Intracellular Maturation of the Mouse Metalloprotease Disintegrin MDC15

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Metalloprotease disintegrins are a family of membrane-anchored glycoproteins that play a role in fertilization, myoblast fusion, neuronal development, and cleavage of the membrane-anchored cytokine tumor necrosis factor-α. Here, we report the cloning and cDNA sequencing of the mouse metalloprotease disintegrin MDC15 and an analysis of its processing in the secretory pathway. A notable difference between mMDC15 and its putative human orthologue (hMDC15, metargin) is the presence of the peptide sequence TDDC instead of the RGDC found in the disintegrin domain of hMDC15. In a Western blot analysis the majority of mMDC15 was found to lack the pro-domain in all mouse tissues examined. Pulse-chase experiments in transiently transfected COS-7 cells suggest that mMDC15 is processed by a pro-protein convertase in a late Golgi compartment, since (i) addition of brefeldin A or monensin blocks pro-domain removal, (ii) all detectable processed mMDC15 is endoglycosidase H-resistant, and (iii) a recombinant soluble form of the trans-Golgi network pro-protein convertase furin can mimic mMDC15 processing in vitro. Cell-surface trypsinization revealed that more than half of mature mMDC15 is intracellular. Immunolocalization provided evidence for a strong perinuclear accumulation in a region resembling the trans-Golgi network and/or endosomal compartments. This study provides the first characterization of the intracellular processing of a metalloprotease disintegrin, and highlights the potential role of pro-protein convertases in removal of the inhibitory pro-domain. These results further suggest possible intracellular functions for mMDC15, such as in protein maturation, in addition to a potential role in cell-surface proteolysis or cell adhesion.

Proteolytic processing in the secretory pathway is often a crucial step in the maturation and activation of secreted and membrane-anchored proteins. Pro-protein convertases such as furin play critical roles in protein maturation and have been implicated in the cleavage and activation of several different types of proteins, including pro-hormones (1–3), viral fusion proteins (4–7), integrin α chains (8), transforming growth factor-β (9), the human insulin proreceptor (10), and specific membrane-anchored matrix-type metalloproteases (11, 12). In the case of the matrix metalloprotease stromelysin 3, intracellular proteolytic processing of thezymogen by furin results in activation of the protease (11). Several members of a different family of metalloproteases, the metalloprotease disintegrins, also contain cleavage sites for pro-protein convertases in their extracellular domain. This suggests that proteolytic processing in the secretory pathway plays a role in the maturation and activation of these proteins (13–15).

Metalloprotease disintegrins (also referred to as MDC proteins, metalloprotease/disintegrin/cysteine-rich, or as ADAMs, a disintegrin and metalloprotease) are a family of membrane-anchored glycoproteins that have roles in fertilization (16–19), muscle fusion (20), release of tumor necrosis factor-α from the plasma membrane (21–23), and in modulating the function of Notch (15, 24–26). About half of the presently known metalloprotease disintegrins carry a catalytic site consensus sequence for metalloproteases (HEXXH) and are therefore predicted to be catalytically active, whereas the remaining family members do not contain a catalytic site in an otherwise related metalloprotease-like domain and therefore must lack metalloprotease activity (15). Proteolytic processing of metalloprotease disintegrins could thus serve to activate the protease by removing the pro-domain or could alternatively regulate the function of other domains, such as the disintegrin domain which is thought to mediate cell-cell interactions by binding to integrins. Processing could also release soluble extracellular protein domains from the plasma membrane.

The proteolytic processing of the heterodimeric sperm metalloprotease disintegrin fertilin has been studied in some detail. The α subunit of fertilin contains a predicted pro-protein convertase cleavage site (RXKR/R/R) between its metalloprotease domain and disintegrin domain and is processed in the secretory pathway next to this cleavage site before the protein emerges on the cell surface (27). Interestingly, a targeted deletion of the spermatid-specific pro-protein convertase PC4 leads to infertility, which could be due, at least in part, to a lack of fertilin α processing (28). Fertilin β processing occurs on the sperm surface in transit through the epididymis and correlates with the acquisition of fertilization competence in sperm (29). Proteolytic processing of another metalloprotease disintegrin, meltrin α, has also been suggested to play a role in regulating the function of this protein in myoblast fusion (20).

In contrast to fertilin, where the pro-protein convertase...
clefting site directs removal of the metalloprotease domain, several other MDC proteins have a pro-protein convertase cleavage site between their pro- and metalloprotease domain (13, 14, 20–23, 30). This suggests that the pro-domain, but not the metalloprotease domain of these proteins, is removed in the secretory pathway. In this study, we have analyzed the intracellular processing and pro-domain removal of mouse MDC15, a putative homologue of human MDC15 (metargidin) (14), both of which have a pro-protein convertase cleavage site between the pro- and metalloprotease domain. Western blot analysis of mMDC15 with antibodies against the pro-domain and the cytoplasmic domain demonstrate that the majority of the detectable protein indeed lacks the pro-domain in all mouse tissues examined. In mDC15 expressing COS-7 cells, removal of the mMDC15 pro-domain can be inhibited with the secretory pathway inhibitors brefeldin A and monensin, and processed mMDC15 is resistant to the deglycosidase endo H.1 These results indicate that the pro-domain is removed after transist through the medial Golgi compartment. In vitro, the pro-domain of mMDC15 can be removed by furin, suggesting that a furin-type pro-protein convertase is important for the maturation of mDC15 in vivo. Finally we report that mMDC15 has a predominantly perinuclear localization in COS-7 cells, which could correspond to the trans-Golgi network and/or endosomal compartments. The predominantly intracellular localization raises the possibility that mMDC15 may also have intracellular functions, as a role in intracellular protein maturation, in addition to the previously proposed roles as cell-surface metalloprotease or adhesion protein. These studies provide the first analysis of the intracellular maturation of a metalloprotease disintegrin protein in somatic cells and indicate that metalloprotease disintegrins may represent physiologically important substrates of pro-protein convertases such as furin.

MATERIALS AND METHODS

Reagents—All reagents were obtained from Sigma unless indicated otherwise. α-[35S]dCTP for cDNA sequencing, [α-32P]dCTP for labeling of cDNA probes, and [35S]-labeled cysteine/methionine for metabolic labeling were purchased from NEN Life Science Products.

cDNA Cloning—A mouse lung cDNA library (13) was probed under high stringency conditions with an [α-32P]dCTP-labeled cDNA fragment corresponding to the first 1481 nucleotides of the 5′ end of human MDC15 (14). Positive phage plaques were identified, and the phage carrying a cDNA insert with the apparently longest 5′ end of human MDC15 was identified by PCR, using an antisense MDC15 primer and the T3 primer that anneals to the 3′ end of the cDNA insert. One clone with a cDNA insert of 2833 base pairs was sequenced on both strands (Sequenase, U. S. Biochemical Corp.) and found to contain an open reading frame encoding for a protein of 815 amino acid residues. cDNA sequence assembly and analysis were performed with AssemblyLino and MacVector software programs (Kodak Scientific Imaging Systems, New Haven, CT) or the Editseq and Megalign modules of DNASTAR (Madison, WI).

Northern Blot Analysis—A mouse multiple tissue Northern blot (CLONTECH, Palo Alto, CA) was probed with [α-32P]dCTP-labeled mMDC15 probe corresponding to the first 1050 nucleotides of the 5′ end under high stringency conditions as described previously (31). A human β-actin probe was used as a control for equal RNA loading.

Antibody Production—cDNA constructs encoding for GST fusion proteins with either the 103 C-terminal amino acid residues within the cytoplasmic tail of mMDC15 or with the 93 amino acids within the pro-domain of mouse and of human MDC15 (Asn25 to Asn128) were generated as follows. The appropriate PCR products were produced using gene-specific primers with added restriction sites and ligated in frame into a pGEX plasmid vector (Amersham Pharmacia Biotech, Uppsala, Sweden). All constructs were sequenced to rule out possible mutations introduced by PCR and then transfected into XL1-Blue Escherichia coli cells (Strategene, La Jolla, CA). Bacteria expressing the fusion protein were collected from 1-liter LB cultures and disrupted by French press homogenization. Cell lysates were spun at 30,000 × g for 30 min in a Sorvall centrifuge using an SS-34 rotor, and the supernatant was incubated with glutathione-Sepharose CL4B beads (Pharmacia LKB, Uppsala, Sweden). Bound fusion proteins were eluted with SDS sample loading buffer sampled on 10% sodium dodecyl sulfate polyacrylamide gel, excised after visualizing in Coomassie Brilliant Blue, and recovered by electrophoresis in an Elutrap apparatus (Schleicher & Schuell) in 50 mM NH4CO3, 0.1% SDS. Eluted fusion proteins were lyopholized, resuspended in double distilled H2O, precipitated at -20°C with 10 volumes of acetone, 1 mM HCl, and finally resuspended in phosphate-buffered saline (PBS, pH 7.4). Female New Zealand White rabbits were immunized with the resuspended fusion proteins according to established protocols (32). The resulting antiserum was processed as follows. The antiserum raised against the mMDC15 GST-cytoplasmic fusion protein was first depleted of anti-GST antibodies by incubating with GST protein coupled to CNBr-activated CL4B beads (Pharmacia LKB, Uppsala, Sweden). After depletion of the antibodies reactive with GST, the antiserum was incubated with mMDC15-GST-cytoplasmic fusion protein coupled to CNBr-activated CL4B beads. Beads with bound antibodies were washed three times in PBS, pH 7.4, and the bound affinity purified antibodies were eluted with 100 mM glycine, pH 3.0, and neutralized immediately with 1× Tris, pH 7.4. Depletion of antibodies reactive with GST alone or with the mMDC15-GST-cytoplasmic fusion protein from affinity purification of anti-MDC15 antibodies was confirmed by Western blot analysis (data not shown). The affinity purified antibodies are referred to as mMDC15-cyto-IG, whereas the antibodies depleted of IgG reactive with both GST and the mMDC15-GST-cytoplasmic fusion protein are referred to as control IgG. Antibodies against the mouse MDC15 pro-domain were raised using an essentially identical approach.

Cell Culture Expression of mMDC15, mMDC15-Fc, and hMDC15-Fc in COS-7 Cells—The cDNA encoding full-length mMDC15 was cloned into a pcDNA3 expression vector (Invitrogen, San Diego, CA) between an EcoRI and XhoI restriction site. Fusion proteins consisting of the hMDC15 extracellular domain or the mMDC15 extracellular domain and the Fc portion of human IgG (hMDC15-Fc and mMDC15-Fc, respectively) were generated by using gene-specific PCR primers with added restriction sites to generate the cDNA encoding Met1–Ser395 (hMDC15) or Met1–Leu695 (mMDC15), so that the resulting fusion proteins included the entire extracellular domain of hMDC15 or mMDC15. These constructs were ligated into a pcDNA3 expression vector in frame with the cDNA encoding the human IgG-Fc domain without a signal sequence (kindly provided by J. Orlinik and Dr. M. Chao). The PCR-generated mMDC15 and hMDC15 cDNAs were sequenced to rule out PCR-induced mutations. All cDNAs were transfected into COS-7 cells using LipofectAMINE (Life Technologies, Inc.), and transient protein expression was monitored by Western blot analysis (see below) using a horseradish peroxidase-coupled goat antibody against the human IgG-Fc domain (Promega Biotech, Madison, WI) or by metabolic labeling followed by protein A-Sepharose precipitation.

Western Blot Analysis of Expressed Protein—Cell lysates for Western blot analysis were harvested from C57BL/6 mice that had been euthanized following the guidelines of the American Veterinary Association. Immediately following euthanasia, mouse tissues were dissected and homogenized with a Polytron homogenizer (Kinematica, Littau, Switzerland) using 10 ml of lysis buffer (1% Nonidet P-40, Tris-buffered saline, and protease inhibitors [29]) per g of tissue. COS-7 cells overexpressing mMDC15 were grown to confluence on 6-well tissue culture plates and lysed in 1 ml of lysis buffer per well. All lysates were spun at 13,000 rpm in a Sorvall tabletop centrifuge for 15 min. MDC15 contains several potential N-linked glycosylation sites and is thus predicted to be a glycoprotein. In order to enrich for glycoproteins for Western blot analysis, supernatants of tissues or cells containing full-length mMDC15 were incubated with 100 μl of ConA-Sepharose lectin beads (Pharmacia LKB, Uppsala, Sweden) per 10 ml of extract for 1 h at 4°C. Samples containing hMDC15-Fc were incubated with protein A beads. Bound proteins were eluted in sample loading buffer for 5 min at 95°C, were subsequently reduced by addition of 10 μl dithiothreitol where indicated, separated by SDS-PAGE, and transferred to nitrocellulose (Schleicher & Schuell). After blocking in 5% dry milk dissolved in PBS for 1 h, the primary antibodies (monoclonal secondary horseradish peroxidase-coupled antibodies against either mouse or human MDC15) were added for 1 h each, and bound antibodies were visualized after four washes in PBS, 0.5% Tween using a chemiluminescence detection kit (Amersham Pharmacia Biotech) and Kodak XAR autoradiography film.

Metabolic Labeling—COS-7 cells were transfected with mMDC15, mMDC15-Fc, hMDC15-Fc, or as a control with the pcDNA3 vector as a way inhibitors brefeldin A and monensin, and processed mMDC15 is resistant to the deglycosidase endo H.1 These results indicate that the pro-domain is removed after transist through the medial Golgi compartment. In vitro, the pro-domain of mMDC15 can be removed by furin, suggesting that a furin-type pro-protein convertase is important for the maturation of mDC15 in vivo. Finally we report that mMDC15 has a predominantly perinuclear localization in COS-7 cells, which could correspond to the trans-Golgi network and/or endosomal compartments. The predominantly intracellular localization raises the possibility that mMDC15 may also have intracellular functions, as a role in intracellular protein maturation, in addition to the previously proposed roles as cell-surface metalloprotease or adhesion protein. These studies provide the first analysis of the intracellular maturation of a metalloprotease disintegrin protein in somatic cells and indicate that metalloprotease disintegrins may represent physiologically important substrates of pro-protein convertases such as furin.
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described above. One day post-transfection, cells were preincubated for 30 min with growth media lacking the amino acids cysteine and methionine followed by incubation with 200 μCi/ml of Express Protein labeling mix (NEN Life Science Products) (70% [35S]methionine, 30% [35S]cysteine) for 20 min. Cells were then either left untreated or incubated in unlabeled Opti-MEM-1 low protein media (Life Technologies, Inc.) for an additional 3, 6, or 9 h prior to lysis. For pulse-chase experiments in the presence of secretory pathway inhibitors, monensin was added to a final concentration of 2 μg/ml, and brefeldin A was added to a final concentration of 5 μg/ml. The serine protease inhibitor Pefabloc SC (4-2-aminoethylbenzenesulfonyl fluoride hydrochloride, Boehringer Mannheim) was added to hMDC15-Fc expressing cells in the chase period only at a final concentration of 400 μM in Opti-MEM. At this concentration Pefabloc does not alter the maturation of the 75-kDa nerve growth factor receptor overexpressed in COS-7 cells (data not shown). mMDC15 was immunoprecipitated from 32P-labeled cell extracts with the mMDC15-cyto IgG, the mMDC15 pro-domain IgG, or control antibodies as indicated, whereas the mMDC15-Fc and hMDC15-Fc fusion proteins were immunoprecipitated with protein A beads. All immunoprecipitated materials were separated by SDS-PAGE. Following electrophoresis, the gels were fixed in 10% acetic acid and 50% methanol for 15 min, incubated for 15 min with Enhance solution (DuPont), dried, and then exposed to Kodak XAR autoradiography film.

RESULTS

Cloning and Sequencing of Mouse MDC15 cDNA—We have previously reported the cDNA sequence of human MDC15 (metargidin), the first metalloprotease disintegrin with an RGD sequence in its predicted integrin binding site (14). In this study, a putative mouse orthologue of human MDC15 was identified by screening a mouse lung cDNA library with a 32P-labeled human MDC15 cDNA probe (as described under “Materials and Methods”). A cDNA clone of 2833 nucleotides was isolated, sequenced in both orientations, and found to encode a protein consisting of 815 amino acid residues. 82% of the amino acid residues deduced from the cloned mouse cDNA are identical to those found in human MDC15 (Fig. 1). Due to this relatively high degree of sequence identity, and because the GenBankTM data base of expressed sequence tags currently contains no mouse cDNA sequences that are more closely related to hMDC15 than the cloned mouse cDNA, we termed the deduced mouse protein mMDC15. mMDC15 has an identical domain structure to hMDC15 and other metalloprotease disintegrin proteins: an N-terminal signal sequence is followed by a non-membrane-permeable biotinylation reagent NHS-LC-biotin (Pierce) in PBS for 45 min at 4 °C. After labeling, the cells were washed in PBS, incubated with 0.1 M glycine to quench the labeling reaction, and lysed directly on the plate in 1 ml of lysis buffer per well (see above), and the resulting cell extracts were spun at 13,000 rpm for 15 min in a Sorvall tabletop centrifuge. To differentiate between proteins that are bound to mMDC15 covalently or non-covalently, certain samples were adjusted to a final SDS concentration of 1.25%, heated to 95 °C for 5 min, and subsequently diluted to a final SDS concentration of 0.15% with cell lysis buffer prior to immunoprecipitation. All other samples were diluted such that the final volume was equal prior to immunoprecipitation. Immunoprecipitated material was run on SDS-PAGE, transferred to nitrocellulose, and processed for Western blot analysis as described above.

Cell Surface Labeling and Immunoprecipitation—COS-7 cells were transiently transfected with mMDC15 in pcDNA3 or as a control with a pcDNA3 vector (see above) in 6-well tissue culture plates as described above. Two days post-transfection, cells were washed in PBS and incubated with the non-membrane-permeable biotinylation reagent NHS-LC-biotin (Pierce) in PBS for 45 min at 4 °C. After labeling, the cells were washed in PBS, incubated with 0.1 M glycine to quench the labeling reaction, and lysed directly on the plate in 1 ml of lysis buffer per well (see above), and the resulting cell extracts were spun at 13,000 rpm for 15 min in a Sorvall tabletop centrifuge. To differentiate between proteins that are bound to mMDC15 covalently or non-covalently, certain samples were adjusted to a final SDS concentration of 1.25%, heated to 95 °C for 5 min, and subsequently diluted to a final SDS concentration of 0.15% with cell lysis buffer prior to immunoprecipitation. All other samples were diluted such that the final volume was equal prior to immunoprecipitation. Immunoprecipitated material was run on SDS-PAGE, transferred to nitrocellulose, and probed with horseradish peroxidase-streptavidin, and bound horseradish peroxidase-streptavidin was visualized as described above under Western blot analysis.

Cell-surface Trypsinization—COS-7 cells transiently transfected with mMDC15 cDNA or pcDNA vector were pulse-chase-labeled with Express Protein Labeling Mix (NEN Life Science Products) for 0, 3, or 6 h, or cell-surface biotinylated (see above). Cells were then washed twice in PBS, cooled on ice for 10 min, and then incubated with ice-cold PBS with or without 500 μg/ml trypsin on ice for 15 min. Detached cells were pelleted in a tabletop microcentrifuge at low speeds. Cells were then washed once in 500 μl of soybean trypsin inhibitor in PBS, pelleted again, lysed in cell lysis buffer with protease inhibitors, and then immunoprecipitated and analyzed as described above.

In Vitro Furin Cleavage—COS-7 cells were metabolically labeled and chased for 4 h in the presence of brefeldin A (5 μg/ml, see above). mMDC15 was immunoprecipitated from these cell extracts, and the immunoprecipitated material bound to protein A beads was washed into a modified furin cleavage buffer (20 mM MES, pH 7.0, 1 mM CaCl2, and 0.5% Nonidet P-40) without protease inhibitors (10). Recombinant furin (kindly provided by R. Fuller) was then added to a final concentration of approximately 40, 400, or 4000 units/ml (1 unit of furin is able to cleave 1 pmol of the standard substrate, tert-butoxycarbonyl-Arg-Val-Arg-Arg-methylcoumarin amide per min at 37 °C). The samples were incubated at 37 °C for 20 min (40 and 400 units/ml) or 40 min (4000 units/ml), and the reaction was stopped with sample loading buffer. Where indicated, the samples were further digested overnight with Proteinase K (see above).

Immunofluorescence—COS-7 cells grown on glass coverslips in 6-well tissue culture plates were transiently transfected with mMDC15 cDNA or pcDNA vector. One day post-transfection, cells were washed in PBS and subsequently fixed in 3% paraformaldehyde in PBS for 30 min. Cells were then permeabilized and blocked in Tris-buffered saline with 0.2% Triton X-100 and 5% normal goat serum for 30 min, and all subsequent washes, the coverslips with fixed cells were mounted on glass microscope slides with Immum Flouore Mounting Medium (ICN, Costa Mesa, CA), which was allowed to settle overnight. The cells were viewed and photographed with a Zeiss Axioshot microscope.

2 R. Fuller, personal communication.
lung glycoproteins, separated by SDS-PAGE under non-reducing conditions, the mMDC15-cyto-IgG recognize a band of 80 kDa (Fig. 3A, lane 2). In an identical sample run under reducing conditions, the mMDC15-cyto-IgG bind to prominent bands of 90, 25, and 23 kDa (Fig. 3A, lane 3), which are not recognized by the preimmune serum (Fig. 3A, lane 1) or by the control IgG (Fig. 3A, lane 4). The shift in apparent molecular weight of mMDC15 separated under non-reducing compared with reducing conditions is a characteristic feature of cysteine-rich MDC proteins. Because the 25- and 23-kDa bands are only visible in the reduced sample, they most likely represent products of a membrane-proximal cleavage that are still disulfide-linked to the larger fragment and therefore co-migrate with the full-length protein at 80 kDa under non-reducing conditions.

Western blots of ConA-enriched glycoproteins isolated from mouse tissue extracts were probed with the mMDC15-cyto-IgG and revealed predominantly a single band of 90 kDa in heart, brain, spleen, and skeletal muscle tissues under reducing conditions.
immunoprecipitation resulted in a 90-kDa band, residual amounts of the 110-kDa bands, and an ~115-kDa band that migrated slightly slower and more diffuse than the 110-kDa band seen in the first time point (Fig. 4A, upper panel, lane 3). In contrast, after a 3-h chase, the mMDC15-pro-domain IgG mainly immunoprecipitated bands between 110 and 115 kDa and very little of the 90-kDa band (Fig. 4A, lower panel, lane 3). Immunoprecipitation after a chase of 6 and 12 h with the mMDC15-cyto-IgG showed that the 110- and 115-kDa bands gradually disappeared and are most likely chased into the 90-kDa band. The 115-kDa band was still visible in an immunoprecipitation with the mMDC15-pro-domain IgG after 6 but not after 12 h.

Taken together, these data suggest that pro-domain removal of mMDC15 occurs over a course of 3–12 h in mMDC15 expressing COS-7 cells. The small amount of the 90-kDa protein that can be immunoprecipitated with the anti mMDC15-pro-domain IgG most likely represents a form of the protein where the pro-domain has been cleaved but remains non-covalently attached to the metalloprotease domain (see below). The 25- and 23-kDa bands that can be detected by Western blot of COS-7 cells expressing mMDC15 are not seen in the immunoprecipitated material, perhaps due to the low methionine/cysteine content of the cytoplasmic tail and transmembrane domain, a slow cleavage rate, or the relatively small fraction of mMDC15 molecules that are cleaved at this site.

When the samples that had been immunoprecipitated at different time points were treated with endoglycosidase H, the 110-kDa band migrated faster at ~100 kDa (Fig. 4B, lanes 1 and 2), whereas the 115- and the 90-kDa bands visible at later time points were not detectably affected (Fig. 4B, lanes 3–8). Since resistance to endo H treatment is acquired by conversion of high mannose glycans into complex carbohydrates in the medial Golgi network, these data suggest that removal of the mMDC15 pro-domain occurs in the secretory pathway after passage through this compartment. Consistent with the behavior of the protein overexpressed in COS-7 cells, the 90-kDa band corresponding to mMDC15 in mouse brain tissue is resistant to endo H but sensitive to PNGase F, which removes most or all N-linked carbohydrate residues from glycoproteins (data not shown). Sialidase (neuraminidase) treatment of samples that were immunoprecipitated after a 3-h chase revealed that the 115-kDa band is sialylated, whereas the processed 90-kDa bands is not (Fig. 4, lanes 11 and 12). The three bands around 110 kDa that are immunoprecipitated immediately after labeling are not affected by sialidase treatment (Fig. 4, lanes 9 and 10). As the processed mMDC15 does not appear to be sialylated, the apparent shift of pro-MDC15 from 110 to 115 kDa is likely due to O-linked carbohydrate addition to the pro-domain, which contains no N-linked glycosylation sites.

To define further the subcellular compartment in which removal of the pro-domain occurs, mMDC15 was immunoprecipitated from mMDC15 expressing COS-7 cells after a 3-h chase in the presence of brefeldin A or monensin, two inhibitors of the secretory pathway. Brefeldin A blocks traffic to the Golgi by retrieving the Golgi apparatus to the endoplasmic reticulum (34–36), whereas the ionophore monensin is expected to interfere with transport to late Golgi compartments (37, 38). Although a significant amount of mMDC15 was processed to the 90-kDa form after 3 h in a pulse-chase experiment (Fig. 4C, lane 2), both brefeldin A (Fig. 4C, lanes 3 and monensin (Fig. 4C, lane 4) completely blocked mMDC15 pro-domain removal. Together with the deglycosylation experiments described above, these data demonstrate that the pro-domain of mMDC15 is removed in a late Golgi compartment or after passage through the late Golgi.

Cleavage of mMDC15 by Furin in Vitro—The pro-protein con-
vertase furin, which resides and functions primarily in the trans-Golgi network (39, 40), is a good candidate protease for removal of the mMDC15 pro-domain because a consensus furin cleavage site exists between the pro- and metalloprotease domains of mMDC15 (see above). To test the ability of furin to cleave the mMDC15 precursor, we generated 35S-labeled pro-mMDC15 by pulse-chase in the presence of brefeldin A and then added recombinant furin to pro-mMDC15 immunoprecipitated from the cell lysates. Addition of furin to a final concentration of 40 or 400 units/ml to the immunoprecipitated pro-mMDC15 for 20 min at 37 °C resulted in a 90-kDa band (Fig. 4D, lanes 1, 3, and 4) or not reduced (lane 2) prior to electrophoresis. The blotted samples were probed with preimmune antiserum which were prepared as described under “Materials and Methods.” A, ConA-enriched mouse lung glycoprotein samples were either reduced with dithiothreitol (DTT) (lanes 1, 3, and 4) or not reduced (lane 2). B, Western blot of reduced glycoproteins that are immunoprecipitated by both pro-domain and cytoplasmic tail antibody yield a similar band pattern (Fig. 5A, lane 6), although the 90-kDa band was somewhat weaker compared with immunoprecipitations with the mMDC15-cyto-antiserum. This result confirmed that the 115-kDa band corresponds to pro-mMDC15, as opposed to a distinct protein that could be associated with processed mMDC15.

To test whether any of the other immunoprecipitated bands of lower molecular weight might be associated with mMDC15, an aliquot of the cell-surface biotinylated sample was heated to 95 °C in the presence of 1.25% SDS and subsequently diluted to a final SDS concentration of 0.15% prior to immunoprecipitation. After treatment with SDS, the mMDC15-cyto-IgG immunoprecipitated proteins of 115, 90, 50, and 23 kDa, whereas the molecules of 27, 25, and 21 kDa were no longer observed (Fig. 5A, lane 4). This result suggests that the 23-kDa band most likely corresponds to the cytoplasmic domain and a small portion of the extracellular domain of mMDC15, whereas the other three lower molecular weight bands (27, 25, and 21 kDa) are associated with mMDC15. Immunoprecipitation of the SDS-treated extract with the mMDC15-pro-domain IgG revealed the 115-kDa protein and all four lower molecular weight proteins (Fig. 5A, lane 8). Taken together, these results indicate that the four lower molecular bands are differently processed forms of the mMDC15 pro-domain which remain associated with the processed protein but can be removed by treatment with SDS. After SDS treatment, immunoprecipitations with both the pro-domain and the cytoplasmic tail antibody yield a 25-kDa band, suggesting that one processed form of the pro-domain may co-migrate with the cytoplasmic tail fragment. A continued association of the pro-domain with the processed mMDC15 after cleavage would also explain why some processed mMDC15 of 90 kDa can be immunoprecipitated with the pro-domain antibody. The identity of the ~50-kDa proteins that are immunoprecipitated by both pro-domain and cytoplasmic tail antibody at the final SDS concentration of 0.15% prior to immunoprecipitation.
Fig. 4. Pulse-chase analysis of mMDC15 maturation in COS-7 cells. COS-7 cells transiently transfected with mMDC15 cDNA or control cDNA were labeled for 20 min with [35S]methionine/cysteine (NEN Life Science Products) and chased with cold media for the indicated periods. Cells were lysed at each time point, and mMDC15 was immunoprecipitated from lysates with mMDC15-cyto-IgG or mMDC15-pro-domain IgG as indicated. All samples were reduced prior to electrophoresis. A, comparison of samples immunoprecipitated with mMDC15-cyto-IgG (top panel) or with mMDC15-pro-domain IgG (lower panel) at different time points during the pulse-chase experiment from COS-7 cells expressing mMDC15 (lanes 2–5) or transfected with pcDNA3 vector as a control (lane 1). B, endoglycosidase H and sialidase (neuraminidase) treatment of mMDC15 immunoprecipitated at different time points during a pulse-chase experiment. Prior to deglycosylation, or mock treatment as a control, the immunoprecipitated material was heated to 95 °C in sample loading buffer in the presence of dithiothreitol for 5 min, then adjusted to pH 5.5 (lanes 1–8) or pH 4.5 (lanes 9–12) with sodium citrate, and then incubated overnight at 37 °C with either 500 units of recombinant endo H (lanes 2, 4, 6, and 8) or 50 units of sialidase (lanes 10 and 12). C, immunoprecipitation of mMDC15 from pulse-chase labeled mMDC15 expressing COS-7 cells after a 0- (lane 1) or 3-h chase (lanes 2–4) in the presence of 5 μg/ml brefeldin A (lane 3) or 2 μg/ml monensin. D, pro-mMDC15 was immunoprecipitated from brefeldin A-treated cells (lanes 1–4) or from untreated cells (lane 5) after a 3-h chase. mMDC15 immunoprecipitated from the brefeldin A-treated cells was incubated in vitro with 40 (lane 2) or 400 units/ml (lane 3) recombinant furin for 20 min at 37 °C or with 4000 units/ml for 40 min (lane 4) to compare the resulting products with the mMDC15 processed in vivo. To determine whether the slight difference in apparent molecular weight between the sample treated in vitro with furin and the sample seen after 3 h in vivo was due to differences in carbohydrate modifications, identical samples to those in lane 2 (brefeldin A, 40 units/ml furin) and lane 4 (mMDC15 after a 3-h chase) were adjusted to pH 7.5 with sodium phosphate and incubated at 37 °C overnight in the presence (lanes 7 and 8) or absence (lane 6) of PNGase F/ml.
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FIG. 5. Cell-surface biotinylation and trypsinization of mMDC15-overexpressing COS-7 cells. A, analysis of cell-surface labeled forms of mMDC15. Lane 1 shows a Western blot of mMDC15 expressing COS-7 cells identical to those used for cell-surface labeling, probed with mMDC15-cyto-IgG. Lanes 3 and 4, extracts from cell-surface biotinylated COS-7 cells expressing mMDC15 were subjected to immunoprecipitation with mMDC15-cyto-IgG or the corresponding preimmune IgG (lanes 2 and 5), or with mMDC15-pro-domain-IgG (lanes 6 and 8), or the corresponding preimmune IgG (lane 7). In an attempt to distinguish between non-covalently attached proteins and different forms of mMDC15 or its pro-domain, an aliquot of the cell-surface-labeled material was treated with 1.25% SDS at 95 °C for 5 min and subsequently diluted to a final SDS concentration of 0.15% prior to immunoprecipitation (lanes 4, 5, and 8). Samples were detected by probing the immunoprecipitated material after transfer to nitrocellulose with streptavidin-coupled horseradish peroxidase. B, probing the accessibility of cell-surface biotinylated mMDC15 to trypsin. Cell-surface biotinylated cells were placed on ice and subsequently incubated with 500 μg/ml ice-cold trypsin (lane 2) or mock-treated for 30 min on ice (lane 1). The PBS used to wash the cells after trypsinization and the cell lysis buffer contained 500 μg/ml soybean trypsin inhibitor. C, probing the accessibility of metabolically labeled mMDC15 to trypsin. After pulse-chase labeling of mMDC15 expressing COS-7 cells with [35S]Met/Cys for the indicated duration, immunoprecipitations were performed from extracts of cells that had been treated as described above on ice in the presence (lanes 2, 4, and 6) or absence (lanes 1, 3, and 5) of 500 μg/ml trypsin.

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mic domain antibodies in untreated and in SDS-treated samples remains to be determined.

Cell-surface Trypsinization and Immunolocalization—A comparison of the Western blot in Fig. 5A, lane 1, and the surface-biotinylated material that was immunoprecipitated from identical cells (Fig. 5A, lanes 3, 4, 6, and 8) showed a clear discrepancy between the predominantly processed mMDC15 seen on the Western blot and the comparable amounts of unprocessed and processed mMDC15 seen in immunoprecipitations. One explanation for this discrepancy could be that the majority of processed mMDC15 may not reside on the cell surface. We therefore decided to probe the cell surface accessibility of mMDC15 to trypsin at different time points during a pulse-chase experiment. As a positive control, trypsin was shown to completely remove all immunoprecipitable material that can be biotinylated on the cell surface (Fig. 5B, lanes 1 and 2). In a [35S]-labeled pulse-chase experiment, trypsin apparently had no access to the precursor of mMDC15 seen immediately after labeling, as would be expected for this intracellular form of the protein (Fig. 5C, lanes 1 and 2). Of the different forms of mMDC15 that are observed after a 3- and 6-h chase, the glycosylated and sialylated 115-kDa protein appears to be largely removed by trypsin treatment (Fig. 5C, lane 4, arrow), whereas the processed 90-kDa band was somewhat reduced in intensity but was not removed. The residual amounts of the 110-kDa protein in lanes 3 and 4 do not appear to be accessible to trypsin. This result suggests that the glycosylated precursor of mMDC15 becomes accessible to trypsin on the cell surface, whereas a significant amount of the processed form is protected from trypsinization.

To evaluate the subcellular localization of mMDC15 expressed in COS-7 cells, immunofluorescent staining analysis was performed on fixed and permeabilized cells with mMDC15-cyto-IgG. Fig. 6 shows three examples of the observed staining pattern in transiently transfected COS-7 cells expressing different levels of mMDC15, and one example of the staining pattern of cells transfected with the vector as control. In all mMDC15-transfected cells, the strongest staining was apparent in the perinuclear region that could correspond to the trans-Golgi network or endosomal/lysosomal compartments. In addition, at higher expression levels, an increase in staining in the periphery of the cell was also observed, consistent with an increase in staining on or near the cell surface. Only weak background staining was visible in the cells transfected with vector control. When similar experiments were performed on NIH-3T3 cells, where endogenously expressed mMDC15 can be detected by Western blot of ConA-enriched glycoprotein samples, no staining above background levels could be seen. Presumably the expression level of mMDC15 in these cells is too low for detection by immunofluorescent labeling (data not shown).

Processing of a Soluble Mouse or Human MDC15-Fc Fusion Protein—In order to examine whether the processing of the MDC15 precursor occurs in the secretory pathway or alterna-
The main purpose of the present study was to use mouse MDC15 as a model to follow the intracellular processing and maturation of a metalloprotease disintegrin protein. Many MDC proteins contain a furin consensus cleavage site between their pro-domain and metalloprotease domain, suggesting that removal of the pro-domain is mediated in the secretory pathway. Metalloprotease disintegrins with a catalytic site consensus sequence (HEXXXH) also contain an additional odd-numbered cysteine residue in their pro-domain which is not found in metallo-
protease disintegrins lacking the catalytic site consensus sequence. This presumably unpaired cysteine residue in the pro-domain is thought to regulate the metalloprotease activity via a cysteine-switch mechanism (49). Analogous to the snake venom metalloprotease adamalysin II (50), and to membrane-anchored matrix type metalloproteases (49), removal of the pro-domain is presumably a prerequisite for the protease to become active. This does not preclude other additional means of regulating protease activity once the pro-domain has been removed.

In all mouse tissues examined, the predominant form of mMDC15 lacks the pro-domain. The results of the present study further suggest that cleavage of mMDC15 occurs in the secretory pathway in or after passage through a late Golgi compartment. In COS-7 cells expressing mMDC15, processing is blocked in the presence of brefeldin A, which blocks transport from the endoplasmic reticulum to the Golgi (34–36), and monensin, which is thought to inhibit transport to the late Golgi (37, 38). Furthermore, whereas most of the precursor of mMDC15 is sensitive to endo H in a pulse/chase experiment, a small amount of the precursor is both sialylated and resistant to endo H. Acquisition of endo H resistance and sialylation are thought to occur in the medial Golgi and trans-Golgi network, respectively. Finally, all of the detectable processed mMDC15 is endo H-resistant.

Processing in or after a late Golgi compartment would be consistent with the action of a pro-protein convertase such as furin. Increasing concentrations of furin resulted first in the appearance of a band of ~85 kDa and at the highest concentration of an additional faster migrating band of 75 kDa. After removal of N-linked carbohydrates with the deglycosidase PNGase F, the processed form of mMDC15 generated in vitro by furin co-migrated with the processed form of mMDC15 immunoprecipitated from untreated cells. This result indicates that furin cleaves mMDC15 at or very near the cleavage site used in vivo in the secretory pathway.

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| Lane | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
|------|---|---|---|---|---|---|---|---|---|----|----|
| Chase (hrs) | 3 | 3 | 1 | 3 | 6 | 1 | 3 | 6 | 1 | 3 | 6 |

Culture supernatant

FIG. 7. Pulse-chase analysis of COS-7 cells expressing a soluble mouse or human MDC15-extracellular domain fused to an IgG Fc domain (mMDC15-Fc, hMDC15-Fc). Lane 1 shows [35S]Met/Cys metabolically labeled material isolated from a lysate of COS-7 cells expressing mMDC15-Fc immediately after labeling with protein A-Sepharose beads. To allow a direct comparison between the intracellular and the secreted form of mMDC15-Fc, lane 2 shows protein A-bound 35S-labeled material isolated from the culture supernatant of hMDC15-Fc expressing COS-7 cells after a 3-h chase. Lanes 3–11 contain protein A-bound material from lysates (lower panel) or cell supernatants (upper panel) of COS-7 cells expressing human MDC15-Fc (lanes 3–8) or mouse MDC15-Fc (lanes 9–11) isolated at different time points during pulse-chase labeling. Lanes 6–8 show the effects of adding 0.4 μM of the general serine protease inhibitor Pefabloc (PFB) to COS-7 cells expressing mMDC15-Fc.

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The pulse-chase analysis described above, and the finding that no pro-mMDC15 can be detected on a Western blot, suggests that processed mMDC15 should be the predominant form present on the cell surface. However, mMDC15 that was immunoprecipitated from cell-surface biotinylated cells consisted of approximately equal amounts of pro-mMDC15 and processed mMDC15. It should be noted that cell-surface biotinylation is a very sensitive technique that can allow detection of relatively small amounts of proteins on the cell surface. Since pro-MDC15 is not seen on a Western blot of whole cell extracts, but pro-MDC15 is the predominant form of mMDC15 that can be labeled on the cell surface, this suggests that the amount of mMDC15 which is accessible to cell-surface biotinylation may only represent a small percentage of the total mMDC15 in the cell. In that case, most of the processed mMDC15 would not reside on the surface of COS-7 cells expressing mMDC15.

This idea was tested by probing the accessibility of mMDC15 on the cell surface with trypsin. Although the cell-surface biotinylated forms of mMDC15 were almost completely digested after
addition of trypsin on ice, the majority of the metabolically labeled processed mMDC15 was resistant to trypsin treatment. Furthermore, immunofluorescent labeling of COS-7 cells expressing mMDC15 revealed a perinuclear staining pattern which in most cells resembled the staining pattern of proteins found in the endosome or trans-Golgi network. This subcellular localization pattern corroborates that the majority of expressed mMDC15 is intracellular. In a small number of cells expressing higher levels of mMDC15, staining was also visible in the periphery of the cell, consistent with a cell-surface staining pattern. One interpretation of this result is that higher expression levels could saturate a putative mechanism by which mMDC15 is retained in a perinuclear compartment, thus resulting in higher levels of escape to the cell surface in these cells.

Since pro-mMDC15 can be labeled on the cell surface, and because the sialylated precursor form appeared largely accessible to trypsin on the cell surface, it seemed possible that mMDC15 processing may require endocytosis from the cell surface. To distinguish further between processing in the secretory pathway and in the endocytic pathway, secreted forms of mouse and human MDC15 consisting of all extracellular domains, but lacking the transmembrane domain, were expressed in COS-7 cells. In both cases, approximately equal amounts of the precursor and the processed form of the soluble extracellular domains were secreted into the supernatant. This suggests that processing of MDC15 can occur in the secretory pathway and does not necessarily require endocytosis. In the presence of the general serine protease inhibitor Pefabloc, only the precursor of hMDC15-Fc was secreted, further supporting the role of serine proteases in processing and most likely activating MDC15. Further studies will be necessary to determine whether a “first pass” cleavage of membrane-anchored mMDC15 in the trans-Golgi network favors retention in an intracellular compartment, in which case the precursor form on the cell surface may have simply escaped processing due to overexpression. Alternatively, transport of pro- or processed mMDC15 to the cell surface may be an integral step in the maturation of this protein.

The results presented here provide the following picture of the intracellular maturation of mMDC15 (see also the model in Fig. 8). After removal of the signal sequence, core glycosylation, and proper folding, mMDC15 exits the endoplasmic reticulum and is transported through the medial Golgi apparatus, where endo H resistance of N-linked carbohydrate residues is acquired. The pro-domain of mMDC15 is subsequently removed by a furin-type pro-protein convertase, presumably in the trans-Golgi network. At least some pro-mMDC15 is sialylated in the pro-domain prior to removal of the pro-domain. In both cases, approximately equal amounts of the precursor and the processed form of the soluble extracellular domains were secreted into the supernatant. This suggests that processing of MDC15 can occur in the secretory pathway and does not necessarily require endocytosis. In the presence of the general serine protease inhibitor Pefabloc, only the precursor of hMDC15-Fc was secreted, further supporting the role of serine proteases in processing and most likely activating MDC15. Further studies will be necessary to determine whether a “first pass” cleavage of membrane-anchored mMDC15 in the trans-Golgi network favors retention in an intracellular compartment, in which case the precursor form on the cell surface may have simply escaped processing due to overexpression. Alternatively, transport of pro- or processed mMDC15 to the cell surface may be an integral step in the maturation of this protein.

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surface may be an integral part of its maturation. An alternative possibility is that some pro-MDC15 leaks to the cell surface due to overexpression in COS-7 cells. In both cases the precursor that is found on the cell surface could be processed at a later stage after endocytosis. Further studies using cells that endogenously express MDC15 will be necessary to resolve this issue. However, expression of soluble mouse and human MDC15 clearly shows that at least some pro-domain removal can occur in a first pass through the secretory pathway without endocytosis.

In addition to the proteolytic removal of the pro-domain by a pro-protein convertase, MDC15 undergoes additional proteolytic cleavage steps. One cleavage occurs relatively close to the plasma membrane but can only be detected after reduction, indicating that it results in two disulfide-linked fragments of MDC15. Furthermore, the pro-domain is apparently cleaved in several positions that are N-terminal of the furin cleavage site. These additional pro-domain cleavages could conceivably play a role in releasing the pro-domain after processing by a pro-protein convertase.

While the exact intracellular localization of MDC15 remains to be determined, the observation that the majority of MDC15 expressed in COS-7 cells does not reside on the cell surface raises the possibility that MDC15 may function as a metalloprotease in the secretory and/or endocytic pathway. A localization both intracellularly and on the cell surface is reminiscent of the pro-protein convertase furin, which cycles between the cell surface and the endosome/trans-Golgi network but is mainly localized in the trans-Golgi network (40). It should be noted that a predominant localization of MDC15 in the trans-Golgi network/endosome does not rule out potential proteolytic or adhesive functions on the cell surface, which may require only a relatively small percentage of the total MDC15 molecules in a cell. Other possibilities include that transport to the cell surface could be regulated in certain cells or that MDC15 is only retained on the cell surface under certain conditions, such as by engaging a cell-surface receptor. Nevertheless, in light of the mainly intracellular localization of MDC15 expressed in COS-7 cells, it will be interesting to determine whether MDC15 might be involved in the intracellular cleavage of substrate proteins and, if so, whether this occurs in the constitutive and/or in the regulated secretory pathway.

In summary, this study provide the first analysis of the intracellular maturation of a metalloprotease disintegrin and suggests that MDC15 is processed in the secretory pathway in or close to the trans-Golgi network by a pro-protein convertase such as furin. Pro-protein convertases have been linked to the processing of several different classes of proteins, including prohormones, viral fusion proteins, and certain matrix metalloproteases. Based on the results presented here, it seems likely that pro-protein convertases also play an important role in the maturation and activation of MDC15 and other metalloprotease disintegrins.

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