Metalloprotease-disintegrin MDC9: Intracellular Maturation and Catalytic Activity*

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Metalloprotease disintegrins are a family of membrane-anchored glycoproteins that are known to function in fertilization, myoblast fusion, neurogenesis, and ectodomain shedding of tumor necrosis factor (TNF)-α. Here we report the analysis of the intracellular maturation and catalytic activity of the widely expressed metalloprotease disintegrin MDC9. Our results suggest that the pro-domain of MDC9 is removed by a furin-type pro-protein convertase in the secretory pathway before the protein emerges on the cell surface. The soluble metalloprotease domain of MDC9 cleaves the insulin B-chain, a generic protease substrate, providing the first evidence that MDC9 is catalytically active. Soluble MDC9 appears to have distinct specificities for cleaving candidate substrate peptides compared with the TNF-α convertase (TACE/ADAM17). The catalytic activity of MDC9 can be inhibited by hydroxamic acid-type metalloprotease inhibitors in the low nanomolar range, in one case with up to 50-fold selectivity for MDC9 versus TACE. Peptides mimicking the predicted cysteine-switch region of MDC9 or TACE inhibit both enzymes in the low micromolar range, providing experimental evidence for regulation of metalloprotease disintegrins via a cysteine-switch mechanism. Finally, MDC9 is shown to become phosphorylated when cells are treated with the phorbol ester phorbol 12-myristate 13-acetate, a known inducer of protein ectodomain shedding. This work implies that removal of the inhibitory pro-domain of MDC9 by a furin-type pro-protein convertase in the secretory pathway is a prerequisite for protease activity. After pro-domain removal, additional steps, such as protein kinase C-dependent phosphorylation, may be involved in regulating the catalytic activity of MDC9, which is likely to target different substrates than the related TNF-α-convertase.

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1 The abbreviations used are: MDC, metalloprotease/disintegrin/cysteine-rich proteins; ADAMs, a disintegrin and metalloprotease(1); TNF, tumor necrosis factor; TNFR, TNF receptor; PMA, phorbol 12-myristate 13-acetate; TACE, TNF-α convertase; β-APP, β-amyloid precursor protein; PAGE, polyacrylamide gel electrophoresis; PNGase F, peptide N-glycosidase; PBS, phosphate-buffered saline; TGF-α, transforming growth factor-α; MMP, matrix metalloproteinase; MP, metalloprotease; endo H, endoglycosidase H; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)-3-ethyl]glycine; SPA, scintillation proximity assay; Dnp, dinitrophenyl; MALDI-TOF, matrix-assisted laser-desorption/ionization time-of-flight; CHO, Chinese hamster ovary.

In this study, we have evaluated the biosynthesis and in vitro catalytic activity of the widely expressed metalloprotease...
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disintegrin MDC9 (33). Our results demonstrate that the pro-domain of MDC9 is removed in the secretory pathway, most likely by a furin-type pro-protein convertase, and that MDC9 is phosphorylated when cells are stimulated with PMA. Furthermore, this study provides the first evidence that the soluble metalloprotease domain of MDC9 is catalytically active, has a different peptide substrate specificity than the related TNF-α convertase, and that the catalytic activity of MDC9 can be inhibited by hydroxamic acid-based metalloprotease inhibitors. These results are discussed in the context of a potential role of MDC9 in protein ectodomain shedding.

MATERIALS AND METHODS

Cell Culture—COS-7 cells were grown in Dulbecco’s modified Eagle’s medium (10% fetal calf serum, 1% glutamine, 1% penicillin/streptomycin). Transient transfections of COS-7 cells were performed using LipofectAMINE (Life Technologies, Inc.) or via electroporation (Bio-Rad GenePulser II).

Western Blot Analysis—Cells were lysed in TBS containing 1% Nonidet P-40 and protease inhibitors (4) on ice for 5 min, and centrifuged for 10 min in a Sorvall tabletop centrifuge. Cleared lysates were mixed with sample loading buffer, heated for 5 min at 95 °C in 50 mM dithiothreitol, separated by SDS-PAGE, transferred to nitrocellulose, probed with antibodies, and blotted as described (34). The nitrocellulose filters were blocked with 5% reconstituted dry milk, incubated with primary and secondary antibodies, and bound antibodies were visualized using the ECL chemiluminescence detection kit as described (34). Where indicated, the samples were deglycosylated with endoglycosidase H or PNGase F as described previously (35).

Cloning and Site-directed Mutagenesis—The cDNA encoding the soluble MDC9 metalloprotease (consisting of nucleotides 1–1253) was synthesized as follows: a cDNA encoding for a Myc epitope tag (EQKLI-SEEDL) and an XbaI restriction site was added to the 3′ end of the cDNA by polymerase chain reaction. The resulting polymerase chain reaction product was cleaved with EcoRI and XbaI and subcloned into a pcDNA3 vector (Invitrogen, San Diego, CA). The transformer site-directed mutagenesis kit (CLONTECH Laboratories Inc.) was used to introduce specific mutations, which were confirmed by DNA sequencing.

Cell-surface Biotinylation and Immunoprecipitation—Transfected COS-7 cells were grown to confluency on 6-well plates, washed in PBS at 4 °C, and incubated with the non-membrane-permeable biotinylation reagent NHS-LC-biotin (Pierce) for 45 min on ice. After washing with 0.1% glycine in PBS, the cells were lysed directly on the dish with cell lysis buffer (see above). Cleared cell lysates were subjected to immunoprecipitation as described (33). The immunoprecipitated material was eluted in 100 mM glycine, pH 3, neutralized immediately with 1 M Hepes, pH 7.5, and concentrated with a Centricon-10 concentrator (Amicon). The N terminus of the purified soluble MDC9 metalloprotease was identified as A206VLPQTR, which corresponds to the sequence immediately following the putative pro-protein convertase cleavage site RRRK in mouse MDC9 (33).

Inhibin B-chain Cleavage Assay—Purified soluble MDC9 MP-Myc, or recombinant purified TNF-α convertase (TACE) (18), or purified recombinant MMP-1 (39) were incubated with 50 μM oxidized insulin B-chain in 100 μl of reaction buffer (50 mM Tricine, pH 7.4, 200 mM NaCl, 10 mM CaCl2, 1 mM EDTA, 0.1% Triton X-100, 1,10-phenanthroline). The reaction was stopped by addition of trifluoroacetic acid. The insulin B-chain and its cleavage products were detected by matrix-assisted laser-desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry using a Reflex III instrument (Bruker Franzen, Bremen, Germany) as described (40).

Inhibitor Titration of MDC9 to Determine Enzyme Concentration—The MMP peptide substrate dinitrophenyl-proline cyclohexylalanine-GC(Me)HK(N-methylanilinic acid)NH2 (41), which is cleaved by MDC9 (see Table I), was diluted from a Me2SO stock to 30 μM in 10 mM Hepes, pH 7.5, containing 0.0015% Brij-35 (Buffer A). 60 μM of substrate was then added to 30 μl of CGS27023 (a potent hydroxamic acid-based inhibitor of MDC9, see below and Table III) at concentrations ranging from 0 to 10 μM. The reaction was initiated by addition of MDC9 (10 μl), so that the final dilution was 100–3000-fold and the reactions were run for 1–4 h at 22 °C. Initial velocities were calculated by measuring the fluorescence units of the reaction time and dividing by the fluorescence units when complete turnover of the substrate was observed (final reaction time). The initial velocities were plotted as a function of inhibitor concentration, and the curve was fit to Equation 1, a modified version of the Morrison equation (42). As an approximation, the K_i was proposed to be comparable to the K_s as the substrate concentration was at least 5-fold below the K_m. Note: an absolute number for the K_m could not be determined as the substrate was not soluble at high concentrations.

\[ v = \frac{k_{cat}}{K_m} \cdot \frac{[S] - K_{app}}{[S] + K_{app}} \]  

\[ K_{app} = K_s / (1 + S/K_m) \]  

Incubation of Candidate Cysteine Peptides with MDC9 and Determination of Cleavage Sites and Kinetics—Peptides carrying from 12 amino acid residues surrounding the membrane proximal cleavage sites of selected proteins that are known to be released from the plasma membrane by metalloproteases (see Table I) were synthesized by Synpep (Dublin, CA). All peptides contained a dinitrophenyl (Dnp) group at their N terminus to facilitate detection at 350 nm. The peptide sequences and the reported cleavage sites are presented in Table I. MDC9 (1 nm) was incubated with each of the putative peptide substrates (30 μM) in 10 mM Hepes, pH 7.2, containing 0.0015% Brij (Sigma). Reactions were timed to allow approximately 5–20% turnover of the substrate. Reactions were quenched using 1% heptfluorobutyric acid, and products were separated by high pressure liquid reverse phase chromatography (C18 column, Vydac, Hesperia, CA) with absorbance monitored at 350 nm. Turnover was quantitated by integrating peak areas of the substrate and product(s). Liquid chromatography-mass spectrometry was used to determine the masses of the products and therefore the cleavage site(s) recognized by MDC9. Briefly, digestion mixtures were passed over a hypersil C18 column, and after UV detection at 350 nm, the sample was routed into the ionspray source of a Sciex API-III triple quadrupole mass spectrometer. Specificity constants were calculated from initial velocities using the equation: k_{cat}/K_m = (% turnover/100)/(t_initial). Conditions of k_{cat}/K_m were verified by running the reactions at more than one substrate concentration. The enzyme concentration for MDC9 was determined as described above. For the TNF and the MMP peptide substrates, two products were formed, and individual specificity constants were determined for each of the products since they were generated independently from one another (data not shown).

Evaluation of the Inhibitory Potential of Cysteine Switch Peptides—Synpep Corp. (Dublin, CA) synthesized all of the Cys-switch peptides (see Table IV). All solvents and buffers used were sparged with argon to avoid oxidation of the thiol group. Peptides were dissolved in dimethyl formamide to yield a stock solution of 50 mM and were diluted as described below such that the final concentration of dimethyl formamide in the assay was 1%. MDC9 activity was measured using streptavidin-coated scintillation proximity assay (SPA) beads and a biotin-
labeled peptide corresponding to the sequence of the cleavage site in pro-TNF-α. The digests were performed by the addition of 25 μl of titrated inhibitor in 3% dimethyl formamide (10 mM Hepes, pH 7.5) to 25 μl of substrate (biotin-SP-LQA*VRRSPPTP[H] 8-NH2) in Hepes with 0.1% bovine serum albumin. Reactions were initiated by addition of 25 μl of recombinant MDC9 (in Hepes with 0.1% bovine serum albumin). The digestion was quenched by addition of streptavidin SPA beads (Amer sham Pharmacia Biotech) in EDTA. The final concentration of substrate in the assay was 200 nM. Enzyme was 1.0 nM, and cysteine-switch peptides were titrated from 100 μM with 3-fold dilution, 11 points per curve. Under the above conditions the substrate concentrations are significantly less than K<sub>m</sub> and the K<sub>i</sub> can be determined directly by plotting percent inhibition versus the log of the inhibitor concentration, where K<sub>i</sub> = K<sub>m</sub>(1 + [S]/K<sub>m</sub>). No effects on the K<sub>i</sub> were observed whether 0.5 mM cysteine was included or not.

**Inhibition Constants Against Batimastat, Marimastat, CGS 27023, and TNF-α Protease Inhibitor—Synthesis of batimastat, CGS 27023, and the TNF-α protease inhibitor has been described (18, 43). The substrate Dnp-PChaGC(Me)/HK(NMA)NH2 (41) was diluted from a Me<sub>6</sub>SO stock solution to a concentration of 30 μM in buffer containing 10 mM Hepes, pH 7.5, and 0.0015% Brij-35 (Buffer A). A 60-μl aliquot was removed and added to a black Polyfiltronics 96-well plate. Inhibitor was diluted 30-fold into Buffer A. An aliquot of the inhibitor concentration, where K<sub>i</sub> = K<sub>m</sub>(1 + [S]/K<sub>m</sub>). The inhibitor was diluted 30-fold into Buffer A. A 60-μl aliquot was added. and to a black Polyfiltronics 96-well plate. MDC9 was diluted 60–300-fold into Buffer A, and 10 μl was added. Fluorescence was monitored at an excitation of 343 nm and emission of 450 nm. Reactions were run from 0.5 to 4 h at 22 °C and liquid chromatography/mass spectrometry analysis was performed to determine cleavage sites in the substrate.

In a similar manner, K<sub>i</sub> values were determined for 19-kDa truncated collagenase (MMP-1), 20-kDa truncated collagenase 3 (MMP-13), stromelysin-1 (MMP-3), and 50-kDa truncated gelatinase B (MMP-9) using the same fluorogenic substrate. Assays were conducted in a total volume of 0.180 ml of assay buffer (200 mM NaCl, 50 mM Tris, 5 mM CaCl<sub>2</sub>, 10 μM ZnSO<sub>4</sub>, 0.065% Brij 35, pH 7.6) in each well of a black 96-well microtiter plate. 19-kDa collagenase-1, 20-kDa collagenase-3, stromelysin-1, and 50-kDa gelatinase B concentrations were adjusted to 500 pm, 30 pm, 5 nm, and 100 pm, respectively. A dose response was generated using an 11-point 3-fold serial dilution with initial starting concentrations of 100, 10, or 1 μM. Inhibitor and enzyme reactions were incubated for 30 min at room temperature and then initiated with 10 μM fluorogenic substrate. The product formation was measured after 45–180 min. Finally, K<sub>i</sub> values for these inhibitors against TACE were determined using the same SPA assay as described earlier for the cysteine-switch peptides. In all cases, the conditions were such that the substrate concentrations were significantly less than K<sub>m</sub> and the K<sub>i</sub> can be determined directly by plotting percent inhibition versus the log of the inhibitor concentration, where K<sub>i</sub> = K<sub>m</sub>(1 + [S]/K<sub>m</sub>). Phorbol Ester Treatment of CHO Cells—To evaluate the biosynthesis and maturation of mouse MDC9 in the secretory pathway, pulse-chase experiments were performed using transiently transfected COS-7 cells expressing MDC9. Immunoprecipitations at different time points show that MDC9 is first synthesized as a precursor of 110 kDa (Fig. 1A) which is later processed to an 84-kDa form. Only the processed form of MDC9 with a molecular mass of 84 kDa is immunoprecipitated from cell-surface biotinylated cells, demonstrating that the pro-domain removal most likely occurs intracellularly (Figs. 1C and 2B, and see Ref. 33). The subcellular localization of pro-domain removal was further defined by assessing the effect of the secretory pathway inhibitors brefeldin A and monensin on MDC9 processing. Brefeldin A, which blocks vesicle budding in the endoplasmic reticulum (44), completely blocked processing of MDC9 at 5 μg/ml. Monensin, which is thought to prevent transport past the medial-Golgi apparatus (45), partially blocked MDC9 processing at 2 and 10 μg/ml and completely blocked processing at 25 μg/ml (Fig. 1B). Western blot analysis of an endoglycosidase H-treated sample of MDC9-expressing COS-7 cells showed that both the precursor and mature forms of MDC9 were sensitive to endo H (Fig. 1C, lane 2). Treatment of an identical sample with PNGase, which removes most or all N-linked carbohydrate residues, resulted in a faster migrating mature form of MDC9 compared with the endo H-treated sample. In contrast, the MDC9 precursor appeared to comigrate in the endo H- and PNGase-treated samples. The cell surface-labeled processed form of MDC9 displayed a similar change in migration on SDS-PAGE after endo H and PNGase treatment compared with the processed form of MDC9 detected by Western blot. This result suggests that at least one, but not all, of the N-linked carbohydrate moieties of mature MDC9 acquires resistance to endo H treatment through the conversion of high mannose glycans into complex carbohydrates in the medial-Golgi network. Taken together, these results suggest that MDC9 is proteolytically processed in the secretory pathway after passage through the medial-Golgi apparatus.

Since MDC9 contains a consensus cleavage sequence for the pro-protein convertase furin between its pro- and metalloprotease domain, and because furin resides predominantly in the trans-Golgi network (45), furin or related pro-protein convertases are good candidates for processing MDC9 in the secretory pathway. To provide direct evidence for a potential role of furin in processing MDC9, we immunoprecipitated the precursor of full-length MDC9 from brefeldin A-treated COS-7 cells and incubated it with recombinant furin (36) (Fig. 1D). The furin-treated MDC9 precursor co-migrated with the MDC9 as it is processed in vivo in COS-7 cells. Increasing amounts of furin also generated an additional faster migrating band, perhaps due to an additional cleavage site that is not accessible to the membrane-anchored form of furin or a related pro-protein convertase in vivo. Alternatively, this site may be less efficiently cleaved and therefore only utilized when relatively high concentrations of furin are added in vitro.

To evaluate the sequence requirements for intracellular processing and metalloprotease activity of MDC9, the following mutations were introduced into both a soluble and a membrane-anchored form of MDC9 by site-directed mutagenesis (see Fig. 2A): 1) removal of the furin cleavage site (Δ4R) between the pro- and metalloprotease domains; 2) conversion of the putative pro-domain cysteine-switch residue (46, 47), which is predicted to inhibit the protease during biosynthesis by binding to the active site, into an alanine (pro-Cys → Ala); 3) mutation of the “catalytic” glutamic acid in the metalloprotease active site (MP-Glu → Ala) (48).

The effects of the mutations on intracellular processing were first tested by comparing the relative amounts of MDC9 precursor and processed forms in Western blots and in cell-surface biotinylated samples (Fig. 2B). On Western blots, the 110-kDa precursor of MDC9 and the processed form of 84 kDa can both be detected (Fig. 2B). However, only the processed form of 84 kDa can be immunoprecipitated from cell-surface-labeled samples (Figs. 1C and 2B, see also Ref. 33). Both removal of the predicted furin cleavage site, and the Glu → Ala mutation in the catalytic site did not detectably affect MDC9 processing and appearance on the cell surface (Fig. 2B). However, removal of the putative cysteine-switch residue in the pro-domain led to a dramatic decrease of the processed 84-kDa form of MDC9 in the cell lysate as well as on the cell surface. The Δ4R and the Pro-Cys → Ala mutations also resulted in small amounts of additional faster migrating bands compared with wild-type.
MDC9, which most likely result from aberrant processing. Similar results were obtained with a recombinant soluble pro- and metalloprotease domain of MDC9 (Fig. 2C). The wild-type soluble protein and all three mutant forms were detected as proteins of ~47 kDa in Western blots of COS-7 cell lysates (Fig. 2A, predicted molecular mass of soluble wild-type MDC9 is 44.29 kDa). The supernatant of cells expressing the soluble wild-type and the MP Glu3Ala mutant contained mainly a ~28-kDa protein, suggesting that the pro-domain had been removed intracellularly (Fig. 2C, lower panel, lanes 2 and 5). N-terminal sequence analysis of the affinity purified soluble wild-type MDC9 metalloprotease domain (Fig. 2D, lane 1) confirmed that processing had occurred immediately after the predicted pro-protein convertase cleavage site in MDC9 (RRRR205-AVLPQTR, see “Materials and Methods”). Soluble MDC9 Δ4R was secreted both as an unprocessed precursor of 47 kDa and as a processed form of ~33 kDa (Fig. 2C, lane 3). Evidently processing of MDC9 can occur in the absence of a furin cleavage site at an adjacent position. This finding prompted a re-evaluation of the processing of the full-length MDC9 Δ4R mutant by comparing its migration to that of the wild-type MDC9 on a Western blot of proteins that had been separated on a 7.5% SDS-polyacrylamide gel. Under these conditions the full-length Δ4R mutant migrated slightly slower than the wild-type protein (Fig. 2B, lanes 6 and 7), consistent with a cleavage at an adjacent but distinct site. Similar to the behavior of the full-length pro-Cys3Ala mutant, very little of the soluble pro-Cys3Ala MDC9 mutant could be detected in the supernatant, although the protein was clearly present in the cell lysate (Fig. 2C, lane 4).

**Catalytic Activity of Soluble MDC9, TACE, and MMP-1 on the Insulin B-chain**—To determine whether MDC9 is catalytically active, the soluble wild-type metalloprotease was affinity purified from COS-7 cell supernatants (Fig. 2D). The affinity purified material was incubated with the insulin B-chain peptide, a generic protease substrate consisting of 30 amino acid residues (49). The resulting insulin B-chain fragments were identified by mass spectrometry (Fig. 3, A–C). After 1-h incubation with MDC9, cleavage between insulin B-chain residues Tyr16 and Leu17, and Tyr26 and Thr27 could be detected (Fig. 3A–C).
After 6 h, the substrate had been completely converted into products resulting from cleavage between Tyr16 and Leu17, and Tyr26 and Thr27. No processing was seen after 6 h in the presence of the metalloprotease inhibitor 1,10-phenanthroline (Fig. 3A) or after co-incubation of the insulin B-chain for 6 h with a similar amount of the MP Glu → Ala catalytic site mutant purified under identical conditions (data not shown). When the insulin B-chain was incubated with purified soluble TACE (Fig. 3B) or MMP-1 (Fig. 3C) under identical conditions, in each case only one cleavage between Tyr16 and Leu17 was observed. As would be expected, 1,10-phenanthroline inhibited TACE- and MMP-1-mediated cleavage of the insulin B-chain (Fig. 3, B and C). Under the conditions used in this study, the apparent cleavage specificity of MDC9, TACE, and MMP-1 on the insulin B-chain is thus more restricted than what has been reported for most other proteases, including related snake venom metalloproteases (49, 50).

**Kinetic Analysis of Candidate Substrate Peptide Cleavage by MDC9**—Several different types of membrane proteins are known to be released from the plasma membrane by metalloproteases. To evaluate further the cleavage specificity of MDC9 and to test whether MDC9 might in principle have a role in intracellular maturation and catalytic activity of MDC9.
Fig. 3. MALDI-TOF analysis of insulin B-chain cleavage products produced by soluble MDC9. Matrix-assisted laser-desorption/ionization time-of-flight (MALDI-TOF) mass spectrometric traces of products generated from incubating the oxidized insulin B-chain with affinity purified soluble MDC9 for 1, 6, or 6 h in the presence of 1,10-phenanthroline (A); recombinant TACE (B); and MMP-1 for 6 h or 6 h in the presence of 1,10-phenanthroline (C). The numbers above the major peaks indicate the experimental monoisotopic masses (m/z). D, sequence of the insulin B-chain and the predicted protonated monoisotopic masses of the oxidized insulin B-chain and of the corresponding cleavage products. The predicted protonated monoisotopic masses were calculated with Procomp software, assuming that both cysteine residues in the insulin B-chain have the mass of the oxidized form of cysteine, cysteic acid (C(=O)H). The cleavage sites are marked by arrowheads.
protein ectodomain shedding, the soluble MDC9 metalloprotease was incubated with peptides corresponding to cleavage sites of specific membrane proteins that are known to be released from the membrane by metalloproteases (21). Table I shows that MDC9 was able to cut the cleavage site peptide of the β-amyloid precursor protein (β-APP) (28), tumor necrosis factor α (TNF-α) (51), the p75 TNF receptor (p75-TNFR) (52), and the c-kit ligand-1 (KL-1) (53). To determine the cleavage sites, the fragments of peptides that were processed by affinity purified MDC9 were analyzed by mass spectrometry. As indicated in Table I, MDC9 did not cleave any of the peptides tested here at the same site that is known to be used by sheddases in cells in vivo. The c-kit ligand and TNF-α peptide were cut in more than one position by recombinant MDC9. Furthermore, we found that affinity purified MDC9 was able to process an MMP substrate peptide that is not processed by TACE.2 Finally, affinity purified soluble MDC9 did not process cleavage site peptides for the interleukin-6 receptor (54), the p55 TNF receptor (p55-TNFR) (55), pro-transforming growth factor α (TGF-α) (56), and L-selectin (57) under the conditions used here.

The cleavage kinetics of the β-APP peptide, the TNF-α peptide, and also of the MMP peptide by MDC9 were determined as described under “Materials and Methods.” The kinetic analysis revealed that the $k_{cat}/K_m$ of β-APP cleavage by MDC9 is 4.3-fold higher than the $k_{cat}/K_m$ for cleavage of the P1 site in TNF-α, 6.8-fold higher than the $k_{cat}/K_m$ for the P2 site in TNF-α, and 21-fold higher than the $k_{cat}/K_m$ for cleavage of the MMP substrate peptide by MDC9 (Table II).

**Hydroxamic Acid Derivatives and Cysteine-switch Peptides as Inhibitors of MDC9 Metalloprotease Activity—**Since TACE can be inhibited by hydroxamic acid-based metalloprotease inhibitors, we asked whether (a) hydroxamic acid derivatives are also inhibitors of the MDC9 metalloprotease activity, and (b) whether there is some specificity of four previously described hydroxamic acid-based inhibitors toward MDC9 or TACE. Table III shows the $K_i$ of different inhibitors toward TACE and MDC9, as well as toward the matrix-type metalloproteases MMP-1, MMP-3, MMP-9, and MMP-13. Under the in vitro cleavage conditions used here, CGS 27023 was the most selective for MDC9 over TACE (~50-fold), whereas marimastat was the most selective for TACE over MDC9 (~12-fold). These data further demonstrate that MDC9 has a unique inhibitor profile which does not resemble that of TACE or any of the MMPs tested here.

**DISCUSSION**

This study provides the first analysis of the intracellular maturation and catalytic activity of the metalloprotease disintegrin MDC9. Several lines of evidence demonstrate that MDC9, like MDC15 (35), is processed in a late compartment of the secretory pathway, most likely by a pro-protein convertase.
such as furin. First, processing of MDC9 can be prevented by addition of the secretory pathway inhibitors brefeldin A (44) and monensin (45), suggesting that MDC9 is processed after passage through the medial-Golgi apparatus. Second, incubation of pro-MDC9 with recombinant furin in vitro results in pro-domain removal. Third, N-terminal sequence analysis of a soluble secreted MDC9 metalloprotease domain confirmed that processing occurs next to the predicted furin cleavage site (RRRR) in cells. Finally, after deletion of the furin cleavage site, both full-length membrane-anchored MDC9 and a soluble pro- and metalloprotease-domain construct appear to be processed at a different site than the wild-type proteins. Nevertheless, removal of the furin site did not noticeably affect the efficiency of processing of full-length membrane-anchored MDC9. One explanation for this result could be that additional cleavages in the MDC9 pro-domain normally occur after processing at the furin site, something that has also been observed for mMDC15 (35). Such additional cleavage sites in the prodomain may facilitate its removal after cleavage by furin and may only become apparent after the furin cleavage site is eliminated.

The first evidence for catalytic activity of MDC9 was obtained by incubating affinity purified soluble MDC9 with the insulin B-chain, a generic protease substrate. Our results demonstrate that MDC9, TACE, and MMP-1 can all cleave the insulin B-chain, albeit with somewhat different specificity. Cleavage of the insulin B-chain and of the general protease inhibitor α2-macroglobulin (58) may thus be useful initial assays to assess the catalytic activity of other metalloprotease

### Table III

Inhibition constants were determined for each enzyme using the MMP fluorescent substrate assay. For TACE, which does not cleave the MMP fluorescent substrate, the TNF SPA peptide assay was used instead (see “Materials and Methods”).

| Structure | Compound | MDC-9 | TACE | MMP-1 | MMP-3 | MMP-9 | MMP-13 |
|-----------|----------|-------|------|-------|-------|-------|--------|
| Chiral    | marimastat | 274  | 22  | 1  | 68  | 1  | 0.1    |
| Chiral    | batimastat | 14  | 11  | 1.4 | 3    | 0.4  | 0.5    |
| Chiral    | TAPI     | 17  | 8.8 | 6  | 68  | 0.5  | 0.2    |
| Chiral    | CGS 27023 | ~1  | 54  | 11 | 16  | 3   | 5     |

### Table IV

IC50 (µM) values for various Cys-switch peptides were determined against TACE and MDC9 using the SPA TNF peptide assay (see “Materials and Methods”). (>150) denotes those peptides for which an IC50 could not be determined but where the IC50 is higher than 150 µM since in all cases inhibition of the enzyme by these Cys peptides was less than 20% at 100 µM. h, human; m, mouse.

| Cys-switch Peptide | Enzyme | KtACE (µM) | mMDC9 |
|--------------------|---------|------------|--------|
| Human TACE (PKVCGYLK) | 43  | 4           |
| Human MDC9 (PLRGCVSN) | 105 | 37          |
| Mouse MDC9 (PLRGCVSN) | 52  | 10          |
| Mouse ADAM12 (QGLCGSH) | >150 | >150        |
| Human ADAM10 (QQGCADHS) | >150 | >150        |

![FIG. 4. Phorbol ester-dependent phosphorylation of MDC9. Immunoprecipitation of MDC9 from [35S]Met/Cys-labeled (lane 2) or from [32P]labeled CHO cells stably expressing MDC9, incubated in the presence (lane 5) or absence of PMA (lane 4). Control immunoprecipitations were performed on CHO stably transfected with the expression vector and labeled with [35S] (lane 1) or [32P] (lane 3).](image-url)
Because metalloprotease disintegrins have been hypothesized to function in protein ectodomain shedding, we evaluated the ability of soluble MDC9 to cleave selected candidate substrate peptides that mimic the cleavage sites of proteins that are shed from the plasma membrane by a metalloprotease activity. Of the eight peptides tested here, the β-APP peptide was the most efficiently cleaved, followed by the pro-TNF-α, p75-TNFR, and KL-1 peptides. MDC9 did not process peptides mimicking the cleavage site of the p55-TNFR, L-selectin, pro-TGF-α, and the interleukin-6 receptor. Mass spectrometric analysis of the products generated by MDC9 revealed that none of the four substrate peptides were cleaved at the site that is used during release of the corresponding protein from cells. Therefore MDC9 is most likely not the secretase responsible for processing the proteins that were included in the present study. Indeed, TACE can process TGF-α, L-selectin, and p55-TNFR peptides in the correct position in vitro. Furthermore, a targeted deletion of TACE results in the inability of cells to release TNF-α, TGF-α, and L-selectin from the cell surface, although it remains formally possible that some of these substrates are cleaved by another protease that needs TACE to become activated (17, 59).

With respect to β-APP processing, TACE is apparently the major protein kinase C-dependent α-secretase in cultured cells, and it also cleaves the β-APP peptide at the major α-secretase site in vitro (28, 60). Although MDC9 clearly does not target the major α-secretase site in vitro, in light of the relatively efficient processing of the β-APP peptide observed here it will be interesting to determine whether MDC9 may have some role in β-APP processing, perhaps restricted to certain cell types. In this context it is worth pointing out that MDC9 cleaves β-APP within a peptide sequence (HHQK) which apparently plays a role in inducing neurotoxic microglia (61). Finally, we note that a standard MMP peptide substrate is cleaved in a similar fashion by MDC9 and MMPs -1, -3, -9, and -13, but not by TACE, and that MDC9 cleaves the TNF-α peptide at the same two sites as MMP-1 and MMP-9. It will thus also be interesting to evaluate whether MDC9 may have substrates in the extracellular matrix in vivo.

These results provide the first evidence for differences in the substrate specificity and cleavage site selection between two distinct metalloprotease disintegrins, at least as far as soluble peptide substrates and soluble proteases are concerned. In addition to any inherent substrate specificity of metalloprotease disintegrins in vitro, additional factors may determine which substrates are cleaved in vivo. These determinants may include potential targeting or recognition events between the substrate and other parts of the metalloprotease disintegrin protein, such as the disintegrin domain, cysteine-rich region, or epidermal growth factor repeat (20). Furthermore, access to substrates may also depend on regulated or constitutive co-localization in the same subcellular compartment(s) (35).

An evaluation of the inhibitory potential of different hydroxamic acid-based metalloprotease inhibitors, which are known to potently inhibit TACE (17, 18, 62), revealed that these compounds are also potent inhibitors of MDC9 and of certain matrix-type metalloproteases. Furthermore, we observed differences in the selectivity of the compounds tested here toward TACE or MDC9. An extension of this approach may therefore yield even more selective inhibitors of these or other metalloprotease disintegrins.

To address whether removal of the prodomain, for example by furin, is a prerequisite for catalytic activity (33, 35, 63, 64), we tested whether peptides corresponding to the cysteine-switch sequence of various metalloprotease disintegrins inhibit the catalytic activity. In matrix type metalloproteases (46, 47) and the snake venom metalloproteases adamalysin II (47), the free sulfhydryl of the cysteine-switch residue in the pro-domain is thought to bind to the Zn$^{2+}$ ion in the catalytic site. Our results suggest that MDC9 and TACE are indeed both inhibited by their respective cysteine-switch peptides. The inhibitory potential of the mouse MDC9 cysteine-switch peptide for mouse MDC9 is similar to that of the adamalysin II cysteine-switch peptide for adamalysin II (47). The relatively potent inhibition of mouse MDC9 by its cysteine-switch peptide may explain why mutating the cysteine-switch residue strongly decreased transport of full-length and soluble MDC9 to the cell surface. In analogy to the protease subtilisin, where the prodomain is thought to function as intramolecular chaperone (65), the interaction of the free sulfhydryl residue with the active site may be necessary for the pro- and metalloprotease domains of MDC9 to fold properly. If so, removing the cysteine-switch residue might prevent productive folding, resulting in retention of the mutant protein by endoplasmic reticulum-resident chaperones and subsequent degradation (66). In contrast to MDC9, mutating the cysteine-switch residue in soluble human ADAM12 (meltrin α) did not affect its secretion (58), suggesting differences in the contribution of the cysteine-switch peptide to how these two proteins fold.

Finally, we demonstrate that MDC9 is phosphorylated after addition of PMA to cells. Since protein ectodomain shedding can be triggered with phorbol esters (21, 57), this result raises the intriguing possibility that the metalloprotease activity of MDC9 could be regulated by inside-out signaling due to protein kinase C-dependent phosphorylation of the cytoplasmic tail. However, since PMA stimulation can result in the phosphorylation of a variety of different proteins, the physiological significance of this result remains to be determined.

In conclusion, this study demonstrates that MDC9 is made as a precursor which is processed by a pro-protein convertase in the secretory pathway. Processing removes the presumably inhibitory pro-domain of MDC9 and is thus most likely a prerequisite for the prodomain to become active. After pro-domain removal, there may be additional mechanisms to regulate protease activity, such as phosphorylation of the cytoplasmic tail by protein kinase C. Furthermore, this study provides the first evidence that MDC9 is catalytically active, that it has a different substrate specificity than TACE, and that it can be inhibited by hydroxamic acid-based inhibitors. The catalytically active metalloprotease domain of MDC9 will be an important tool for further biochemical and functional analysis of this widely expressed metalloprotease disintegrin protein.

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