Cell-surface Processing of Pro-ADAMTS9 by Furin*

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Processing of polypeptide precursors by proprotein convertases (PCs) such as furin typically occurs within the trans-Golgi network. Here, we show in a variety of cell types that the propeptide of ADAMTS9 is not excised intracellularly. Pulse-chase analysis in HEK293F cells indicated that the intact zymogen was secreted to the cell surface and was subsequently processed there before release into the medium. The processing occurred via a furin-dependent mechanism as shown using PC inhibitors, lack of processing in furin-deficient cells, and rescue by furin in these cells. Moreover, down-regulation of furin by small interference RNA reduced ADAMTS9 processing in HEK293F cells. PC5A could also process pro-ADAMTS9, but similarly to furin, processed forms were absent intracellularly. Cell-surface, furin-dependent processing of pro-ADAMTS9 creates a precedent for extracellular maturation of endogenously produced secreted proproteins. It also indicates the existence of a variety of mechanisms for processing of ADAMTS proteases.

Zinc metalloendopeptidases, like most proteases, are synthesized aszymogens, and the N-terminal propeptide is usually excised. Propeptide excision usually leads to enzymatic activation, an important regulatory event, and it can occur intracellularly, at the cell surface, or extracellularly through a variety of proteolytic mechanisms. In one such mechanism, the propeptide is proteolytically excised by serine proteases of the mammalian subtilisin-like proprotein convertase (PC) family (1–5). This mechanism is used by some MMPs (6, 7), many ADAMs (8–10), and all ADAMTS proteases studied thus far (11, 12). In these proteases, removal of the propeptide appears to be mediated by the most widely distributed PC, furin, and occurs within the constitutive secretory pathway, specifically in the trans-Golgi network (TGN) (7–9, 13, 14).

Furin is the best studied of the seven PCs implicated in proprotein processing within the constitutive secretory pathway, and it is present in virtually all cells (1, 15). It is a type I transmembrane protein that is itself synthesized as a zymogen that undergoes autocatalytic intramolecular activation (16). Furin cleaves on the carboxyl side of a consensus recognition site that is rich in basic residues (e.g. Arg-Xaa-Arg/Lys-Arg) (2, 4, 5, 17). Most furin resides in the TGN, but some is present at the plasma membrane and shuttles between the cell surface and the TGN (18–20). Furin is also shed from cells and may be functional in the extracellular space (21). Microbial toxins such as the anthrax protective antigen and diphtheria toxin are processed by cell-surface furin, with important implications for their toxicity (20, 22). However, the physiological role of cell-surface or secreted furin in processing endogenous cellular products has remained elusive.

The ADAMTS proteases are a family of 19 secreted enzymes, of which some have critical physiological functions and have been implicated in inherited human disorders, namely Ehlers-Danlos syndrome type VIIC (ADAMTS2), Weill-Marchesani syndrome (ADAMTS10), and inherited thrombocytopenic purpura (ADAMTS13) (23–26). Overexpression of ADAMTS5 and ADAMTS4 is implicated in proteolytic loss of aggrecan, a major cartilage component, in arthritis (27). ADAMTS proproteases (except ADAMTS13, the von Willebrand factor–proprotease, whose propeptide contains 41 amino acid residues) are synthesized with propeptides of ~220–240 amino acids, which are larger than the MMP and ADAM propeptides. Almost all ADAMTS proproteases have consensus sites for the attachment of N-linked oligosaccharide (Asn-Xaa-Thr/Ser, where Xaa is any amino acid except Pro). Consensus recognition sequences for PCs are present at the junction of the propeptide and catalytic domain, and ADAMTS proteases may have additional, more N-terminal PC-processing sites within their propeptides. Previous studies of ADAMTS1, ADAMTS4, and ADAMTS7 suggested that, like ADAMs and the furin-processed MMPs, ADAMTS proteases were processed in the TGN by PCs, although some cell-surface processing of ADAMTS7 was also observed (11–14, 28).

ADAMTS9 is one of only two ADAMTS proteases that is highly conserved during evolution (29–31). Its mRNA is widely expressed (29, 30), especially during embryonic development (32). ADAMTS9 is upregulated by inflammatory cytokines in chondrocytes (33). It may have a role in atherosclerosis and arthritis, because it can proteolytically process the proteoglycans versican and aggrecan (30), which are important constituents of the vessel wall, and cartilage, respectively. This potential biological significance of ADAMTS9 prompted an in-depth analysis of the processing of its propeptide. In the initial characterization of ADAMTS9, we demonstrated that it could be processed at more than one site (30). In continuing these studies, we have made the unexpected observation that ADAMTS9 processing is exclusively extracellular and occurs at the cell surface in cells that express high levels of furin. These results establish a precedent for the cell-surface activation of precursor endogenous proteins by furin and are possibly of broad biological relevance.

EXPERIMENTAL PROCEDURES

Expression Plasmids and Site-directed Mutagenesis—Plasmids for expression of full-length ADAMTS9, or a truncated form containing the
signal peptide, propeptide, and catalytic domain (Pro-Cat) with C-terminal Myc and His tags were described previously (30). Due to low expression levels of full-length ADAMTS9, we excised its open reading frame with the Myc-His tags as a NotI/Pmel fragment and then recloned this into pCEP4 (Invitrogen) digested with KpnI and blunt-ended followed by NotI digestion. To insert a FLAG tag between Thr276 and Arg279 in the propeptide, site-directed mutagenesis (QuickChange kit, Stratagene) was performed using the forward primer 5'-AATAAGACGGAACACACAGACTAACAGAGCTAGGACAGAAGAGAGAAAGAGGACCCAC-3' (with FLAG encoding site underlined) and the reverse primer 5'-GTGGTCTCCTTTTTGTCTCTGGTCTCATCGTCTTTGTAGTCTGTGTCCGTCTTTAAT-3' (FLAG encoding site underlined). Plasmids for full-length ADAMTS1 or the propeptide and catalytic domain of ADAMTS7 (ADAMTS7-Pro-Cat) were previously described (11, 28).

Antibodies and Immunoblotting—The peptides RP1 and RP4 representing different regions of the ADAMTS9 propeptide were synthesized using Fmoc-(N-(9-fluorenyl)methoxycarbonyl) chemistry and conjugated to KLH. New Zealand White male rabbits (7–8 pounds) were immunized with the conjugates at biweekly intervals for 8 weeks. After an initial injection in Freund’s complete adjuvant, subsequent injections were given in incomplete adjuvant. Antibody titer was measured by enzyme-linked immunosorbent assay using free peptides. Affinity-purified antibodies were prepared using the respective immobilized antigens. Anti-penta-His monoclonal antibody, anti-Myc monoclonal antibody 9E10, and anti-FLAG M2 monoclonal antibody were obtained from commercial sources (Invitrogen and Sigma-Aldrich). Anti-furin monoclonal antibody was purchased from Alexis Biochemicals (San Diego, CA). Immunoblotting was done using denaturing SDS-PAGE and electroblotting to polyvinylidene fluoride membrane followed by detection of the bound antibody using enhanced chemiluminescence (Amersham Biosciences).

Cell Culture, Transfection, and Cell Treatments—HEK293F cells stably transfected with ADAMTS9 or Pro-Cat, COS-1 cells, LoVo cells (ATCC no. CCL-299), CHO-K1 cells, CHO RPE40 (35), and rat chondrosarcoma RCS-LTC cells (36) were maintained as described previously (30) or as per vendors’ instructions. Transient transfections were done using FuGENE6 (Roche Diagnostics, Indianapolis, IN) as per the manufacturer’s recommendations. For inhibition of PCs, cells were treated with the PCR inhibitor mixture, Roche Diagnostics) for 1 h at 4 °C and centrifuged. The soluble portion of the lysate was transferred to a fresh tube and incubated overnight with anti-FLAG-agarose (Sigma-Aldrich) at 4 °C with rotation. Anti-FLAG-agarose was pelleted by centrifugation at 1000 rpm in a microcentrifuge and washed six times in lysis buffer. The supernatant was discarded, and the bound protein was eluted with 0.1 M glycine/HCl (pH 3.5) from the resin. The eluted samples were analyzed by Western blotting with anti-furin monoclonal antibody (Alexis Biochemicals) or RP4 antibody.

Metabolic Labeling and Pulse-Chase Analysis—HEK293F cells stably expressing Pro-Cat were grown to confluence and incubated in Dulbecco’s modified Eagle’s medium without cysteine and methionine (Sigma-Aldrich). After 24 h, the medium was replaced with cysteine-methionine-free medium containing radioactive cysteine/methionine (50 μCi/well in 6-well plates, Pulse medium) for 15 min using EXPRESS35S35S (PerkinElmer Life Sciences). For pulse-chase analysis, the pulse medium was removed, and the cells were washed three times on ice with phosphate-buffered saline containing 0.5 mM MgCl2 and 0.5 mM CaCl2, followed by incubation for 0, 15, 30, 60, or 120 min in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (Chase medium) at 37 °C. At the end of the chase period, cells were placed on ice, the medium was collected, and the cells were washed four times with phosphate-buffered saline and extracted with lysis buffer. In parallel, samples were processed for removal of cell-surface proteins in these assays by treatment with trypsin/EDTA for 20 min on ice, and trypsin was subsequently inactivated with 10% fetal bovine serum supplemented medium.

RNA Interference—HEK293F cells stably expressing Pro-Cat were transfected with 20 pmol of Furin ShortCut siRNA Mix (New England Biolabs) using Lipofectamine 2000 (Invitrogen). After 48 h of incubation without antibiotics, the medium was changed to 293 SFM-II medium (Invitrogen), and cells were incubated for a further 24 h. The conditioned medium and cell lysate were analyzed by Western blotting with anti-Myc and anti-RP4. Cell-surface biotinylation was done as above.

The medium was taken for analysis at successive time points. In parallel experiments purified Pro-Cat was incubated with cell-free conditioned medium from HEK293F cells to investigate whether the processing was cell-mediated or not. The samples were analyzed by Western blotting with anti-Myc.

Enzymatic Deglycosylation—Removal of N-linked carbohydrate with peptide N-glycosidase F (PNGase F, New England Biolabs, Beverly, MA) was performed as previously described (30).

Co-transfection of Pro-Cat and Proprotein Convertases—Furin-deficient CHO RPE.40 cells (35) and LoVo cells were transfected with Pro-Cat alone or together with plasmids encoding the proprotein convertases furin, PACE4, and PC5A (kindly provided by Dr. Nabil Seidah). 48 h after transfection, cells were incubated in 293 SFM-II medium for 24 h, and Western blotting of the cell lysate and medium was performed with anti-Myc.

Cell-surface Cross-linking and Immunoprecipitation—48 h after transfection with the Pro-FLAG-Cat plasmid, HEK293F cells were washed four times with phosphate-buffered saline and treated with the thiocleavable, membrane-nonpermeable cross-linker 3,3′-dithiobis(sulfo-succinimidylpropionate) (Pierce Endogen, Rockford, IL) to cross-link molecules at the cell surface. The cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 150 mM NaCl, 1% Nonidet P-40, protease inhibitor mixture, Roche Diagnostics) for 1 h at 4 °C and centrifuged. The soluble portion of the lysate was transferred to a fresh tube and incubated overnight with anti-FLAG-agarose (Sigma-Aldrich) at 4 °C with rotation. Anti-FLAG-agarose was pelleted by centrifugation at 1000 rpm in a microcentrifuge and washed six times in lysis buffer. The supernatant was discarded, and the bound protein was eluted with 0.1 M glycine/HCl (pH 3.5) from the resin. The eluted samples were analyzed by Western blotting with anti-furin monoclonal antibody (Alexis Biochemicals) or RP4 antibody.

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RESULTS

The Proteolytically Processed ADAMTS9 Propeptide Is Present in the Conditioned Medium but Not in the Cell Lysate—The ADAMTS9 propeptide (Fig. 1, A and B) has five consensus furin-recognition sequences (Arg–Xaa–Xaa–Arg). Arg–Lys–Asp–Arg35 and Arg–Thr–Arg–Arg74 are near the signal peptidase processing site, whereas the remaining three sites (Arg–Glu–Lys–Arg286, Arg–Thr–His–Arg283, and Arg–Thr–Lys–Arg287) overlap and are clustered near the junction of the propeptide and catalytic domain. We previously showed that mutation of Arg74 and Arg287 abrogated processing at these sites, whereas mutation of Arg280, which compromised two putative sites, was without effect (30). The propeptide also contains three sequence motifs for attachment of N-linked oligosaccharides (Fig. 1B), whereas the catalytic domain has none.

Two nonoverlapping synthetic peptides from the propeptide (RP1 and RP4; sequences are not provided for proprietary reasons of Triple Point Biologics) within the propeptide were used for the production of polyclonal antiserum. In HEK293F cells stably expressing full-length ADAMTS9 (Fig. 2A), anti-RP4 recognized the expected ~180-kDazymogen in the cell lysate. Smaller polypeptides (37 kDa and a ~20–22-kDa doublet) were recognized in the conditioned medium. These bands were not seen in untransfected HEK293F cells (Fig. 2A). When the medium was deglycosylated using PNGase F, the 37-kDa band and the ~22-kDa doublet increased in mobility and migrated at 28 and 15 kDa, respectively, demonstrating the presence of N-linked carbohydrate (Fig. 2B), characteristic of propeptide fragments. Notably, the ~22-kDa doublet converted to a ~15-kDa doublet, indicating two protein species rather than glycoforms of the same fragment. When these experiments were conducted using an ADAMTS9 active site mutant (ADAMTS9 Glu381→Ala, a widely used metalloprotease inactivating mutation (12, 38)), no differences were seen (Fig. 2B); thus, the anti-RP4-reactive fragments were not autoproteolytic in origin. In Western blotting with anti-RP1 the medium showed a weakly immunoreactive 37-kDa band but not the ~22-kDa doublet (Fig. 2C), whereas PNGase F treatment clearly resolved the 28-kDa band seen with RP4 and a 15-kDa doublet also seen with RP4. The comparable results with RP4 and RP1 as well as the presence of glycosylation identified these fragments in the medium as originating from the propeptide. The RP1 peptide is adjacent to an N-linkage site, and the carbohydrate may mask reactivity of the ~22-kDa fragments without prior PNGase F treatment. Because anti-RP4 provided more robust immunoreactivity, it was used in subsequent experiments.

Because the large size and extensive glycosylation of full-length ADAMTS9 (30) precludes accurate resolution in gels, the above studies were repeated using an expression plasmid encoding the ADAMTS9 signal peptide, propeptide, and catalytic domain (Pro-Cat) with a Myc–His tag at the C terminus (Fig. 2D). The results were identical, i.e. anti-RP4 detected the intact Pro-Catzymogen (55 kDa) only in the cell lysate, and 37- and 20–22-kDa fragments were found only in the conditioned medium (Fig. 2D). Similarly, anti-Myc detected only the unprocessed 55-kDa Pro-Catzymogen in the cell lysate, and only the processed catalytic domain (29 kDa) in conditioned medium (Fig. 2D). Incubation of conditioned medium with PNGase F enhanced the mobility of propeptide fragments to the same extent as with full-length ADAMTS9 (Fig. 2E). That these fragments originated from the propeptide was further verified by insertion of a FLAG epitope tag between Thr276 and Arg277, i.e. upstream of the furin processing site at Arg381. Western blotting with anti-FLAG provided similar results to that with anti-RP4, with differences (Fig. 2F) reflecting distinct locations of the FLAG and RP4 epitopes within the propeptide. These results demonstrate that the propeptide is part of the intactzymogen in HEK293F cells, but that it is present as a distinct, proteolytically derived entity in their conditioned medium.

We conclude that the 37-kDa band seen with all propeptide antibodies corresponds to the complete propeptide. When deglycosylated, its size (28 kDa), is compatible with processing at the most N- and C-terminal PC processing sites, i.e. Leu34–Arg287 (28 kDa). The site at which the processing of the prodomain to the 20- and 22-kDa RP4-reactive fragments occurs is not established, but it may represent cleavage at the Arg–Arg285–Ser. This site is not a typical furin processing site, but it has certain attributes that favor PC processing, such as His at the P6 position and Ser at the P1’ position.

We asked whether the conditioned medium of cells endogenously expressing ADAMTS9 or from other transfected cell types had similar processing profiles. RCS-LTC chondrosarcoma cells endogenously expressing ADAMTS9 demonstrated an anti-RP4-reactive 22-kDa band in their conditioned medium, but not in the cell lysate, where only a 180-kDa band corresponding to the intactzymogen was detected (Fig. 3A). When COS-1 and CHO-K1 cell lines were transiently transfected with Pro-Cat, anti-RP4 detected only the unprocessedzymogen in cell
lysate, but both the secreted, unprocessed zymogen and proteolytically processed pro-domain in the conditioned medium (Fig. 3, B and C, left panels). Western blotting with anti-Myc detected the intact zymogen in cell lysate and the 29-kDa processed catalytic domain in the conditioned medium (Fig. 3, B and C, right panels). Thus, in all the cell types examined, the ADAMTS9 propeptide was not processed within the cell.

Varying amounts of unprocessed Pro-Cat were detected in conditioned medium of transfected COS-1 and CHO-K1 cells (Fig. 3, B and C, left panels) but little in HEK293F cells (Fig. 2). Together, the results suggest that the ADAMTS9 propeptide is intrinsically resistant to removal within the secretory pathway, because it is not processed intracellularly regardless of the cell type.

**ADAMTS9 Processing Differs from That of Other ADAMTS Proteases**—Previous studies demonstrated intracellular processing of ADAMTS1, ADAMTS4, and ADAMTS7 (11, 13, 14, 28). We directly compared ADAMTS9 processing with ADAMTS1 and ADAMTS7 processing. ADAMTS1 and ADAMTS9 Pro-Cat were transfected into QBI-HEK 293A cells, followed by metabolic labeling with radioactive amino acids and immunoprecipitation with anti-ADAMTS1 and anti-penta-His. Fluorography of the cell- and medium-derived proteins demonstrated ADAMTS1 zymogen (110 kDa) and the processed enzyme (87 kDa) in the cell but only the unprocessed 55-kDa ADAMTS9 Pro-Cat in cells (Fig. 4A). In a second set of experiments, ADAMTS9-Pro-Cat was compared with ADAMTS7-Pro-Cat. Cell lysate from ADAMTS7-Pro-Cat-transfected HEK293F cells showed both the intact Pro-Cat and the predicted furin-processed catalytic domain, whereas ADAMTS9-Pro-Cat transfected cells showed only the unprocessed zymogen (Fig. 4B). These results demonstrate that differences in biosynthetic mechanisms exist in the ADAMTS family.

**ADAMTS9 Is Processed at the Cell Surface**—The presence of only the intact pro-ADAMTS9 in cells and of the processed propeptide exclusively in the conditioned medium, suggested that propeptide processing occurred extracellularly, i.e. at the cell surface or in the medium. We utilized a variety of approaches to examine this possibility further. First, cell-surface proteins were labeled with biotin. Biotinylated proteins were purified from detergent-extracted cells using streptavidin-agarose capture and analyzed by immunoblotting with anti-Myc. As a control, cells were treated with trypsin to remove cell-surface proteins and pro-
teoglycans. Intact Pro-Cat (55 kDa) was present at the surface of HEK293F cells (Fig. 5A).

Because biotinylation is very sensitive and may reveal only a small fraction of the total secreted ADAMTS9, we undertook pulse-chase analysis of all newly synthesized protein and followed the labeled protein into the medium. The proportion of cell-surface Pro-Cat at any point during the chase was determined by digesting away cell-surface proteins with trypsin. Over the period of the chase, the total cellular levels of the 55-kDa Pro-Cat zymogen diminished slightly, but the trypsin-sensitive protein accounted for a significant fraction of it 30 min after synthesis and almost all the cellular protein by 2 h (Fig. 5B, left and center panels). This observation was complemented by analysis of the processed catalytic domain in conditioned medium. It was detectable by 30 min after biosynthesis and increased steadily throughout the chase (Fig. 5B, right panel). No zymogen was detected in the conditioned medium. This suggested that the majority of synthesized zymogen was located at the cell surface and that it was processed there prior to release of the catalytic domain into the medium.

To confirm that this was indeed the fate of cell-surface Pro-Cat, biotinylated cells were transferred to fresh medium, and the biotinylated proteins were chased at hourly intervals over an 8-h period in conditioned medium and cells. As early as 2 h into the chase, detectable levels of processed RP4-reactive propeptide (37 kDa) and Myc-reactive catalytic domain (29 kDa) were detected in the conditioned medium, with a plateau attained at 6 h (Fig. 5C, left and center panels, respectively). Notably, very little intact Pro-Cat was detected in the medium. The levels of cell-surface Pro-Cat gradually declined and were minimal by 6 h (Fig. 5C, right panel). The 20–22-kDa fragments did not appear until 6 h (Fig. 5C, left panel), suggesting they follow, and may be dependent on, initial PC processing and that the proteolytic activity responsible for these fragments is within the conditioned medium.

To ask whether exogenously added Pro-Cat was processed by the cell-surface proteolytic machinery, Pro-Cat purified from detergent-solubilized cell lysate using nickel-chelating resin was added to untransfected HEK293F cells, and processing was evaluated by Western blotting of the medium. No alteration was seen in conditioned medium incubated in cell-free conditions, but an increase in processing was observed after Pro-Cat was incubated with cells (Fig. 5D).
**Proprotein Convertases Mediate Propeptide Processing of ADAMTS9 Zymogen**—We previously showed that furin processed ADAMTS9 after Arg287 (30). To determine if PCs have an exclusive role in ADAMTS9 zymogen processing, cells were treated with increasing concentrations of the membrane soluble PC inhibitor dec-RVKR-cmk. Processing of Pro-Cat was inhibited at concentrations as low as 10 μM and was essentially abolished by 50 μM (Fig. 6A). In a second approach, HEK293F cells expressing Pro-Cat were treated with increasing amounts of the furin inhibitor, α1-PDX (39), which substantially reduced the processing of Pro-Cat in a 2-h experiment (Fig. 6B). We next investigated which widely expressed PCs associated with the constitutive secretory pathway could mediate processing. When Pro-Cat was expressed in CHO RPE.40 cells, the intact 55-kDa zymogen was detectable in the conditioned medium and cell lysate, and no fully processed catalytic domain was detected in conditioned medium (Fig. 6C, upper panel). When furin was co-transfected with Pro-Cat, the 29-kDa processed catalytic domain appeared in the conditioned medium (Fig. 6C, upper panel). Transfection of PC5A (Fig. 6C, upper panel), and PC7 (data not shown) also led to appearance of the 29-kDa His-tagged catalytic domain in the conditioned medium, but not in cell lysate. On the
other hand, PACE4 did not produce the fully processed catalytic domain (Fig. 6C, upper panel). When similar experiments were conducted in LoVo cells, the outcomes were comparable to those in CHO RPE.40 cells (Fig. 6C, lower panel). The ability of PACE4 to process Pro-Cat efficiently to a 35-kDa Myc-reactive fragment argues for the presence of a PACE4-susceptible site in the propeptide, possibly, Arg-Arg209-Ser, but argues that PACE4 does not process ADAMTS9 following Arg287. Overall, the most efficient conversion was by furin.

FIGURE 6. PC-dependent processing of pro-ADAMTS9. The zymogen (Z), processed propeptide fragments (P), and processed catalytic domain (C) are indicated. A, dose-dependent inhibition of Pro-Cat processing by the PC inhibitor dec-RVKR-cmk. Cells were treated with 0–100 nM inhibitor, and conditioned medium (CM) was analyzed by Western blotting using anti-RP4 or anti-Myc. Note decreasing pro-domain and catalytic domain fragments with increasing concentration of inhibitor. The precise identity of the molecular species migrating between 40 and 50 kDa is unknown, but presumably represents processing intermediates. B, HEK293F cells stably transfected with Pro-Cat were incubated for 2 h with increasing concentrations of α1-PDX as shown. Immunoblotting of conditioned medium showed a dose-dependent reduction of zymogen processing. C, analysis of Pro-Cat processing by furin, PACE4 and PC5A in CHO RPE.40 cells. Pro-Cat was transfected alone or co-transfected with furin or other PCs in furin-deficient CHO RPE.40 cells (top panel) or LoVo cells (bottom panel). Western blotting was done with anti-Myc. Note that, when seen in the CM, the zymogen is consistently 1–3 kDa larger than the main band in cell lysate, indicating the addition of complex terminal carbohydrate structures. Notice significant processing of the zymogen in CM from the furin- and PC5A-transfected cells but not PACE4-transfected cells.

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successful (data not shown), suggesting that furin was not directly responsible for retention of Pro-Cat at the cell surface. Furthermore, when furin was co-expressed with Pro-Cat in CHO RPE.40 cells, there was not an increase in cell-surface Pro-Cat that would be expected if furin was directly responsible for its sequestration. Instead, the cell-surface ADAMTS9 Pro-Cat was decreased when co-expressed with furin, suggesting that furin contributed directly to processing but not sequestration at the cell surface (Fig. 7C).

Furin Is Essential for ADAMTS9 Propeptide Processing at the Surface of HEK293F Cells—Because the gain-of-function experiments that were done by transfecting furin-deficient cells suggested that furin could most efficiently process the ADAMTS9 propeptide, we asked whether it is responsible for the processing in HEK293F cells in a loss-of-function experiment. To test whether reduction of furin levels in HEK293F cells leads to reduction in cell-surface processing, the cells were transfected with furin siRNA, which significantly reduced total cellular furin (Fig. 8A). In these cells, neither the total amount of ADAMTS9 Pro-Cat nor the total level of cell-surface Pro-Cat were altered (Fig. 8B). However, in contrast to cells that were not siRNA-treated, the conditioned medium contained a significant amount of unprocessed zymogen (Fig. 8C).

DISCUSSION

We previously investigated PC processing of several ADAMTS zymogens (11, 13, 28, 30, 40). The absence of mature ADAMTS9 in cell lysates that was noted in a previous study reporting the complete primary structure of ADAMTS9 (30) led us to carry out the present comprehensive analysis of its maturation. We examined ADAMTS9 propeptide processing using various approaches, none of which provided any evidence for intracellular processing. Both gain-of-function and loss-of-function approaches supported the conclusion that furin processes the ADAMTS9 propeptide. Furthermore, the data suggest that the cell surface is a major site of ADAMTS9 propeptide processing in HEK293F cells or cells that express high levels of cell-surface-processing activity. In contrast, cells expressing no furin activity (CHO RPE.40 or LoVo) or lower levels of furin than HEK293F cells (e.g. COS-1 and CHO-K1) secrete varying amounts of unprocessed pro-ADAMTS9. Whether pro-ADAMTS9 is processed at the cell surface or potentially in the extracellular space, therefore, appears to depend on the relative quantity of furin or other PCs present at these locations. Although CHO RPE.40 and LoVo cells may express other PCs, they processed ADAMTS9 inefficiently. Indeed, the widespread expression of ADAMTS9 (32) is compatible with physiological processing by furin, because this is a ubiquitously distributed convertase. However, there is overlap of ADAMTS9 expression (32) with PC5A during mouse develop-
opment (17) suggesting that processing by this enzyme may occur in vivo as it did in co-transfection experiments.

These data raised the issue of whether or not furin might directly mediate cell-surface binding of ADAMTS9. We argue that it does not. When transfected into CHO RPE 40 cells, furin does not increase the amount of cell-surfacezymogen, as one might expect if it contributed significantly to cell-surface binding, but decreases it, presumably because it processes ADAMTS9, and the processed forms are released. In addition, although we could co-precipitate furin and ADAMTS9 after chemical cross-linking of cell-surface molecules, we could not demonstrate an interaction between them without cross-linking. This suggests the existence of a cell-surface complex that contains furin, ADAMTS9, as well as other molecules. However, because unprocessed zymogen is ultimately released into the medium in cells with low levels of furin activity, the binding is likely to be of low affinity. Significantly, neither the cleaved propeptide nor the processed catalytic domain were everdetected at the cell surface. This suggests that once processed, by furin, the cell-surface interactions in which the Pro-Cat zymogen participates are abruptly disrupted. Thus, properties of both the propeptide and the catalytic domain, or the junctional region between them might be crucial in cell-surface binding of this construct.

We propose that cell-surface interactions of ADAMTS9 Pro-Cat could be one way of targeting its prolytic activity to the pericellular space. Although the function of ADAMTS9 is presently unknown, it is present at the cell surface in cultured cells (30), and its homologs are known to be involved in cell migration (41, 42), which typically requires cell-surface proteolysis (43). Thus, retention of the propeptide may be a mechanism for sequestration of ADAMTS9 at the cell surface, although the ADAMTS9 ancillary domain is also likely to have a role in this context (30).

Traditionally, PCs have been shown to cleave their substrates intra-cellularly. This is particularly true for furin, the best known member of this protease family (1). Molecular shedding events that take place at the cell surface are mostly attributable to PC-activated cell-surface pro-teases such as the ADAMs and membrane-type-MMPs (44, 45). Furin is known to exist at the cell surface (19, 46) and other PCs such as PACE4 and PC6/SA are known to be secreted and anchored in the extracellular matrix (47) and are therefore presumed to have extracellular substrates. Protective antigens of anthrax and diphtheria toxin are cleaved at con-"
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