways are both involved in the internalization and the recycling of MT1-MMP (14,15). In the pathway of MT1-MMP through the cell compartment, the proteolytically potent MT1-MMP accumulates in the microtubulin cytoskeleton-organizing centers, the centrosomes. Our earlier work suggested that both human and canine pericentrins were cleavage targets of centrosomal MT1-MMP (18). Pericentrin is an integral centrosomal protein and it is essential to the normal functioning of centrosomes and to mitotic spindle formation (48). The expression of MT1-MMP in the centrosomes of either human or canine cells correlated with the presence of the proteolytic fragments of pericentrin. In addition, these events correlated with the induction of mitotic spindle aberrations and aneuploidy in non-malignant MDCK cells (18,23). The sequence alignment of the putative cleavage site in human and canine pericentrin (ALRRLLG1156↓L1157FG and ALRRLLS2068↓L2069FG, respectively) supported our biochemical and cellular experiments because both Gly and Ser are compatible with the known cleavage preferences of MT1-MMP. This alignment also suggested that the corresponding sequence region of murine pericentrin (ALRRLLD948↓L949FGD) is protected from MT1-MMP proteolysis because of the Asp-948 at the P1 position (underlined in the peptide) in the murine sequence. Consistent with this hypothesis, murine pericentrin was resistant to MT1-MMP. Based on this knowledge, we reconstructed the human cleavage site in the murine pericentrin sequence. As expected, the D948G mutant that exhibited the human cleavage site was cleaved by MT1-MMP. We believe that our experiments prove that MT1-MMP cleaves pericentrin directly and we suggest that centrosomal MT1-MMP, through the cleavage of pericentrin, plays a unique role in human cells. In addition, the presence of the proteolytic fragments of pericentrin in the tumor biopsies, which express high levels of MT1-MMP, supports an important role of the MT1-MMP/pericentrin axis in cancer (18). Our data imply the pericellular function of MT1-MMP appears to be common across the species, while the intracellular, pericentrin-cleaving, function of MT1-MMP is absent in mice. These hypotheses add another level of
complexity to be overcome in our attempts to understand completely the tumorigenic functions of MT1-MMP in humans and they warrant additional studies of the genetically redesigned animal models, so that they will fully recapitulate human tumorigenesis.

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Legends to Figures

**Fig. 1.** The cleavage of pericentrin-derived peptides by MT1-MMP. The cleavage control peptide SGRIGF↓LRTA and the pericentrin peptides which span the putative MT1-MMP cleavage site, were each cleaved by MT1-MMP. The intact and digest samples were analyzed by mass-spectrometry. The mass of intact peptide is underlined. The sequence alignment of the putative MT1-MMP cleavage site in human, canine and murine pericentrin-2 (GenBank™ Accession Numbers O95617, XP_548735 and P48725, respectively) is presented in the upper part of the figure. Arrows indicates the scissile bonds.

**Fig. 2.** Mutant pericentrin D948G is cleaved by cellular MT1-MMP in glioma U251 cells. Murine pericentrin-2 and its D948G mutant were each transfected into mock cells (mock/PER-MUR and mock/PER-D948G, respectively) and co-expressed with MT1-MMP (MT/PER-MUR and MT/PER-D948G, respectively). In addition, murine pericentrin and its D948G mutant were co-expressed with the siRNA construct, which silenced the expression of MT1-MMP (siMT/PER-MUR and siMT/PER-D948G, respectively). For the analysis of MT1-MMP, cells were surface labeled with biotin, biotin-labeled proteins were immunocaptured on streptavidin-beads and the precipitated samples were analyzed by Western blotting with the MT1-MMP antibody AB815 (middle panel). Pericentrin was analyzed by Western blotting of the cell lysates. The mixture of the 4b and M8 antibodies was used to detect pericentrin (upper panel). The MT1-MMP-dependent activation of MMP-2 was determined by subjecting medium aliquots to gelatin zymography (bottom panel). For gelatin zymography analyses, cells were grown in serum-free medium. Positions of the molecular weight markers are on the left. The pericentrin sequence is shown above the panels.

**Fig. 3.** Pericentrin D948 mutant co-localizes with MT1-MMP in the centrosomes. Pericentrin and MT1-MMP were stained with the antibodies M8 and 5D1, respectively, in permeabilized MT/PER-D948G U251 cells. Nuclei were counterstained with DAPI. Arrows indicate the centrosomes.
HUMAN PERICENTRIN   ALRRLLG$^{1156}$ LFG
CANINE PERICENTRIN   ALRRLLS$^{2068}$ LFG
MURINE PERICENTRIN  ALRRLLD$^{948}$ LFG

CONTROL PEPTIDE SGRIGFLRTA

ALRRLLG
ALRRLLG
ALRRLLD LFG

Figure 1
Figure 2
