Shedding of Collagen XXIII Is Mediated by Furin and Depends on the Plasma Membrane Microenvironment

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Collagen XXIII belongs to the class of type II oriented transmembrane collagens. A common feature of these proteins is the presence of two forms of the molecule: a membrane-bound form and a shed form. Here we demonstrate that, in mouse lung, collagen XXIII is found predominantly as the full-length form, whereas in brain, it is present mostly as the shed form, suggesting that shedding is tissue-specific and tissue-regulated. To analyze the shedding process of collagen XXIII, a cell culture model was established. Mutations introduced into two putative proprotein convertase cleavage sites showed that altering the second cleavage site inactivates much of the shedding. This supports the idea that furin, a major physiological protease, is predominantly responsible for shedding. Furthermore, our studies indicate that collagen XXIII is localized in lipid rafts in the plasma membrane and that ectodomain shedding is altered by a cholesterol-dependent mechanism. Moreover, newly synthesized collagen XXIII either is cleaved inside the Golgi/trans-Golgi network or reaches the cell surface, where it becomes protected from processing by being localized in lipid rafts. These mechanisms allow the cell to regulate the amounts of cell surface-bound and secreted collagen XXIII.

The group of collagenous transmembrane proteins consists of type XIII, XVII, XXIII, and XXV collagens and several related proteins such as ectodysplasin A, the class A macrophage scavenger receptors, the MARCO1 receptor, and the group of colmedins. These are type II transmembrane proteins that contain at least one collagenous triple helical domain (summarized in Ref. 1). Collagens XIII, XXIII, and XXV are of unknown function and consist of three collagenous domains that are flanked and separated by non-collagenous domains. Whereas collagen XVII is more distant related, types XIII, XVII, XXIII, and XXV all exist in two forms: a transmembrane form and a shed ectodomain form. Whereas collagen XVII is shed from the surface by TACE (tumor necrosis factor-α-converting enzyme), a member of the ADAM (a disintegrin and metalloproteinase) family (2), mutation analysis of collagens XIII and XXV demonstrated that the protease furin produces the shed forms (3, 4). Initial cell culture studies suggested an involvement of furin either directly or indirectly in the cleavage of collagen XXIII as well (5). Furthermore, the co-existence in tissues of both the transmembrane and shed forms of collagen XXIII was suggested from immunoblot analyses (6).

The shedding of an ectodomain amplifies the possible functional role of a protein because different forms, i.e. full-length cell surface-bound or soluble, likely have different biological activity. The "sheddes" furin is a member of a proprotein convertase family. Among other functions, furin participates in the maturation and activation of proteins at the cell surface, endowing the cell with the ability to change its functional behavior (7–9). Moreover, conversion by proteolytic cleavage can be tightly regulated (10, 11). Furin-dependent shedding has been implicated in the activation of growth factors, initiating protease cascades as well as affecting pathogen entry into cells. With regard to the latter, it is notable that the virulence of pathogens such as anthrax, Ebola virus, and influenza virus is influenced by the presence of furin cleavage sites in viral proteins (9). Furthermore, there is at least one instance demonstrating that pathology results if shedding does not occur. A mutation in the furin cleavage site of the transmembrane collagen-like molecule ectodysplasin A is responsible for 20% of the cases of X-linked hypohidrotic ectodermal dysplasia (12). The consensus sequence for furin cleavage is Arg-X-(Lys/Arg)-Arg (13), with the most critical position being Arg at position 1. Less conserved sequences such as Arg-X-X-Arg also serve as furin cleavage sites, but with 10-fold lower efficiency than the consensus sequence. Furin is activated by two autocatalytic cleavage processes in the trans-Golgi network (TGN) (14). As a mature enzyme, furin cycles between the cell surface and the TGN in a well-regulated clathrin-dependent fashion (8).

2 The abbreviations used are: TGN, trans-Golgi network; AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride; hydrochloride; cmk, chloromethyl ketone; MBCD, methyl-β-cyclohexane; RT, reverse transcription; HEK, human embryonic kidney; EBNA, Epstein-Barr virus nuclear antigen; DMEM, Dulbecco's modified Eagle's medium; PLAP, phosphatidylinositol-linked placental alkaline phosphatase; HTR, human transferrin receptor; PBS, phosphate-buffered saline; PC, proprotein convertase; α1-PDX, α1-antitrypsin Portland.
To regulate which form of a transmembrane molecule is present in a cell’s environment, control is exerted not only by activation of the sheddase, but also by accessibility of the sheddase enzyme to the cell-surface protein to be shed. In this context, the plasma membrane microenvironment has a crucial regulatory function for the shedding of a variety of transmembrane proteins such as Alzheimer amyloid precursor protein (15), CD30 (16), the interleukin-6 receptor (17), and collagen XVIII (18). Lipid rafts are small, dispersed, cholesterol-rich, and sphingolipid-rich microdomains that are fluid, but tightly packed (19). The lipid raft and non-raft microdomains have different association capabilities and therefore accumulate and separate membrane proteins. Lipid rafts play an important role in signal transduction, cell-cell interaction, and endocytosis of certain proteins (20, 21).

Here we present evidence for processing of collagen XXIII directly by furin. We also show that ectodomain shedding is influenced by the plasma membrane microenvironment: lowering the cellular cholesterol level significantly increases the shedding of collagen XXIII. This, together with partial shedding already occurring in the secretory pathway, likely represents the mechanism used by cells to regulate the ratio of the membrane-bound to released ectodomain forms of collagen XXIII. In addition, shedding is also tissue-dependent: in brain, collagen XXIII is present predominantly as the shed form, and in other tissues such as skin and lung, the full-length molecule is the prevailing form.

**EXPERIMENTAL PROCEDURES**

**Reagents**—The following protease inhibitors were used: AEBSF (4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride; Merck); antipain, 1,10-ortho-phenanthroline, aprotinin, E-64 (trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane), pepstatin A, leupeptin, and chymostatin (Sigma); the furin inhibitor decanoyl-Arg-Val-Lys-Arg-chloromethyl ketone (cmk; Bachem); the hydroxamate inhibitor TAPI-0 (Calbiochem-Novabiochem GmbH); and the membrane-impermeable furin inhibitor α1-PDX (Calbiochem). Other chemicals used were methyl-β-cyclodextrin (MβCD) and filipin III from Streptomyces filipensi (Sigma), brefeldin A (Epigentic Biotechnologies), and 3,3′-dithiobis(sulfosuccinimidylpropionate) (Pierce).

**Cloning of Full-length α1(XXIII) and Furin cDNAs and Site-directed Mutagenesis**—Reverse transcription (RT)-PCR was used to clone the full-length mouse α1(XXIII) and furin cDNAs. Primers were designed according to the mRNA sequences provided for GenBank accession numbers NM153393 (α1(XXIII)) and NM011046 (furin). The full-length α1(XXIII) cDNA was amplified from mouse embryonic day 15.5 cDNA using the primer pair K500/K501 (see the list of PCR primers in supplemental Table S1). The full-length construct was ligated into a modified pCEP-Pu vector carrying a 3′-His8 tag. The full-length furin cDNA was amplified from mouse embryonic day 15.5 reverse-transcribed mRNA using the primer pair P185/P186. Additional furin cDNA was ligated into a modified pCEP-Pu vector carrying a 5′-SPARC/BRM40 signal peptide and a 3′-FLAG tag. Site-directed mutagenesis was carried out using the QuikChange II site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions. Amino acids of the two potential furin cleavage sites within non-collagenous domain 1 of collagen XXIII were altered. The primer pair M515/M517 was used to introduce the mutation R86Q (termed A), and the primer pairs M506/M507 and M508/M509 were used to insert the mutations R96S (B1) and R99G (B2), respectively.

**Semiquantitative RT-PCR**—Total RNA was extracted from various tissues and primary cells of newborn and adult C57BL/6J mice using the RNeasy fibrous tissue mini kit (Qiagen Inc.). Aliquots (2 µg) were transcribed into cDNA using random hexamer primers and the enzymes SuperScript II and III (Invitrogen). The relative RT-PCRs were performed in the linear range of amplification. The mouse α1(XXIII) primers M630 and K501 produced a 727-bp product, and the control primers for γ-actin (M542 and M543) produced a 569-bp product. Products were run on agarose gels containing ethidium bromide. On-line quantification of the bands was performed using a Diana III advanced imaging system (Raytest).

**Real-time RT-PCR**—Primers and probes were designed using open source Primer3 web software (frodo.wi.mit.edu/cgi-bin/primer3/primer3 www.cgi) and were evaluated by Blast searches at NCBI (www.ncbi.nlm.nih.gov/blast/). All primers and probes were synthesized by Metabion. For detection and quantification of the PCR assays, the Mx3000P real-time PCR system (Stratagene) was employed. For relative quantification, 18 S rRNA served as an internal control. All primer/probe combinations were designed intron-spanning. The primer/probe combinations RT1-RT2/RTprobe1 for collagen XXIII and RT3-RT4/RTprobe2 for 18 S RNA were used (see the list of PCR primers in supplemental Table S1). The real-time PCR amplification was performed in a final reaction volume of 20 µl containing primers (300 nM) and probe (200 nM) mixed with the appropriate volume of Eppendorf RealMasterMix (Eppendorf AG) and 4 µl of cDNA. The reaction mixture was preheated at 95 °C for 2 min, followed by 45 cycles at 95 °C for 20 s and 60 °C for 1 min. All experiments were evaluated by performing three identical runs. To avoid quantification bias, a standard curve of every assay on every single run was carried out to ascertain the specific amplification efficiency. Briefly, the relative mRNA expression level was determined setting the cycle threshold against the standard curve in each case. Normalization against the internal control 18 S RNA was followed by calculating the case-specific calibrated gene expression. The mean value of normalized gene expression of skin mRNA served as a calibrator.

**Isolation and Cultivation of Cells and Cell Culture-based Assays**—Keratinocytes were isolated from newborn mouse skin and placed in primary culture according to established procedures (22). They were either kept under low calcium conditions to sustain their proliferative potential or treated for 2 h with 2 mM CaCl2-containing medium to induce the formation of cell-cell contacts. Full-length collagen XXIII cDNA (ligated into the pCEP-Pu vector) was used to transfect human embryonic kidney (HEK) 293 cells expressing Epstein-Barr virus nuclear antigen-1 (HEK 293-EBNA cells; Invitrogen) and HT1080 cells using FuGENE 6 reagent (Roche Applied Science) according to the manufacturer’s instructions. The medium used was Dul-
becco’s modified Eagle’s medium (DMEM)/nutrient mixture F-12 with GlutaMAX™ (Invitrogen) containing 10% fetal calf serum (Biochrom AG). Supplements of 250 μM L-ascorbic acid and 450 μM L-ascorbic acid 2-phosphate (Sigma) were added every day. Stable transfectants were selected with puromycin (1.25 μg/ml; Sigma). For shedding assays, the stably transfected cells were incubated in serum-free DMEM/nutrient mixture F-12 supplemented with 250 μM ascorbate, followed by incubation with the indicated chemicals for specified periods. The media and cell lysates were prepared and processed as described below.

**Protein Isolation from Cells and Tissue and Western Blot Analysis**—Protein isolation from tissue and subsequent immunoprecipitation and detection of collagen XXIII by Western blot analysis were performed as described previously (6). For protein extractions from cultured cells, the media and cell layers were processed separately as described (23). The medium was collected on ice; protease inhibitors (1 mM AEBSF and 10 mM EDTA) were added immediately; and cell debris were removed by centrifugation. To the remaining cell layer was added chilled extraction buffer (Tris-buffered saline, 1% Nonidet P-40, 2 mM EDTA, and Complete proteinase inhibitor mixture (Roche Applied Science)), and the sample was incubated for 30 min at 4 °C. The material was then collected using cell scrapers, and insoluble material was removed by centrifugation. The supernatant was precipitated with acetone or methanol/chloroform, and normalized aliquots were run on gels for immunoblotting with the previously described guinea pig anti-collagen XXIII antibody (6). Normalization was accomplished 1) by working with same cell numbers, 2) by determination of the protein content of the cell lysates and application of specified protein amounts and comparable volumes of cell supernatant for analysis, and 3) by analyzing the Western blot signals of cell supernatants with respect to the signals of the corresponding cell lysates. For quantitation, densitometry of signals was done using the Gel-Pro Express program (Media Cybernetics) or by performing on-line signal quantification with a Diana III advanced imaging system.

**Immunofluorescence Staining and Antibody-induced Patching**—Antibody-induced patching was performed as described previously (18). Briefly, HEK 293-EBNA cells cultured on coverslips were cotransfected with full-length α1(XIII) cDNA and PLAP or HTTR cDNA. 24 h after transfection, cells were washed with PBS and incubated with 0.25 mM 3,3′-dii-thiobis(sulfo)succinimidyl propionate) for 30 min at room temperature. Alternatively, cells were treated with 10 mM MβCD prior to cross-linking. The reaction was stopped with 50 mM Tris-HCl (pH 7.5). The cells were lysed (1% Nonidet P-40, 0.1 mM NaCl, 25 mM Tris-HCl (pH 7.4), and inhibitor mixture set III (Calbiochem)) and immunoprecipitated with guinea pig anti-collagen XXIII monoclonal antibodies. The precipitated proteins were eluted from antibody-coupled beads, and the cross-linker was reduced by heating for 10 min at 95 °C in SDS sample buffer containing 50 mM dithiothreitol. Subsequently, the eluates were separated on a 10% SDS-polyacrylamide gel and immunoblotted with guinea pig anti-collagen XXIII polyclonal, anti-PLAP monoclonal (Sigma), and anti-HTTR monoclonal antibodies.

**Cell-surface Biotinylation**—Cell-surface biotinylation was carried out as described by Franzke et al. (2). Briefly, cell-surface proteins were biotinylated with 6.6 mM biotin-X-NHS (Calbiochem) for 5 min at room temperature. After washing five times with PBS, the cells were cultured in fresh medium supplemented with ascorbate. At the desired time points, the media were collected, and cell lysates were prepared as described above. Biotinylated proteins from both the medium and the cell layer extract were precipitated with streptavidin-agarose (Novagen) and immunoblotted for reaction with guinea pig anti-collagen XXIII antibody to detect biotinylated collagen XXIII.

**Quantification of Cellular Cholesterol and Protein Content of Cell Lysates**—To normalize cell lysates for Western blot analysis, the protein content was determined with a bicinotic acid assay kit (Uptima). Cellular cholesterol content was assayed spectrophotometrically using a cholesterol quantitation kit (BioVision, Inc.) following the manufacturer’s instructions.
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RESULTS

Shedding of Collagen XXIII Is Tissue-dependent—We demonstrated previously the tissue distribution of collagen XXIII in mouse skin, kidney, lung, and brain (6). Preliminary indications from immunoblots suggested that the protein is shed in some tissue and full-length in others. Using brain and lung as examples, immunoblot analysis of immunoprecipitated collagen XXIII revealed that the largest (full-length) form of collagen XXIII was predominant in lung, whereas in brain, three additional bands were detected. In the corresponding lanes loaded with collagenase-treated protein, the signal was absent. The arrow indicates the molecular mass of the full-length protein, and there were also signals at the molecular mass of the ectodomain (closed arrowhead) and at an even lower molecular mass (open arrowhead). For comparison, the lysate and conditioned medium of HT1080 cells stably transfected with \( \alpha_1(XXIII) \) cDNA are shown. B, semiquantitative tissue distribution of mouse \( \alpha_1(XXIII) \) mRNA. Fragments of mouse \( \alpha_1(XXIII) \) or \( \gamma \)-actin mRNA were amplified and detected after 31 cycles (in the linear range) for \( \alpha_1(XXIII) \) and after 27 cycles (in the linear range) for \( \gamma \)-actin. col, collagen; E, embryonic day. C, quantitative analysis of collagen XXIII mRNA levels by real-time PCR. The signals obtained were normalized against the internal control 18S RNA, and the relative levels are displayed in comparison with signals in skin.

To study the shedding process in a cell culture system, a collagen XXIII-containing tissue (i.e. skin) (6) with cells that could be easily separated and cultured was utilized for the next set of studies. To our surprise, semiquantitative PCR showed that primary keratinocytes cultured from mouse skin and stimulated for various periods with calcium contained sharply reduced amounts of \( \alpha_1(XXIII) \) mRNA, and cultured dermal fibroblasts gave an mRNA signal that was barely detectable compared with mRNA levels from tissues (Fig. 1B). The drastic down-regulation of \( \alpha_1(XXIII) \) mRNA expression in cultured keratinocytes compared with skin was confirmed by real-time PCR (Fig. 1C). For comparison, real-time PCR was performed with RNA from muscle, a tissue containing only minimal amounts of collagen XXIII mRNA and protein (6). Therefore, to manipulate collagen XXIII in an in vitro culture system, we employed immortalized cell lines (HEK 293-EBNA and HT1080) that we stably transfected with full-length \( \alpha_1(XXIII) \) cDNA.

Subcellular localization of collagen XXIII in the transfected cell lines was analyzed by immunofluorescence staining in comparison with the low expression levels in primary mouse keratinocytes. In keratinocytes cultivated under low calcium conditions and kept at a low passage number, weak signals for collagen XXIII that co-localized with the Golgi marker protein GM130 could be detected (Fig. 2A). Similarly, co-localization was found in the transfected cell lines (Fig. 2, B and C). Also, cell-surface localization of collagen XXIII was detected in non-permeabilized primary keratinocytes that were stimulated with calcium to form cell–cell contacts in non-permeabilized transfected cells, emphasizing detection of the molecule at the cell–cell boundaries (Fig. 2, D and E). Therefore, because the expression pattern of collagen XXIII in transfected cultures is similar to that in keratinocytes, we used transfected cell cultures to study shedding.
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FIGURE 2. Subcellular localization of collagen XXIII in primary mouse keratinocytes and cell lines transfected with α1(XXIII). Primary mouse keratinocytes (A and D), α1(XXIII)-transfected HT1080 cells (B and E), and α1(XXIII)-transfected HEK 293-EBNA cells (C and F) were stained with anti-collagen (col) XXIII polyclonal antibody (red) after (A–C) or prior (D–F) to fixation and solubilization of the cells. Counterstaining (A–C) was performed with antibody against the Golgi marker protein GM130 (green). Merging the images indicated intracellular co-localization (yellow) of collagen XXIII with the Golgi marker (A–C). Staining for collagen XXIII in non-permeabilized cells (D–F) identified its localization at the plasma membrane as well as its concentration at cell-cell boundaries. Scale bars = 20 μm.

FIGURE 3. Effect of protease inhibitors on the release of the collagen XXIII ectodomain. Stably α1(XXIII)-transfected HEK 293-EBNA cells were cultured in serum-free DMEM supplemented with protease inhibitors, including 50 μM decanoyl-Arg-Val-Lys-Arg-cmk (CMK), 50 μM antipain, 45 μM TAPI-0, 100 μM chymostatin, 10 μM E-64, 10 μM leupeptin, 10 μM pepstatin A, 200 μM 1,10-ortho-phenanthroline, 1 mM AEBSF, 200 μM EDTA, and 10 μM aprotinin. Shedding in the presence or absence of inhibitors was analyzed by immunoblotting the precipitated media, followed by semiquantitative densitometry of the signals. The histogram shows the release of the collagen XXIII ectodomain as percent ± S.D. (n = 3) relative to the controls.

Furin/Proprotein Convertases Are the Major Class of Enzymes Responsible for Collagen XXIII Shedding—It has been suggested previously that furin likely plays a role in the processing of collagen XXIII (5). To further explore the enzymes involved in the shedding of collagen XXIII, stably transfected HEK 293-EBNA cells were cultured in the presence of protease inhibitors, and the release of the ectodomain was analyzed by immunoblotting. As shown in Fig. 3, the ectodomain shedding was efficiently inhibited (~95%) only with the furin/proprotein convertase (PC)-specific agent decanoyl-Arg-Val-Lys-Arg-cmk. It was also partially inhibited (~45%) by the broad-range sulfonyl fluoride-type serine protease inhibitor AEBSF. Very minimal inhibition (~5%) was seen with antipain, leupeptin, and chymostatin, three primary serine protease/cysteine protease inhibitors, and with E-64, a cysteine protease inhibitor. No inhibition was observed with the aspartic protease-directed inhibitor pepstatin A, with the metalloprotease inhibitors 1,10-ortho-phenanthroline and EDTA, or with the hydroxamate-derived inhibitor TAPI-0. These last data excluded the potential involvement of matrix metalloproteinases or ADAM proteins in the ectodomain shedding of type XXIII collagen in the cell culture model system.

For cleavage, most PCs require the consensus sequence RX(K/R)R. For processing by furin, the minimal amino acid sequence RXR is sufficient (13, 24). The translated amino acid sequence of mouse collagen XXIII cDNA revealed two potential furin/PC cleavage sites in non-collagenous domain 1: 81LERLLR86 and 94KIRTVR99 (Fig. 4A). Edman degradation sequencing of the purified ectodomain revealed the major cleavage site to be after RTVR. No cleavage was observed in the recombinant protein after the predicted sequence RLLR. Interestingly, a very minor amount of recombinant shed protein commenced with sequence RGDPG, which follows a potential furin cleavage site (RGKPG) in collagenous domain 1. Whether such a cleavage occurs in vivo could not be verified because of the lack of abundance of the shed product isolated from any tissue. Therefore, the cell culture system, being the only tool available to date to study the mechanism of shedding, was used to concentrate on furin cleavage of collagen XXIII. To analyze whether the more upstream furin site is cleaved less frequently or whether there is a cooperative interaction between the two sites that might result in a double cleavage, we altered the recognition sites by site-directed mutagenesis and assessed their influence on shedding. The mutation R86Q (termed A) was introduced into the first potential cleavage site, and the mutations R96S (B1) and R99G (B2) were introduced into the second potential cleavage site (RGKPGR) in collagenous domain 1. The altered recognition sites by site-directed mutagenesis revealed the major cleavage site to be after RTVR. No cleavage was observed in the recombinant protein after the predicted sequence RLLR. Interestingly, a very minor amount of recombinant shed protein commenced with sequence RGDPG, which follows a potential furin cleavage site (RGKPG) in collagenous domain 1. Whether such a cleavage occurs in vivo could not be verified because of the lack of abundance of the shed product isolated from any tissue. Therefore, the cell culture system, being the only tool available to date to study the mechanism of shedding, was used to concentrate on furin cleavage of collagen XXIII. To analyze whether the more upstream furin site is cleaved less frequently or whether there is a cooperative interaction between the two sites that might result in a double cleavage, we altered the recognition sites by site-directed mutagenesis and assessed their influence on shedding. The mutation R86Q (termed A) was introduced into the first potential cleavage site, and the mutations R96S (B1) and R99G (B2) were introduced into the second potential cleavage site (Fig. 4A). The mutated cDNAs were transfected into HT1080 cells for expression. Mutant and wild-type proteins were extracted from the cells and shown to form disulfide-linked trimers by Western blotting of nonreduced SDS-polyacrylamide gels (supplemental Fig.
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A. Schematic depiction of the domain structure of mouse collagen XXIII highlighting the potential furin/PC cleavage sites and displaying the mutations introduced. B, effect of the mutations on the shedding of collagen XXIII. HT1080 cells stably expressing wild-type (wt) collagen (col) XXIII and mutant forms were cultured in serum-free DMEM/nutrient mixture F-12 supplemented with ascorbate. The collagen XXIII content of the cell lysate (L) and conditioned medium (S) was analyzed by Western blotting, followed by densitometric evaluation of the signals. The histogram shows the release of the collagen XXIII ectodomain as percent ± S.D. (n = 3) relative to the signal from the corresponding cell lysates and wild-type controls.

To get an idea whether other PCs are able to compensate for collagen XXIII shedding, A, schematic depiction of the domain structure of mouse collagen XXIII, stability was tested by resistance to trypsin digestion (supplemental Fig. S1B). The results indicated that all mutant proteins and the wild-type triple helical protein have comparable stability. We examined the relative level of the full-length form of collagen XXIII in cell lysates and the shed ectodomain form in cell medium. The B1 and B2 mutants were nearly completely retained on the cell surface as indicated by the strong signal in the conditioned medium (Fig. 4B). In addition, mutation of the first furin/PC cleavage site (RLLR to RLLQ; A2) led to a reduction in shedding and therefore less accumulation of the ectodomain in the conditioned medium (Fig. 4B), although not nearly to the extent caused by the B mutations in the RTVR recognition site.

To get an idea whether other PCs are able to compensate for furin activity, LoVo cells, which are known to lack furin but still contain active PACE4 and PC7 (4, 25, 26), were employed. Upon transfection with full-length α1(XXIII) cDNA, they showed strongly reduced shedding of collagen XXIII compared with other transfected cell lines (supplemental Fig. S2). These data, the enzyme inhibition assays, and the mutation analysis together indicate that collagen XXIII is predominantly but not exclusively processed by furin.

Localization of Collagen XXIII in the Membrane Microenvironment Regulates Shedding—Lipid rafts are small local regions of cholesterol- and sphingolipid-rich plasma membrane. These microdomains can be visualized with antibodies that laterally cross-link microdomain-specific marker proteins. This causes redistribution of the molecules, forming patches on the cell surface (15, 18, 27). Antibody-induced clustering was used to elucidate the membrane microdomain location of collagen XXIII and furin on the cell surface in our cell culture model system. PLAP is a component of lipid rafts, whereas HTrR is localized outside of rafts (15, 28). The cDNAs for these marker proteins (PLAP and HTrR) and α1(XXIII) or furin were transiently cotransfected into HEK 293-EBNA cells, and patching was obtained by antibody cross-linking. Reactions with primary antibodies against 1) collagen XXIII and PLAP, 2) collagen XXIII and HTrR, 3) furin and PLAP, or 4) furin and HTrR, followed by incubation with appropriate secondary antibodies, were performed for 60 min at 12 °C to minimize the metabolic activity of the cells. The antibody-induced clustering showed that collagen XXIII co-localized in patches with PLAP, but segregated from HTrR (Fig. 5A), whereas furin co-localized predominantly in patches with HTrR, but segregated from PLAP (Fig. 5B). For quantitative analysis of the co-patching extent, the localization of individual membrane patches positive for collagen XXIII or furin in relation to PLAP- or HTrR-positive patches was assigned into three categories: co-localization (100% overlap), partial co-localization, and segregation (0% overlap). The percentage of co-localization compared to the percentage of segregation clearly showed co-patching of collagen XXIII with PLAP and co-patching of furin with HTrR (Table 1). This indicates the localization of collagen XXIII mainly in lipid rafts and the localization of furin predominantly outside lipid rafts. To confirm these findings, a second method that avoids the clustering of lipid rafts prior to analysis was employed (29). Cell-surface proteins on HEK 293-EBNA cells transiently cotransfected with α1(XXIII) and marker protein cDNAs were cross-linked with 250 μM 3′,3′-dithiobis(sulfosuccinimidyl propionate). This agent is a reducible, membrane-impermeable, and short-range (1.2 nm) cross-linker. Immunoprecipitation with guinea pig anti-collagen XXIII antibody, followed by immunoblotting with antibodies against the marker proteins, revealed that PLAP, but not HTrR, was in near association with the collagen (Fig. 5C). When the cells were treated with MβCD prior to cross-linking, the co-precipitated PLAP signal was consistently significantly reduced (Fig. 5C). For furin, this method is not applicable, as the necessary high expression levels of the protease seem to be cytotoxic for the cells.

The presence of collagen XXIII in lipid rafts and its main shedding, furin, outside of rafts suggests that furin-mediated shedding is regulated by the plasma membrane microenvironment. To further understand the controlling mechanism,
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MβCD was used to deplete cholesterol from cells stably transfected with α1(XXIII) cDNA. MβCD is not incorporated into the membrane, but contains a central non-polar cavity that binds cholesterol molecules, leading to disintegration of lipid rafts (30). Treatment of HT1080 or HEK 293-EBNA cells with increasing concentrations of MβCD (5–20 mM) for 60 min led to a concentration-dependent reduction in cellular cholesterol levels (Fig. 6A). The highest concentration reduced cholesterol levels by ~50%. The effect of this treatment on the shedding of collagen XXIII was assessed by Western blot analysis of full-length collagen XXIII in cell lysates and ectodomain released into the conditioned medium. MβCD treatment resulted in a dose-dependent enhancement of the release of the collagen XXIII ectodomain from stably transfected HEK 293-EBNA cells (Fig. 6B) as well as from stably transfected HT1080 cells (Fig. 6C). Shedding was increased by 4-fold in transfected HT1080 cells treated with 20 mM MβCD and by 5-fold in transfected HEK 293-EBNA cells treated with 10 mM MβCD. To validate the implication that shedding is mediated by furin, HT1080 cells stably transfected with the B1 mutation of α1(XXIII) cDNA were subjected to MβCD cholesterol depletion (Fig. 6C, lower panel). The shedding of the mutant protein was minor and was seen to be only slightly increased in a dose-dependent manner by MβCD treatment. Further evidence that furin is the major sheddase came from treatment of the stably transfected HEK 293-EBNA cells with the furin-specific inhibitor decanoyl-Arg-Val-Lys-Arg-cmk (50 μM), which lowered the shedding activity in untreated cells by ~60%. The increase in shedding with cholesterol depletion could be attenuated by ~65% in the presence of decanoyl-Arg-Val-Lys-Arg-cmk (Fig. 6D). In addition, the effects of another cholesterol-binding agent, filipin, which acts via a different mechanism, were tested. Filipin, a polyeole macrolide antibiotic, is a sterol-binding agent that interacts with cholesterol in the plasma membrane, destabilizing lipid rafts by interfering with the cholesterol-sphingolipid interaction (31, 32). Stably transfected HT1080 cells expressing full-length α1(XXIII) cDNA were treated with 10–30 μg/ml filipin. This resulted in a concentration-dependent increase in collagen XXIII shedding of up to 3-fold (Fig. 6E). Taken together, these results indicate that lowering the cholesterol content of the membrane, thereby disrupting lipid rafts, significantly enhances the furin-mediated shedding of collagen XXIII.

Golgi-localized Furin Cleave Collagen XXIII—Because collagen XXIII exists in a cell-surface membrane-bound form as well as a soluble shed form and because the cell-surface molecules are at least predominantly protected from furin shedding due to their localization in lipid rafts, we were interested in the dynamics and subcellular localization of the shedding event. To assess the kinetics of shedding from the cell surface, biotinylation of HEK 293-EBNA cells stably expressing full-length collagen XXIII was performed, and biotinylated collagen XXIII was followed in a pulse-chase experiment (Fig. 7). Western blot analysis revealed that the shedding of collagen XXIII from the cell surface occurred slowly. Even after 72 h, biotinylated molecules were detected on the cell surface as assessed by their

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| | Double labeling | Co-localization | Partial co-localization | Segregation |
| | % ± S.D. | % ± S.D. | % ± S.D. |
| Collagen XXIII-PLAP | 57 ± 12 | 24 ± 8 | 19 ± 12 |
| Collagen XXIII-HTrR | 5 ± 4 | 51 ± 9 | 44 ± 10 |
| Furin-PLAP | 6 ± 8 | 43 ± 18 | 51 ± 20 |
| Furin-HTrR | 37 ± 5 | 49 ± 9 | 14 ± 7 |

FIGURE 5. Plasma membrane microdomain localization of collagen XXIII and furin. A, collagen XXIII co-patching with the lipid raft marker protein PLAP and segregation from the non-raft marker HTrR after antibody cross-linking. B, furin co-patching with HTrR and segregation from PLAP after antibody cross-linking. The clustering of proteins was induced by incubation of the cells with the respective combination of anti-HTrR monoclonal antibodies or incubation of anti-PLAP monoclonal antibodies with guinea pig anti-colla- gen XXIII or anti-furin polyclonal antibodies. Scale bars = 0.5 μm. C, co-immunoprecipitation (co-IP) of collagen XXIII with the lipid raft marker protein PLAP after cross-linking of the cell-surface proteins. Cross-linking of cell-surface proteins was performed with the reducible, membrane-impermeable, short-range (1.2 nm) cross-linker 3,3′-dithiobis(sulfosuccinimidyl propionate) (250 μM) for 30 min at room temperature. Expression of the proteins was confirmed by immunoblotting without prior precipitation with anti-collagen XXIII polyclonal antibody. After cross-linking, the lipid raft marker protein PLAP, but not the non-raft marker HTrR, co-precipitated with collagen XXIII, indicating localization in the same membrane microdomain. Alternatively, cells were treated with 10 mM MβCD prior to cross-linking. This resulted in reduced co-precipitation of PLAP.

TABLE 1

Quantification of individual membrane patches positive for collagen XXIII or furin co-patching with the lipid raft marker PLAP or the non-raft lipid marker HTrR.
persistence in the cell lysate. The shed ectodomain of biotinylated collagen XXIII was detectable in the medium only after 24 h and increased in concentration at ensuing time points. Interestingly, biotinylated collagen XXIII from cell lysates, which represents the surface-bound form, appeared as a double band in Western blot analysis, with the lower band having the size of the ectodomain. The ectodomain-sized product at the early time points post-biotinylation is not likely to be the result of cell-surface collagen XXIII being recycling to the Golgi and being cleaved by furin. This form was very abundant at early

FIGURE 6. Cholesterol depletion of the plasma membrane induces the shedding of collagen XXIII. A, alterations in the concentration of HEK 293-EBNA and HT1080 cell plasma membrane cholesterol after 60 min of MβCD treatment. B and C, cholesterol depletion by 0–20 mM MβCD enhances the shedding of wild-type (wt) α1(XXIII) stably transfected into HEK 293-EBNA and HT1080 cells, respectively. In addition, shedding is shown for α1(XXIII) with the B1 mutation stably transfected into HT1080 cells (C). col, collagen; L, cell lysate; S, conditioned medium. D, inhibition of furin decreases collagen XXIII shedding in the presence of the lipid raft disruptor, MβCD. HEK 293-EBNA cells stably transfected with α1(XXIII) cDNA were treated with 10 mM MβCD in combination with the furin-specific protease inhibitor decanoyl-Arg-Val-Lys-Arg-cmk (CMK; 50 mM). E, stimulation of collagen XXIII shedding in stably transfected HT1080 cells by treatment with 0–30 μg/ml filipin. Cells were incubated with MβCD or filipin for 60 min, and the soluble collagen XXIII ectodomain from the culture medium and cellular collagen XXIII were analyzed by immunoblotting with guinea pig anti-collagen XXIII polyclonal antibody. After densitometric evaluation of the signals, histograms show the release of the collagen XXIII ectodomain as percent ± S.D. (n = 3) relative to the signal in corresponding cell lysates.
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FIGURE 7. Time dependence of collagen XXIII ectodomain shedding. HEK 293-EBNA cells stably expressing full-length collagen XXIII were surface-biotinylated, chased with biotin-free medium, and cultured for 72 h. Aliquots of the conditioned medium and cell extract were precipitated with streptavidin-agarose and analyzed by Western blotting with guinea pig anti-collagen XXIII polyclonal antibody.

time points, and one would expect that not all of it could be captured at the cell surface; some would have to appear in the early supernatant time points. Although we expect that recycling does occur, the abundance of the ectodomain-sized product in the cell lysate suggests that the shedding event is derived from full-length collagen XXIII cleaved at the surface of the cell, secreted at the cell surface by some unidentified molecule, or that the shed collagen is not compelled to clip all three chains of a trimeric collagen XXIII molecule at once. If the latter, just one of the three chains in the triple helix molecule, if not clipped by furin (thus retaining its transmembrane domain), would be sufficient to serve as a membrane anchor for the other two α-chains participating in the triple helix, holding them at the cell surface. Whichever the case, it is clear that clipped chains are retained. Taken together, these results indicate that transmembrane collagen XXIII resides on the cell surface with a half-life on the order of days.

To evaluate the extent of furin activity that takes place in the Golgi, HEK 293-EBNA cells stably transfected with α1(XXIII) cDNA were treated with brefeldin A, a macrolide lactone that inhibits vesicle transport from the endoplasmic reticulum to the Golgi and that leads to disassembly of Golgi stacks (33, 34). Western blot analysis after treatment of the cells for 5 h with brefeldin A revealed a sharp decrease in the collagen XXIII ectodomain in the supernatant compared with untreated cells, suggesting that much of collagen XXIII is normally cleaved in the Golgi. In HEK 293-EBNA cells, brefeldin A caused shedding to be reduced by ~95% and caused an increase in full-length collagen XXIII detected in the cell lysate (Fig. 8, A and B). In contrast, if only cell-surface collagen XXIII was assessed (using surface biotinylation), brefeldin A treatment had a less drastic effect, decreasing shedding by ~50% in HEK 293-EBNA cells (Fig. 8C). Comparable results were obtained with stably transfected HT1080 cells (data not shown). Thus, cleavage of collagen XXIII by furin occurs predominantly intracellularly. To evaluate the extracellular shedding of collagen XXIII after it is deposited into the plasma membrane, the membrane-impermeable furin inhibitor α1-PDX (a bioengineered variant of α1-antitrypsin) was employed (35, 36). Biotinylated proteins on HEK 293-EBNA cells were incubated for 6 h in presence of 8 μM α1-PDX and showed a 45% reduction in shedding. Additionally, an increase in biotinylated collagen XXIII molecules in the cell lysate was observed compared with untreated cells (Fig. 9).

In additional experiments, the effect of cholesterol depletion specifically on the cell-surface collagen XXIII molecules was analyzed. Cell-surface biotinylation and cholesterol depletion could not be combined due to the fact that, after 1 h of treatment, insufficient biotinylated ectodomain for streptavidin-agarose precipitation was accumulated in the supernatant and because prolonged treatment with cholesterol-depleting agents adversely affected cell survival. Therefore, co-treatment with brefeldin A and the cholesterol-depleting agent MβCD was employed. As already described, single treatment of HEK 293-EBNA stably expressing collagen XXIII with brefeldin A for 5 h led to a strong increase in the shedding of collagen XXIII. Like-
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alpha1(XXIII)-transfected HT1080 cells (data not shown). These findings indicate that, in particular, the shedding of collagen XXIII localized at the cell surface is altered by a cholesterol-dependent mechanism.

To conclude, the shedding of collagen XXIII predominantly occurs intracellularly. Collagen XXIII molecules that escape intracellular shedding are present at the cell surface. These molecules are proteolytically processed slowly because of their protection inside lipid rafts.

DISCUSSION

Release of an ectodomain by selective proteolysis has turned out to be a common feature of type II transmembrane collagens (3, 5, 23, 37). Previous work suggested that the shedding of collagen XXIII is not pervasive in skin and kidney (6), but is in prostate cancer cells (5). We have shown here that collagen XXIII is shed by cells in culture, by cells in brain tissue, and to a minor degree by cells in lung. Every transmembrane collagen known to date contains potential furin convertase recognition sites, but only collagens XIII, XXIII, and XXV have been shown to be directly processed by furin (this work and Refs. 3 and 4). For collagen XVII, processing by TACE was demonstrated. However, furin is likely to play a role in a protease cascade leading to activation of the sheddase (2).

Collagen XXIII contains what we consider to be two biologically relevant furin/PC recognition sequences. A consensus site in collagenous domain 1 would participate in a triple helical structure and would likely not be available for furin cleavage. For furin cleavage, an arginine is required that defines position 1, and an arginine downstream is required in position 4. Additional basic residues in positions 2 and 6 considerably enhance cleavage by furin (13). In fact, furin is the only PC that is capable of recognizing basic residues at position 6 (24). Other PCs such as PC1 and PC2 require the basic residue at position 2. In collagen XXIII, the most N-terminal potential furin/PC cleavage site contains no additional basic residues, but the more downstream C-terminal cleavage site has a lysine at position 6. Edman degradation of the purified recombinantly expressed ectodomain revealed that the downstream site is the major location for processing of collagen XXIII. Inhibition studies revealed that primary serine proteases or cysteine protease are capable of producing 5% or less of the shed ectodomain. Aspartate proteases and metalloproteases (responsible for collagen XVII shedding) (2) are not involved in processing collagen XXIII. Effective inhibition of shedding is observed only with AEBSF, a broad-range sulfonamide-type serine proteinase inhibitor, and with decanoyl-Arg-Val-Lys-Arg-cmk, a PC-specific protease inhibitor (38). Even though furin itself is a serine protease, it is relatively insensitive to serine protease inhibitors (38), and thus, it is influenced only by AEBSF and not by primary serine protease inhibitors. Although Edman degradation indicated that the more C-terminal furin cleavage site was used, it could not reveal whether processing occurred in steps, such as a first cleavage at the most N-terminal site, followed by a cleavage at the downstream site. Mutation of one arginine in the more N-terminal site decreased shedding, but to a minor degree, suggesting there is not a specific temporal sequence to the processing event. Processing at the most N-terminal site is likely to be less efficient because of the lack of additional basic residues beyond the minimally required arginines. Mutation of the two arginines in the downstream furin cleavage site drastically reduced shedding. Any minor shedding activity observed in this mutant molecule most likely represents cleavage at the upstream, less used furin cleavage site or at a site cleaved infrequently by a primary serine protease or cysteine protease. Processing of collagen XXIII by PCs other than furin is suggested by studies with LoVo cells, which lack furin but still contain active PACE4 and PC7 (4, 24, 25). In these cells, processing of collagen XXIII is strongly reduced, but is not completely absent. In further support of the downstream furin cleavage site as being the major location for furin-mediated shedding, the cDNAs of all species analyzed to date show a strong conservation of the more C-terminal collagen XXIII furin cleavage site, whereas the more N-terminal site is not as conserved. Also, the reported furin cleavage sites in transmembrane collagens XIII and XXV (3, 37)
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demonstrate strong homology to the downstream collagen XXIII furin site. Taken together, these data indicate that furin is the major protease to process collagen XXIII and that the processing occurs after the downstream recognition motif (**KIRTVR**), releasing the ectodomain.

The release of an ectodomain is influenced by the spatial organization of a transmembrane molecule and its particular sheddase within the plasma membrane lipid microenvironment. For example, the β-secretase localized in lipid rafts is able to process the Alzheimer β-amyloid precursor protein that is localized within lipid rafts, but not the precursor protein molecules outside of rafts (15). Transmembrane collagen XVII is localized inside lipid rafts and is therefore less accessible to its sheddase, TACE, which is outside the rafts (18). The activated form of furin is present in the Golgi and the TGN and on the cell surface (7, 39). The fact that processing by cell-surface furin increases the pathogenicity of anthrax toxin PA and *Clostridium* α-toxin highlights the biological relevance of this sheddase (8, 9). Here we used co-patching immunofluorescence analysis of non-permeabilized cells to reveal the plasma membrane microdomain localization of furin and collagen XXIII on the cell surface. Collagen XXIII co-localized with the lipid raft marker PLAP, whereas furin co-localized predominantly with the non-lipid raft marker HTR. To confirm the lipid raft localization of collagen XXIII, we applied a cross-linking technique (29) and avoided using the previously widely used, but currently disputed method of cold Triton extraction for lipid raft preparation (40, 41). Cross-linking of collagen XXIII with the lipid raft marker protein PLAP in non-clustered rafts supported the immunofluorescence results.

The influence of the membrane microenvironment on shedding was demonstrated by disintegration of lipid rafts with MβCD and by hampering lipid raft formation. These cholesterol disturbances led to an increased release of the collagen XXIII ectodomain from the cell surface, presumably by facilitating contact between furin and collagen XXIII, thereby dysregulating natural controls. In agreement with the previous observations for other lipid raft-localized transmembrane proteins (16, 18), a small decrease in cell-surface cholesterol led to a significant enhancement of shedding. Both treatment with the furin inhibitor decanoyl-Arg-Val-Lys-Arg-cmk and mutations in the downstream furin cleavage site inhibited shedding in untreated as well as cholesterol-depleted cells. This indicates that collagen XXIII shedding induced by cholesterol reduction is due to furin action and not to secondary effects. Therefore, the data suggest a model in which cell-surface furin is localized primarily outside lipid rafts, whereas collagen XXIII is located mainly within lipid rafts, inaccessible to furin processing. Upon disruption of lipid rafts, collagen XXIII molecules become accessible to furin, and an increased release of collagen XXIII ectodomain is seen.

Despite the evidence that transmembrane collagen shedding is influenced by modulating membrane cholesterol levels (this work and Refs. 18 and 42), little is known about the dynamics and subcellular localization of the shedding event. Pulse-chase analyses suggested that the shedding of cell-surface collagen XXIII is a limited proteolysis occurring rather slowly, with the half-life of the full-length protein being in the range of days.

This implies that the full-length protein localized on the plasma membrane has a biological function. Moreover, disassembly of the Golgi suggested that a population of collagen XXIII molecules is shed while passing through the secretory pathway and that another population reaches the cell surface as full-length molecules (summarized in the scheme in Fig. 11). Experiments with the membrane-impermeable furin inhibitor α1-PDX confirmed our previous conclusion (6) that shedding at the cell surface does occur, but only at a low rate. Minor shedding is observed at the cell surface, perhaps because a small amount of collagen XXIII or furin molecules may be unrestricted in the plasma membrane subcompartments or because of the activity of the primary serine or cysteine proteases, which have a minimal effect on collagen XXIII. We favor the former idea because the raft/non-raft phase association of membrane proteins is a dynamic process and because lipid rafts themselves are dynamic entities within the cell membrane (43, 44). The effect of Golgi disassembly on cell surface-biotinylated collagen XXIII molecules hints at the possibility that cell-surface collagen XXIII molecules may be internalized to the Golgi/TGN compartment for processing or reinsertion into the cell membrane. This dynamic scenario is consistent with previous findings for other lipid raft-localized proteins (45). Discrimination between ectodomain shedding on the cell surface regulated by recycling to the Golgi/TGN versus that by only lipid raft manipulation is further supported by the fact that cholesterol depletion in the presence of brefeldin A strongly increased the shedding of collagen XXIII.

With the use of one transmembrane molecule, the cell may economically adjust its phenotype 1) by influencing the amount of collagen XXIII shedding in the secretory pathway and 2) either by modulating its lipid raft composition or by recycling to favor or disfavor the shedding of cell-surface collagen XXIII (Fig. 11). The importance of a highly regulated shedding process influencing the cellular phenotype is further highlighted by the observation that cell surface-localized collagen XXIII was concentrated at sites of cell-cell contact (Fig. 2D), where it can
interact with integrin $\alpha_2\beta_1$. Sup-regulation of shedding would enable the cell to liberate any collagen XXIII-mediated cell-cell adhesion and could positively influence the migratory behavior of the cell. Such action could play an important role in effective wound healing. In addition, such action could, in turn, affect neighboring cells, as the soluble ectodomain could act as a competitor for integrin $\alpha_2\beta_1$ binding. Similar observations were made for regulated release of soluble E-cadherin, which, after shedding by ADAM10, decreases cell-cell adhesion and increases migration (46).

The importance of furin cleavage to the function of collagen XXIII is not yet known. However, furin-mediated shedding in vivo has been shown to be vital in at least one instance involving a collagen-like transmembrane molecule: mutations within the furin consensus sequence of transmembrane collagen-related ectodysplasin A impede shedding and cause the X-linked disorder hypohidrotic ectodermal dysplasia (12, 47). Like collagen XXIII, ectodysplasin A is present as both a full-length plasma membrane-localized form and a soluble ectodomain form in cell culture. Mutation of the furin consensus sequence results in abrogation of ectodysplasin A receptor-mediated signaling (48). Collagen XXIII is newly described, and thus, no human mutations have yet been associated with it. It is tempting, however, to speculate that alterations of the furin cleavage site will be found and that preventing the release of the ectodomain will have pathological consequences.

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