Intracellular Activation of Human Adamalysin 19/Disintegrin and Metalloproteinase 19 by Furin Occurs via One of the Two Consecutive Recognition Sites*

When K2R were mutated to AA, the proenzyme was also activated by furin, brefeldin A, which inhibits protein trafficking from the endoplasmic reticulum (ER) and/or the trans-Golgi network. This reactivity was restored by co-expression of furin and hADAM19 in these two cell lines, suggesting that furin and hADAM19 interact in vivo.

The adamalysin, ADAM* (for a disintegrin and metalloprotease), or metalloprotease/disintegrin/cysteine-rich family includes proteins containing disintegrin- and metalloprotease-like domains. These proteinases are involved in diverse processes, such as development, cell-cell interaction, and protein ectodomain shedding (1–5). For example, ADAM10/kuzbanian (KUZ) and ADAM17/tumor necrosis factor-α convertase play key roles in the processing of both Notch1 receptor, which is critical in development, and amyloid precursor protein, which is related to the pathogenesis of Alzheimer’s disease (3, 4, 6). Six different ADAMs, ADAM2, -9, -12, -15, and -28, are able to interact with integrins such as αβ1, αβ3, αβ5, αβ6, αβδ, and αβ, regulating cell-cell interactions in normal and pathological processes (7, 8). In the prodomain of ADAMs, there is a cysteine switch sequence similar to the motif found in metalloproteinases (MMPs) (9, 10), keeping ADAMs in latent forms (2, 3, 11, 12). There are one or more furin cleavage sites between the pro- and metalloprotease domains of almost all members of the ADAM family discovered (2, 13–15), but only several ADAM precursors, including ADAM1, -9, -12, -15, and -17, are cleaved by furin or furin-like protein convertases (16–23).

Adamalysin 19/ADAM19, a type I membrane protein containing an intact zinc-binding site in its metalloprotease domain, was cloned from mice (24, 25) and humans (26, 27). Human adamalysin 19 was recently demonstrated to be an active metalloproteinase through its cleavage of α-M-macroglobulin (α-M) in vitro (27). The endopeptidase activity of adamalysin 19 was blocked by specific antibodies against its catalytic domain and disintegrin domain peptides (28). Mouse ADAM19 cleaved intracellular neuregulin, a member of the epidermal growth factor (EGF) family in vivo (29). Human ADAM19 and its mouse homolog are highly similar, sharing 80.6% identity in nucleotide sequences and 84.1% identity in amino acid sequences (24, 27). Among its many roles, hADAM19 may be important in osteoblast differentiation (24), as a marker for the differentiation and characterization of dendritic cells, in the distinction between macrophages and dendritic cells (26), and in the intracellular processing of neuregulin (29). ADAM19 is synthesized as azymogen, and its mechanism of activation has not been thoroughly investigated.

The adamalysin, ADAM1 (for a disintegrin and metalloprotease), or metalloprotease/disintegrin/cysteine-rich family includes proteins containing disintegrin- and metalloprotease-like domains. These proteinases are involved in diverse processes, such as development, cell-cell interaction, and protein ectodomain shedding (1–5). For example, ADAM10/kuzbanian (KUZ) and ADAM17/tumor necrosis factor-α convertase play key roles in the processing of both Notch1 receptor, which is critical in development, and amyloid precursor protein, which is related to the pathogenesis of Alzheimer’s disease (3, 4, 6). Six different ADAMs, ADAM2, -9, -12, -15, and -28, are able to interact with integrins such as αβ1, αβ3, αβ5, αβ6, αβδ, and αβ, regulating cell-cell interactions in normal and pathological processes (7, 8). In the prodomain of ADAMs, there is a cysteine switch sequence similar to the motif found in metalloproteinases (MMPs) (9, 10), keeping ADAMs in latent forms (2, 3, 11, 12). There are one or more furin cleavage sites between the pro- and metalloprotease domains of almost all members of the ADAM family discovered (2, 13–15), but only several ADAM precursors, including ADAM1, -9, -12, -15, and -17 are cleaved by furin or furin-like protein convertases (16–23).

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The proprotein convertases (PCs) are a large family of serine proteinases that recognize dibasic or RX(K/R)R motifs and cleave the peptide bond on the carboxyl side (30–32). As a major proprotein convertase, furin is concentrated in the trans-Golgi network (TGN) and cycles between this compartment and the cell surface through the endocytic pathway. The autoactivation and intracelluar trafficking of furin are well characterized. Numerous studies have shown that furin activates a large number of proproteins in multiple compartments (30–32). For instance, furin has been demonstrated to mediate the activation of proenzymes, such as β-amyloid-converting enzyme (BACE), some matrix metalloproteinases (MMPs), including MMP-11, -14, -16, and -24, and some ADAMs, including ADAM1, -9, -12, -15, and -17 and ADAMTS1, -4, and -12 (16–23, 30, 31, 33–40). However, the molecular mechanism and pathway by which the cells regulate the potentially important interactions between these proenzymes and the proproteins convertase in cells are not fully understood. In this report, we present evidence that furin is responsible for the activation of hADAM19 and this activation can occur via one of the two consecutive recognition sites and that furin is co-localized with the substrate in the ER-Golgi complex and/or TGN.

**EXPERIMENTAL PROCEDURES**

**Chemicals, Cell Lines, Cell Culture, and Immunological Reagents—** All common laboratory chemicals, proteins, and reagents, were purchased from Sigma. Anti-furin antibodies were from Affinity Bioreagents, Inc. (Golden, CO). Protein A/G PLUS agarose was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The CMK-based furin inhibitor dec-Arg-Val-Lys-Arg-chloromethyl ketone (decRVKR-CMK), a calcium ionophore, A23187; and a metalloproteinase inhibitor, ilomastat (GM6001), were from BACHEM (Philadelphia, PA). Restriction enzymes were from Promega or Invitrogen. COS1, Madin-Darby canine kidney (MDCK), and derivative cells were maintained as described (40–43). The furin-deficient Chinese hamster ovary-K1 strain RPE.40 and furin-deficient COS-7 cell strain P15 were cultured as described (44). Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), penicillin G, and streptomycin were from Invitrogen. α-M was from Roche Molecular Biochemicals. Goat anti-mouse conjugated to horseradish peroxidase was from BioLegend, and Cy-2 labeled goat anti-rabbit antibody and Cy-3 labeled goat anti-mouse antibody were purchased from Invitrogen. 5-Cy3-labeled phalloidin (a rhodamine red dye) was from Jackson ImmunoResearch Laboratory, Inc. (West Grove, PA). Rabbit polyclonal hADAM19 antibodies pAb361 (anti-metalloproteinase domain) and pAb362 (anti-disintegrin domain) were generated by our laboratory as described (28).

**PCR Primers, Mutagenesis, and Expression Constructs—**PCR3.1-ADAM19 wild type and mutants with or without the FLAG tag were generated by polymerase chain reaction with Pfu polymerase (Stratagene) as described (40–43). The primer sequences for wild type ADAM19 were 5’-ACC ATG CGG GCC GCA GGC GCC GGC-3’ (forward primer) and 5’-GATT TTT CGA GCT AAT CAT CCC TCC-3’ (reverse primer). For deletion from the transmembrane domain to the cytoplasmic domain, sequences were 5’-ACC ATG CGA GGC GCA GGC GCC GGC-3’ (forward primer) and 5’-AGG ACC AAT CAT ACT CTC AGG GCC GGC-3’ (reverse primer). For 196KR to AA mutant, sequences were 5’-ACC ATG CCA AGG GCC GCA GGC GCC GGC-3’ (forward primer) and 5’-AGG ACC AAT CAT ACT CTC AGG GCC GGC-3’ (reverse primer). For 199RR to AA mutant, sequences were 5’-CAG ACC AAG GGC GCA CCT CGG AGG GCC-3’ (forward primer) and 5’-CCT GCC AGG TGC CGG CCT GTT CTG-3’ (reverse primer). For 202KR to AA mutant, sequences were 5’-GAG AAG CGA CCT GCC GGC ATG AAA AGG-3’ (forward primer) and 5’-CCT TTT CAT CCT GCC GGC AGG AGG GAA GGC-3’ (reverse primer). For 196KR to AA mutant, sequences were 5’-CAG ACC AAG GGC GCA CCT CGG AGG GCC-3’ (forward primer) and 5’-CCT GCC AGG TGC CGG CCT GTT CTG-3’ (reverse primer). For 199RR to AA mutant, sequences were 5’-GAG AAG CGA CCT GCC GGC ATG AAA AGG-3’ (forward primer) and 5’-CCT TTT CAT CCT GCC GGC AGG AGG GAA GGC-3’ (reverse primer). For 202KR to AA mutant, sequences were 5’-GAG AAG CGA CCT GCC GGC ATG AAA AGG-3’ (forward primer) and 5’-CCT TTT CAT CCT GCC GGC AGG AGG GAA GGC-3’ (reverse primer).

Furin Activates hADAM19

**Transient Transfection into COS1, RPE.40, or 7.P15 Cells—**Cells were seeded in 24- or 6-well plates for 16–24 h at 80% confluence prior to transfection. The cells were then transfected with the indicated plasmids using LipofectAMINE 2000. After 6–10 h, serum-free or 5% FBS DMEM containing 2.5 μM GM6001, with or without CMK, BFA, or A23187 at indicated concentrations, were added for another 24 h. The conditioned media and cell lysates were analyzed by Western blotting. For co-transfection experiments in these cells, the individual plasmids were transfected alone as a control or co-transfected with expression plasmids directing the production of furin, PAC4, pAT, pAP, furin and pAT, or furin and pATp. After 6–10 h, serum-free or 5% FBS DMEM containing GM6001 (2.5 μM) was added to the transfected cells for 16–24 h. Then, the conditioned media and cell lysates were analyzed by Western blotting as described above.

**Glycogenolysis Analysis—**N-Glycosylation in vitro was investigated by endoglycosidase F treatment as described previously (40, 41). Briefly, transfected cells were grown to 80% confluence and incubated in serum-free medium for 24 h. The conditioned media were then collected, and the cells were lysed with RIPA. After centrifugation, the conditioned media or the supernatant from RIPA were treated with endo-H, endo-F, and PNGase F sequentially. The conditioned media and cell lysates were analyzed by Western blotting with anti-human furin and anti-FLAG-M2 antibodies as described (42).

**Western Blotting—**The experiments were carried out as described previously (40, 41). Briefly, cells were grown to 80% confluence and were treated as indicated. After centrifugation at 14,000 × g for 15 min at 4°C to clear any debris, the supernatants from RIPA were treated with SDS-PAGE. The cells were lysed with RIPA (50 mM Tris, pH 7.5, 150 mM NaCl, 0.25% sodium deoxycholate, 1.0% Nonidet P-40, 1 mM phenylmethylsulfonlfuoride, 2.5 μM GM6001, 10 μg/ml aprotinin, 10 μg/ml E64, and 10 μg/ml pepstatin A) for 15 min in ice. The supernatant was collected after centrifugation at 14,000 × g for 20 min at 4°C. After centrifugation, the proteins were transferred onto nitrocellulose membranes and probed with anti-FLAG-M2 or anti-hADAM19 and developed as described (40, 41).

**Purification of Soluble hADAM19 and Protein N-terminal Sequencing—** All proteins were purified on anti-FLAG-M2 affinity columns as described (42, 43). Briefly, cells stably expressing wild type soluble hADAM19 (DS2-6) or 199RR to AA mutant (199RR-D) were grown to 100% confluence, then washed with PBS twice and incubated for 48 h in serum-free medium containing GM6001 (to prevent the degradation of hADAM19). The conditioned media were collected, centrifuged to clear debris, and loaded onto an anti-M2 immuno-affinity column (1 ml of resuspended agarose) prewashed with Tris-buffered saline. The bound materials were extensively washed with Tris-buffered saline, eluted with FLAG peptides, and collected in 20-μl fractions. The fractions were analyzed by Western blot using anti-hADAM19 antibodies or anti-FLAG-M2. The fraction containing the highest hADAM19 protein concentration was prepared for protein N-terminal sequencing. After separation by SDS-PAGE, the samples were transferred to a polyvinylidenefluoride membrane and stained with Coomassie Blue R-250. After staining, the hADAM19 bands were excised and sent to the Bioanalytical Core Facility at the Florida State University for N-terminal amino acid sequencing.

**α-M Trapping Assay to Determine Endopeptidase Activity of hADAM19 Species—** The detailed experimental procedure was previously reported (27, 28). Briefly, 10 μl of the fraction containing purified soluble hADAM19 was mixed with 24 μl of α-M (0.2 unit/ml), adjusted to a total volume of 100 μl by adding HEPES buffer (50 mM HEPES, pH 7.5, 200 mM NaCl, 10 mM CaCl2, 25 μM ZnCl2, 0.05% Brij-35), and incubated at 37°C for 1–5 days. A 20-μl aliquot of the mixture was removed daily, put into 2× SDS-PAGE sample buffer, and boiled. Following SDS-PAGE, the protein bands in the gels were visualized by silver staining.

**Confocal Microscopy—** The procedures have been described in detail previously (38, 40). Briefly, MDCK cells expressing hADAM19 wild type or 199RR mutant were grown on coverslips in six-well plates with or without treatment with CMK, BFA, or A23187. After fixing with Lina’s fixation buffer for 30 min, the cells were permeabilized with buffer A (0.3% Triton X-100, 1% neutural detergent solution, 150 mM NaCl, 0.01% NaN3, in PBS) for 1 h and incubated for 3 h with anti-furin and anti-FLAG-M2 (1:100 dilution in buffer A) for double staining. After washing with PBS three times, secondary antibodies conjugated with either fluorescein isothiocyanate or rhodamine red were added to the cells for 1 h, followed by four washes with PBS. Confocal microscopy experiments were performed at the Biological Science Imaging Resource Facility at Florida State University.
Removal of the hADAM19 Prodomain Is Dependent on Furin Activity—The sequence of hADAM19 contains two potential furin recognition sites (RXKR/RK). 196KRPRRMK203R, between its pro- and catalytic domains (Fig. 1A). To ascertain the role of furin in the cleaving of the hADAM19 prodomain, wild type hADAM19 (F46) with a C-terminal FLAG tag was transfected into COS1 cells alone, or co-transfected with furin, PACE4, α1-proteinase inhibitor (pAT), both furin and pAT, Pittsburgh mutant of α1-proteinase inhibitor (pATp, a specific inhibitor of furin) (37, 45), or both furin and pATp. As shown in Fig. 1B, active hADAM19 forms were increased by the introduction of furin, but not PACE4. Furthermore, pATp blocked the processing of hADAM19; furin could not restore this processing when cells were co-transfected with furin and pATp (lane 8). pAT had little effect on endogenous or furin-induced processing of hADAM19 (lanes 2, 3, and 5). Because pATp did not completely block the hADAM19 processing, the low levels of endogenous processing may also be mediated by other proprotein convertases in addition to furin (lanes 7 and 8).

Interestingly, both the pro- and active forms of hADAM19 were doublets. These doublets may be differentially glycosylated forms. According to protein sequence analyses, hADAM19 has five potential glycosylation sites (27). Indeed, endoglycosidase F converted the doublets into a single pro- or active form, respectively (data not shown). To verify that the active hADAM19 lacks a prodomain, hADAM19 antibodies against the pro-, catalytic, or disintegrin domains (28) were used to probe the proteins in the cell lysates. The results in Fig. 1C clearly showed that the processed 80-kDa hADAM19 came from the removal of its prodomain because it was not recognized by the antibody against the prodomain peptide; however, it was detected with antibodies against its catalytic and disintegrin domains, respectively. These data showed that furin activity played a major role for the intracellular removal of hADAM19 prodomain.

To further demonstrate a direct role for furin in the activation of the hADAM19 zymogen, COS1 cells transfected with wild type hADAM19 (F46) were incubated with dec-Arg-Val-Lys-Arg-CMK (decRVKR-CMK), a widely used inhibitor of furin (17, 38, 40, 46, 47). As shown in Fig. 2A, decRVKR-CMK blocked the activation of hADAM19 in a dose-dependent manner. Because furin is mainly localized in TGN and the autolysis of the hADAM19 prodomain. To isolate soluble hADAM19 protein for enzyme activity assays, a construct encoding the extracellular domain (ectodomain) of hADAM19 containing a C-terminal FLAG tag was generated; this construct was called D52 and lacked the transmembrane domain and cytoplasmic domain.
This hADAM19 ectodomain construct was transfected into COS1 cells. As shown in Fig. 3A, only the active forms of soluble hADAM19 were detected in the media from COS1 cells co-expressing the hADAM19 ectodomain and furin. Both pro- and active forms were detected in the media from the cells co-transfected with PACE4 or transfected with the ectodomain construct alone. Additionally, the active forms were detected in the cell lysates only when cells were co-transfected with furin (Fig. 3A, lanes 5–8).

A dose-dependent activation of soluble hADAM19 activation by decRVKR-CMK was observed (Fig. 3B). However, there was no significant effect of decRVKR-CMK on the intracellular levels of hADAM19. Furthermore, there was no secretion of soluble hADAM19 in the transfected cells treated with either BFA or A23187 (Fig. 3C). Also, pATp dramatically decreased the amount of active forms in the medium when it was expressed in COS1 cells, but pAT failed to do so (Fig. 3C). Once again, no significant differences were seen in response to these treatments in the cell lysates (Fig. 3C). These results show that the soluble forms of hADAM19 were processed in the same manner as the full-length form and are consistent with furin-mediated activation of hADAM19.

There Are Two Alternative Furin Recognition Sites between the Pro- and Catalytic Domain of hADAM19—Upon the examination of the hADAM19 protein sequence, two consecutive furin recognition sites (RX/K/R)R, 196KPR198KR and 199RRM198KR, were found (Fig. 1A). We hypothesized that these two furin recognition sites are alternatively used for the intracellular activation of pro-ADAM19 by furin. To test this hypothesis, three mutants were generated in full-length and ectodomain hADAM19, which converted the 196KR, 199RR, and 202KR into AA, respectively. These were named as 196RA-F, 199RA-D, 199RA-F, 199RA-D, 202RA-F, and 202RA-D, respectively, indicating that one (196RA-F,
furin-transfected COS1 cell culture. The medium from COS1 cells transfected with a blank vector was used as a negative control. As shown in Fig. 4D, soluble furin did not process the 199RA mutant of the soluble hADAM19. However, the wild type and the mutant soluble proteins containing a furin recognition motif were cleaved by furin. This furin-mediated processing was sensitive to decRVKR-CMK inhibition, consistent with the intracellular processing results obtained earlier (Figs. 3A and 4C). These results demonstrated that furin could activate hADAM19 at both furin cleavage sites between the prodomain and the catalytic domain of the zymogen.

FIG. 4. Requirement of furin motifs between the pro- and catalytic domains for the activation of hADAM19. A, a schematic illustration for the wild type expression vector pCR3.1hADAM19 and its mutant constructs. All the constructs have a C-terminal FLAG tag. SP, signal peptide; Pro-, prodomain; Cat-, catalytic domain; Dis-, disintegrin domain; Cys-, cysteine-rich domain; EGF-, EGF-like domain; TM, transmembrane domain; CD, cytoplasmic domain; F, FLAG tag. B, processing of hADAM19 in the 199RR to AA mutant is abolished. COS1 cells were transfected with the blank vector (lane 1), F46 (lane 2), 196RA-F (lane 3), 199RA-F (lane 4), or 202RA-F (lane 5), and grown in 24-well plates for 24–36 h. The cells were lysed with RIPA followed by SDS-PAGE and Western blotting with anti-FLAG-M2. C, inhibition of the processing of the hADAM19 ectodomain in the 199RR to AA mutant. The conditioned media (lanes 1–5) and cell lysates (lanes 6–10) were from COS1 cells transfected with the blank vector (lanes 1 and 6), D52 (lanes 2 and 7), 199RA-D (lanes 3 and 8), 199RA-D (lanes 4 and 9), or 202RA-D (lanes 5 and 10) overnight, followed by incubation in serum-free medium for 24 h. The samples were analyzed by Western blotting with anti-FLAG-M2. D, activation of the soluble hADAM19 by exogenous soluble furin. The condition media from lanes 2–4 in C were mixed with equal volumes of conditioned serum-free media from COS1 cells transfected with the blank vector (lanes 1, 4, 7, and 10) or soluble furin (lanes 2, 3, 5, 6, 8, 9, 11, and 12). After being incubated with (lanes 3, 6, 9, and 12) or without 50 μM CMK (lanes 1, 2, 4, 5, 7, 8, 10, and 11) at 37 °C for 24 h, the samples were analyzed by Western blotting as in C.
Furin Activates hADAM19

FIG. 5. Requirement of the zymogen activation for the proteolytic activity of hADAM19. A, processing of hADAM19 in stably transfected MDCK cells. MDCK cells stably expressing wild type hADAM19 (F46-4; lanes 2–5) or 199RA mutant (199RA-F-9; lane 6) were seeded in six-well plates at 50% confluence. The next day, cells were treated with CMK (100 μM, lane 3), BFA (10 μg/ml, lane 4), or A23187 (0.5 μM, lane 5). Lanes 2 and 6 represent untreated controls. The cells were lysed with RIPA followed by analysis of Western blotting using anti-FLAG-M2.

B, the requirement of furin in the processing of soluble hADAM19 in stably transfected MDCK cells. MDCK cells stably expressing soluble hADAM19 (D52-5) (lanes 2, 3, 6, and 7) or soluble 199RA mutant (199RA-D-6) (lanes 4 and 8) were grown in 24-well plates to 100% confluence. Cells were incubated in serum-free media without (lanes 1, 2, 4, 5, 6, and 8) or with CMK (100 μM, lanes 3 and 7) for 24 h. Both conditioned media (lanes 1–4) and lysates (lanes 5–8) were analyzed by Western blotting as in A. MDCK cells stably transfected with the blank vector were an additional control (lane 1). B, the requirement of furin in the processing of soluble hADAM19. Purified soluble hADAM19 from D52-5 and 199RA-D6 were incubated in reaction buffer alone (lanes 1 and 2) or with α2-M in the absence (lanes 4 and 5) or presence of EDTA (lane 5) for 48 h. α2-M was a control (lane 3). The α2-M/hADAM19 complex and the cleavage products of α2-M by hADAM19 are labeled on the right. Note that pro-soluble hADAM19 (199RA-D-6) did not form the complex with α2-M.

was not removed by furin (Fig. 5B). These results suggest that hADAM19 activation also obeys the cysteine-switch mechanism for zymogen latency and activation. Furthermore, N-terminal sequences of the purified hADAM19 proteins from the media revealed that the processed doublets (Fig. 5B, lane 2) had the identical N-terminal sequences of 204EDLNSMK, suggesting that furin prefers to cleave hADAM19 using the recognition site of 200RRMK203R rather than 197RPR200R. The doublets have different glycosylation patterns as verified by Western blotting as in A. MDCK cells stably transfected with the blank vector were a control (lane 1). C, the proteolytic activity of soluble mature hADAM19. Purified soluble hADAM19 from D52-5 and 199RA-D6 were incubated in reaction buffer alone (lanes 1 and 2) or with α2-M in the absence (lanes 4 and 5) or presence of EDTA (lane 5) for 48 h. α2-M was a control (lane 3). The α2-M/hADAM19 complex and the cleavage products of α2-M by hADAM19 are labeled on the right. Note that pro-soluble hADAM19 (199RA-D-6) did not form the complex with α2-M.

Two Furin-deficient Cell Strains (RPE.40 and 7.P15) Do Not Activate Pro-hADAM19, and the Introduction of Furin into These Cells Restores Zymogen Activation—To further confirm that furin activity was required for the intracellular activation of pro-hADAM19, the wild type of full-length and soluble forms of hADAM19 (F46 and D52) were transfected into RPE.40 cells and 7.P15 cells, two furin-deficient cell strains (44, 51). Processing of hADAM19 to its mature forms was negligible in these cell lines. However, the active forms were clearly detectable when cells co-expressed furin (data not shown). As shown in Fig. 6, there were barely detectable levels of the active forms of soluble hADAM19 in the media of the two D52-transfected cell lines. High levels of active forms were only detected in the media when the cells co-expressed furin, although PACE4 also increased the amount of active forms when it was co-expressed with D52 (Fig. 6, A and B). Curiously, the active forms were only detected in the lysates of cells co-expressing D52 and furin. These data further confirm that furin was responsible for the intracellular activation of hADAM19.

Furin Was Co-localized with hADAM19 in the ER-Golgi Complex and/or TGN—To verify that hADAM19 was a physiologically relevant substrate of furin, the cellular localization of furin and hADAM19 was examined by confocal microscopy using MDCK cells stably expressing hADAM19 (Fig. 7). Untreated cells are marked as F46-4 (control) (Fig. 7, top panels). Co-localization of hADAM19 and furin was clearly observed, and was consistent with ER-Golgi complex and/or TGN localizations (top, middle panel). Furthermore, hADAM19 was also seen at the edges of the plasma membrane (right lane, top panel) where furin was rare (left lane, top panel). Interestingly, a similar pattern of co-localization between the 199RA mutant and furin was also observed (Fig. 7, bottom panels), suggesting that the co-localization was independent of the recognition sites for furin in hADAM19. To test whether the agents that block the activation of hADAM19 could prevent co-localization between furin and hADAM19 (Fig. 5A), the cells were treated with CMK, BFA, or A23187. None of these treatments interfered with the co-localization pattern of furin and hADAM19 (Fig. 7), suggesting that furin may be co-localized with...
RPE.40 cells were transfected with the blank vector (lanes 1 and 5) or D52 alone (lanes 2 and 6) or co-transfected with D52 and plasmids encoding furin (lanes 3 and 7) or PACE4 (lanes 4 and 8). Cells were grown in 24-well plates for 12–16 h followed by incubation in serum-free medium for 24 h. Conditioned media (lanes 1–4) and cell lysates (lanes 5–8) were analyzed by Western blotting with anti-FLAG-M2.

A

B

hADAM19 in perinuclear ER-Golgi complex and/or TGN independent of the furin catalytic activity.

DISCUSSION

Proteolysis of the extracellular matrix and cell surface proteins mediated by metalloproteases, including MMPs and ADAMs, is of vital importance for tissue-remodeling processes during normal and pathological conditions, such as tissue morphogenesis, wound healing, inflammation, and tumor cell invasion and metastasis (3–7, 52, 53). Metalloproteases are synthesized as inactive proenzymes orzymogens, and their latency is maintained by a cysteine-switch residue in the propeptide domain in which the thiol group is coordinated to the active site zinc (II) (2, 9–12). To display any proteolytic activities, the prodomain located N-terminal to the catalytic domain must be removed from the zymogen in most cases. Recently, PCs, such as furin and or furin-like serine peptidases, have been recognized as very important enzymes for the zymogen activation, although various mechanisms have been proposed for the activation of pro-MMPs and pro-ADAMs. Furin or furin-like PCs mediate zymogen activation by recognizing a conserved RXK/R/R motif in the boundary between pro- and catalytic domains. This motif is present in almost all ADAMs and nine MMPs (2, 13, 53).

By analyzing the intracellular activation of hADAM19, we have demonstrated that both furin activity and one of the two consecutive sites in 197RPRMK199R in ADAM19 are required for activation, which is dependent on calcium and proper secretory pathway trafficking. Furthermore, we have provided direct evidence that furin is co-localized with hADAM19 in ER-Golgi complex and/or TGN. This colocalization between furin and hADAM19 is independent of the furin recognition site and is resistant to a variety of treatments, such as CMK, BFA, and A23187, that inhibit furin activity, vesicular trafficking, and calcium signal, respectively. These findings are consistent with the report published recently showing that furin was co-localized with MMP16 independent of their apparent enzyme-substrate relationship (40).

Latency and Activation of ADAMs—The classic cysteine switch mechanism for pro-MMP latency and activation was originally proposed for MMPs (10) and may be applied for many MMPs discovered with the exception of MMP-3, MMP-23, and MMP-26 (41, 54–58). The activation of pro-MMP-3 by a merccurial compound was triggered by a perturbation of the conformation of the precursor rather than a direct disruption of the Cys-zinc interaction (54). A salt bridge in pro-MMP-3 might also contribute to the latency of the proenzyme (55). Organomercuroic treatment failed to activate pro-MMP-26 with a unique cysteine-switch motif, PH4C8GXGD, and when the conserved cysteine-switch sequence, PR81CGXXD, in the prodomain of pro-MMP-26 was restored by mutagenesis, the cysteine-switch activation mechanism was not induced (58).

Regarding the ADAM family members, the active ADAMs, such as ADAM1,-9,-10,-12,-15,-17,-19,-28, and ADAMTS1,-4, and -12, contain a catalytic site consensus sequence (HEXXH) in their metalloprotease domains (2, 11–14, 16–21, 27–29, 53, 59–61). They may also have a putative cysteine-switch residue in their prodomain to keep them inactive (9). For example, the investigation by Leochel et al. (11) demonstrated that the latency and activation mechanism of ADAM12 was similar to the cysteine switch model proposed for MMPs. ADAM9,-15, and -17 showed catalytic activity against their substrates only after their prodomains were removed (12, 21, 22). However, for many ADAMs, including ADAM19, no direct evidence has been provided to support the hypothesis that the Cys-zinc coordination is required for latency. For ADAM17/tumor necrosis factor-α convertase, the prodomain was not only an inhibitor of the catalytic domain, but also appeared to act like a chaperone, facilitating secretion, folding, or both of the ADAM protein (12). In this report, we have demonstrated that, after the removal of the prodomain of hADAM19 by furin, the enzyme has endopeptidase activity against 33-PM. However, furin is unable to cleave the prodomain of the 199PR to AA hADAM19 mutant lacking a furin recognition site in the boundary of the pro- and catalytic domains. This mutant has no
proteolytic activity using an α2-M trapping assay. These results demonstrate that at least one of the furin recognition sites is required for the removal of the propeptide domain by furin to activate pro-hADAM19. The detailed mechanism of pro-ADAM19 latency and activation and the role of the cysteine-switch sequence remain to be further investigated.

For the activation of ADAM zymogens, two mechanisms have been reported. One is the removal of the prodomain by autolysis, but it was shown only in ADAM28 (60). The predominant mechanism for the activation of ADAMs is mediated by furin or furin-like PCs in the secretory pathway. This mechanism has been shown in many ADAMs, including ADAM1, -9, -12, -15, -17, and -19, and ADAMTS1, -4, - and -12, using N-terminal sequencing, specific inhibitors of furin, blockers of protein trafficking from ER to Golgi, exogenous soluble furin in vitro, furin-deficient cell lines, and mutagenesis at the furin recognition site(s) (RXKR/RKR) between the pro- and catalytic domain (Refs. 16–23; this report). In the present report, we provide a thorough investigation of ADAM zymogen activation mediated by furin (Figs. 1–6) and evidence that furin is co-localized with ADAMs in the ER-Golgi complex and/or TGN (Fig. 7), showing that ADAMs are similar to MMPs in these respects (38, 40).

There Are Two Consecutive Furin Recognition Sites in the Boundary of the Pro- and Catalytic Domains of hADAM19—The minimal furin recognition sequence requires basic residues at P1 and P2 (RXR) and in some cases, at the P3 position, an amino acid with a hydrophilic aliphatic side chain is not suitable (31). Typically, there is only one furin recognition site between the pro- and catalytic domain of the substrates of furin as found in most members of the ADAM family, seven MMPs, pro-BACE, and Notch1 receptor (2, 13, 15, 30, 31, 33–40, 53, 62). In this report, we present evidence for the first time that there are two consecutive furin recognition sites, 197RPR200R and 201RMK202R, between the pro- and catalytic domain in hADAM19, which adhere to the rules for efficient cleavage by furin (31). Only pro-forms were detectable in the 199RA mutant, which lacked a furin recognition site between its pro- and catalytic domain, whereas the mutants of both 198RA and 202RA, which possessed recognition sites, were converted into the active forms. Thus, the Arg residue at the P3 site is required for the intracellular hADAM19 maturation mediated by furin (Figs. 4 and 5B). Interestingly, N-terminal sequencing of wild type mature forms (Fig. 5) confirmed that the preferred intracellular cleavage site for hADAM19 activation is the one nearer to the catalytic domain, 200R–201M, as predicted before (26, 27). This motif is conserved in mice as 201RMF202R (24). The distal motif, 197RPR200R in humans, is, however, replaced with 198QPR201R in mice, which is not efficiently cleaved by furin.

A notion that pro-hADAM19 activation by furin may be sequential, i.e. 204R205M is cleaved first followed by 203R204E, seems to be consistent with the partially activated soluble species seen for 198RA-D compared with 202RA-D data in Fig. 4C, where it does not agree with the data shown in Fig. 4B, where it is seen that the presence of furin with the full-length 198RA-F leads to more activated species than 202RA-F. The delicate changes in the interactions between the different mutants that have subtle structural and conformational differences might be partially responsible for the different activation levels observed. Moreover, among all the protein N-terminal sequence data of wild type hADAM19 activated species, only 204EDELNSMK was found; the alternative cleavage site product of 201MKRED was not detected. Most importantly, the minimal furin recognition sequence requires basic residues at P1 and P2 (RXR) and the Arg residue at the P3 site is required for the intracellular hADAM19 maturation mediated by furin (Figs. 4 and 5B). It may not be possible for furin, an endopeptidase, to effectively cleave the product of the 208R209M cleavage because the 201R202E sequence lacks the required Arg at the P3 site. Thus, our data suggest that the 208R209E site is the predominant cleavage site and 208R209M is an alternative cleavage site by furin when the predominant site is missing. This is consistent with the model proposed for wild type MT1-MMP, in which the pro-MT1-MMP is processed primarily at the 108RRKR site to generate the active proteinase and the secondary site within S6KXXRKR is cleaved only when the primary 108RRKR motif was mutated (39).

Notably, there are two potential consecutive furin recognition sites in other metalloproteinase zymogens, including ADAM11 (AB009675, 292LRRK297R), ADAM22 (AF155382, 218RPRSK225R), ADAMTS4 (AF148213, 208RPRRAK215R), MT2-MMP (NM_002428, 125RRRK231R), and MT5-MMP (AJ010262, 118RRRNKK223R). The ones nearer to the catalytic domains are conserved in different species, whereas the distal ones might be acquired later during evolution. Although the significance of the two alternative recognition sites in these precursors remains poorly understood, we may speculate that the processing of these zymogens are crucial for some biological events; the zymogens may be activated by furin at a different cleavage site even if the primary site is abolished by mutation.

Significance of Furin and Its Related PC Pathways in the Processing of Precursors—Furin and its related PCs have been demonstrated as the major enzymes responsible for the maturation of many precursors, such as some ADAMs and MMPs (Refs. 16–23 and 37–40; this report). Furthermore, zymogens of BACE, a major enzyme related to Alzheimer’s disease, and some growth factors and cell surface receptors, such as transforming growth factor β, insulin-like growth factor, hepatocyte growth factor receptor, and Notch1 receptor, are converted into their active forms by these PC pathways (30, 31, 33–36, 62). Thus, this activation mechanism by a PC may play key roles in many physiological and pathological events. In fact, furin knockout mice are embryonic lethal (63), and inhibition of furin results in absent or decreased invasion and tumorigenicity of human cancer cells (64, 65). The inability to activate many types of proproteins, including some pro-ADAMs and pro-MMPs, in furin null mice may contribute to the abnormal phenotypes during early development and morphogenesis in those mice. On the other hand, the design and synthesis of furin specific inhibitors may lead to a new strategy in the treatment of cancer and other diseases, such as Alzheimer’s disease, in the human adult.

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Furin Activates hADAM19

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