Modulation of the Cellular Cholesterol Level Affects Shedding of the Type XIII Collagen Ectodomain*

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Type XIII collagen is a transmembrane protein that also exists as a soluble extracellular variant because of ectodomain shedding by proprotein convertases. Because ectodomain shedding in a growing number of transmembrane proteins has recently been shown to be dependent on their localization in cholesterol-enriched detergent-resistant membrane microdomains, this work aimed at analyzing this aspect of type XIII collagen ectodomain processing. In HT–1080 cells type XIII collagen and its cleaving proprotein convertase furin localized partially in detergent-resistant cholesterol-containing membrane microdomains. Disruption of these domains by lowering either the level or availability of the cellular cholesterol reduced ectodomain shedding, implying that, in such membrane domains correct cholesterol level is important for the regulation of type XIII collagen ectodomain processing. In addition, we show here that ectodomain of type XIII collagen is also shed intracellularly. HT–1080 cells released vesicles from the Golgi apparatus, which contained only the cleaved variant. Intracellular processing and the subsequent entry of the cleaved ectodomain into the vesicles was totally blocked by inhibition of the proprotein convertase function by cell-permeable chloromethylketone, but not with cell-impermeable H9251/antitrypsin Portland. This supports the hypothesis of type XIII collagen ectodomain also being cleaved intracellularly in the Golgi and suggests that the intracellular cleavage may act as a gating event in the vesicle-mediated ectodomain secretion.

Membrane lipid rafts are envisaged as dynamic cholesterol- and sphingolipid-enriched microdomains of various biological bilayer membranes of the cell, e.g. in the plasma membrane, the secretory pathway, and the endocytic compartments (1–4). These domains have been proposed to act as “hot spots” of signal transduction, but other presumed functions include membrane trafficking of proteins, participation in cell adhesion, cytoskeletal organization, and pathogen entry as well as acting as lateral sorting platforms for various membrane proteins that associate with them (1, 4–8). Cholesterol is considered to play a central role in maintaining the raft structures by keeping them in a tightly packed liquid-ordered phase. It thus follows that changes in the cholesterol content are likely to affect the raft function (2, 3, 6). The high lipid content makes these microdomains poorly dissolvable in non-ionic detergents, which quality is utilized in partitioning of the biological membranes into detergent-resistant versus detergent-soluble membrane fractions when defining association of a given protein with these membrane microdomains. Based on the differential solubility to various detergents, membrane microdomains have been proposed to make a heterogenous group as far as their composition is concerned (3–5, 9, 10).

Controlled cleavage of ectodomains of membrane-spanning proteins is an important regulatory process whereby a cell can optimize its plasma membrane protein content and, in response to external stimuli, produce functionally active cleavage products in the neighboring microenvironment (11, 12). Lately, increasing attention has been paid to ectodomain shedding in relation to the detergent-resistant membrane microdomains (DRMs). In fact, in a growing number of transmembrane proteins, ectodomain cleavage is now known to be related to the localization of these proteins in DRMs and to show sensitivity to cholesterol deprivation (13–18). Type XIII collagen (COLXIII), belonging to the subfamily of transmembrane collagens, was initially found as a membrane-bound variant in focal adhesions on plasma membranes (19, 20). In addition to this integral membrane localization, the long ectodomain of COLXIII is known to be enzymatically cleaved by proprotein convertases at the cell surface (21, 22) to become a soluble molecule with specific effects on cell adhesion, spreading, proliferation, migration, and matrix assembly (22–24). In this study we show that the shedding of COLXIII ectodomain is dependent on the partial localization of COLXIII in the lipid-enriched membrane microdomains. Also, we show here that in addition to the cell surface, the ectodomain of COLXIII is also processed intracellularly, supporting our earlier hypothesis of dual cleavage sites.

MATERIALS AND METHODS

Chemicals and Antibodies—Methyl-β-cyclodextrin (MCD), cholesterol, filipin, Lovastatin, mevalonate, phorbol 12-myristate 13-acetate (PMA), Triton X-100 (TX-100), Triton X-114

‡1 The abbreviations used are: DRM, detergent-resistant membrane microdomain; COLXIII, type XIII collagen; MCD, methyl-β-cyclodextrin; PMA, phorbol 12-myristate 13-acetate; TX-100, Triton X-100; TX-114, Triton X-114; CHAPS, 3-[3-cholamidopropyl]dimethy lammonium]–1-propanesulfonic acid; BFA, brefeldin A; α1-PDX, α1-antitrypsin Portland; CMK, chloromethylketone; MES, 4-morpholineethanesulfonic acid; PBS, phosphate-buffered saline.

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Cholesterol Dependence of COLXIII Ectodomain Shedding

(TX-114), CHAPS, Brij96, Brij35, and brefeldin A (BFA) were purchased from Sigma. Fatty acid-free bovine serum albumin was from Serva, α1-antitrypsin Portland (α1-PDX) was from Calbiochem, and decanoyl-Arg-Val-Lys-Arg-chloromethylketone (CMK) was from Bachem. The cell surface biotinylation kit was from Molecular Probes. The kit for determining cellular cholesterol content, aprotinin, and leupeptin were purchased from Roche Applied Science, the Complete™ EDTA-free protease inhibitor kit was from Santa Cruz Biotechnology Inc. Anti-caveolin-1, anti-GM130, and anti-calreticulin (CD71) antibodies were purchased from Santa Cruz Biotechnology Inc. Anti-COLXIII antibody has been described elsewhere (25–28). Protein concentrations were determined using a BCA protein assay kit from Pierce. Anti-COLXIII antibody has been described before (19). Anti-furin, anti-Lyn, and anti-transferrin receptor (CD71) antibodies were purchased from Santa Cruz Biotechnology Inc. Anti-caveolin-1, anti-GM130, and anti-calnexin antibodies were from BD Transduction Laboratories, and anti-Rab5 antibody was from Abcam.

Cell Culture—HT-1080 cells were grown in Dulbecco’s modified Eagle’s medium (Biochrom KG) supplemented with 10% fetal bovine serum (EuroClone).

Analysis of Membrane Microdomains by Density Gradient Ultracentrifugation—Density gradient separation of the membrane microdomains has been described elsewhere (25–28). In each experiment, equal numbers of cells were detached with 5 mM EDTA, resuspended in 25 mM MES, pH 6.0, with 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride supplemented with Complete™ EDTA-free inhibitor mix and either 1% TX-100, 1% CHAPS, 1% Brij96, or 1% Brij35 as a detergent, and kept on ice for 45 min. The cell samples without detergents were resuspended in 150 mM Na2CO3, pH 11, containing 2 mM EDTA, 25 μg/ml aprotinin, 25 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride (29). The cell suspensions were homogenized, sonicated (detergent-free samples), and sheared by passing through hypodermic needles. Equal volumes were mixed with lysis buffer containing 90% sucrose. The resulting 2-ml 45% sucrose layer was overlaid with 2 ml of 35% sucrose, 1 ml of 5% sucrose in an appropriate lysis buffer. The gradients were centrifuged at 150,000 × g for 22 h at +4 °C in a Beckman SW50.1 rotor. All of the steps were carried out at +4 °C. Fractions of 500 μl were collected from the top of the gradient and boiled after the addition of reducing sample buffer and β-mercaptoethanol. Western blotting with equal sample volumes was performed on 8% (COLXIII, furin, CD71) or 12% (caveolin-1, Lyn) SDS-PAGE gels. In additional experiments the cells were treated for 30 min with 20 mM MCD prior to membrane fractionation with various detergents. The unspecific effects of MCD were excluded by treatment of cells with 20 mM MCD-cholesterol complex (1:1) (30).

Whole Cell Shedding Assay—Equal numbers of HT-1080 cells were cultured in serum-free medium supplemented with MCD, MCD-cholesterol complex (1:1) (30), filipin, or Lovastatin-mevalonate-MCD at concentrations presented in Fig. 3 for 30 min. The Lovastatin-mevalonate-MCD treatment was preceded by preincubation of the cells with Lovastatin (1 or 4 μM, depending on the assay) and 0.25 mM mevalonate in 2% fatty acid-free bovine serum albumin-Dulbecco’s modified Eagle’s medium for 24 h. After overnight shedding, the cleaved ectodomain was precipitated from the medium at −20 °C by adding an equal volume of ice-cold methanol, collected by centrifugation, resuspended in a reducing sample buffer, and analyzed by Western blotting (22).

Determination of Cellular Cholesterol Content—The procedure for the determination of cellular cholesterol has been described elsewhere (31, 32). 3 × 106 cells were taken for the isolation of cellular cholesterol in each assay. They were disrupted by three freeze-thaw cycles followed by sonication, and cholesterol was extracted from the lysates with chloroform and methanol by rotation for 15 min at room temperature. After centrifugation, the organic solvent was evaporated under a nitrogen flow. The residual cholesterol was dissolved in 100 μl of ethanol, of which 40 μl was used for the determination of cholesterol content by means of the enzymatic colorimetric test (Roche Applied Science).

Filipin Staining of Cultured Cells—The immunofluorescent staining method has been described elsewhere (33, 34). The cells were grown on coverslips; treated with 5 mM MCD, 5 mM MCD-cholesterol complex (1:1), or a combination of 2 μM Lovastatin, 0.25 mM mevalonate, and 5 mM MCD for 30 min; rinsed with PBS; fixed with 4% paraformaldehyde in PBS for 30 min on ice; and washed with PBS. The cellular cholesterol was stained with 125 μg/ml filipin in PBS for 15 min at room temperature in the dark. The cells were mounted with ImmuMount™.

Cell Surface Biotinylation and Streptavidin Pull-down Assays—Cell surface biotinylation was performed for equal cell numbers in every experiment. After treatment with either 5 mM MCD, 100 ng/ml PMA, 100 μM CMK, or 15 μg/ml α1-PDX, the media were centrifuged and subjected to streptavidin pull-down with avidin-conjugated protein A-Sepharose as described elsewhere (22, 35, 36). The residual media were precipitated with ice-cold methanol. The samples were dissolved in reducing sample buffer and analyzed by Western blotting.

Detection of Cell Surface Furin—Cell surface furin was detected using immunopatching technique. Subconfluent HT-1080 cell cultures were washed with ice-cold PBS and incubated with anti-furin antibody for 45 min at +4 °C to prevent internalization. After thorough washing with PBS, patching was performed by incubating cells with anti-rabbit-Cy2 secondary antibody for 30 min again at +4 °C. Fractions of 500 μl were collected from the top of the gradient and boiled after the addition of reducing sample buffer and β-mercaptoethanol. Western blotting with equal sample volumes was performed on 8% (COLXIII, furin, CD71) or 12% (caveolin-1, Lyn) SDS-PAGE gels. In additional experiments the cells were treated for 30 min with 20 mM MCD prior to membrane fractionation with various detergents. The unspecific effects of MCD were excluded by treatment of cells with 20 mM MCD-cholesterol complex (1:1) (30).

Vesicle Secretion Assays—Recovery and analysis of the secreted vesicles have been described elsewhere (37). Equal numbers of cells were first either biotinylated or left unbiotinylated, depending on the assay. The cells were treated with either 5 mM MCD, 100 ng/ml PMA, 100 μM CMK, or 15 μg/ml α1-PDX. Detached cells were separated by centrifugation, after which the collected media were centrifuged in a Beckman Ti55.2 rotor at 74,000 × g for 3 h. The vesicle pellets were collected from the top of the gradient and analyzed by Western blotting.
were recovered in 150 mM NaCl, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1% TX-100, and 20 mM Tris, pH 7.6. In the case of brefniylated cells, the vesicle suspensions were directed to the streptavidin pull-down. The cell lysates were produced by scraping the cells from the plates and suspending them in the lysis buffer. The samples were analyzed by Western blotting.

**TX-114 Phase Partitioning** — TX-114 phase partitioning has been described elsewhere (38, 39). In short, culture medium was centrifuged to remove detached cells and chilled to +4 °C. TX-114 was added and dissolved at +4 °C to a final concentration of 1% by agitation. Detergent was condensed at +37 °C for 15 min followed by centrifugation at 1700 × g for 20 min; the aqueous phase was removed and cooled; and partitioning was repeated with an addition of fresh TX-114. The aqueous and detergent phases were pooled and precipitated at −20 °C with equal volumes of ice-cold methanol or acetone, respectively. Precipitated proteins were dissolved in a reducing sample buffer and analyzed by Western blotting.

**Cellular Fractionation** — The methods employed for cellular subfractionation and sucrose density gradient fractionation have been described elsewhere (40–42). All of the procedures were carried out at +4 °C. When treated with BFA, the cells were preincubated in 30 μM BFA for 6 h prior to fractionation. For the subcellular fractionation, the cells were homogenized in 0.25 M sucrose, 5 mM MgCl2, and 25 mM Hepes, pH 7.3, with a glass-Teflon homogenizer and centrifuged in a Sorvall SS-34 rotor at 10,000 × g for 10 min. The postnuclear supernatant was centrifuged with a Beckman Ti55.2 rotor at 100,000 × g for 45 min. The total membrane pellet was resuspended in the homogenization buffer, the sucrose content of which was adjusted with 2 M sucrose stock solution to 1.25 M, equivalent to a refractive index of 1.395. This was centrifuged in a discontinuous sucrose gradient in a Beckman SW40 rotor at 100,000 × g for 2.5 h. The Golgi and smooth endoplasmic reticulum (ER) fractions were defined as materials at the 0.25/1.1 M and 1.1/1.25 M interfaces, respectively. The microsomal fraction in the pellet representing the rough ER was resuspended in the homogenization buffer. The Golgi fraction was diluted to a sucrose concentration of 0.25 M and pelleted by centrifugation in a Beckman Ti55.2 rotor at 100,000 × g for 45 min. The pellet was incubated in 10 mM EGTA for 30 min on ice and centrifuged as before. The supernatant fraction represented the peripheral Golgi proteins. The pellet was resuspended in 150 mM Na2CO3, pH 11.5, incubated on ice for 30 min and centrifuged as before. The supernatant contained the luminal Golgi proteins, and the pellet contained the Golgi membranes. For the sucrose density gradient fractionation, the cells were homogenized in 0.25 M sucrose, 10 mM magnesium acetate, and 10 mM Tris, pH 7.4, and centrifuged at 620 × g for 10 min. The postnuclear supernatant was centrifuged in a discontinuous sucrose gradient in a Beckman Ti55.2 rotor at 74000 × g for 2.5 h. Fractions of 1 ml were collected from the top of the gradient, precipitated at −20 °C with an equal volume of ice-cold methanol, resuspended in reducing sample buffer and β-mercaptoethanol, and boiled. All of the samples and fractions were analyzed by Western blotting on 8% (COLXIII, GM130, calnexin) or 12% (Rab-5) SDS-PAGE gels.

**RESULTS**

**COLXIII in the Membrane Microdomains** — We used detergent extraction and isopycnic density gradient centrifugation to study whether COLXIII is associated with DRMs. In detergent-free high pH/carbonate conditions membrane-bound COLXIII and furin and of the proprotein convertases were found to subfractionate partially into the lipid-enriched light density membrane fractions, positive for caveolin-1 and Src kinase Lyn, and enriched with cholesterol (Figs. 1 and 2A). However, because detergent-free conditions are regarded as nonselective for isolating membrane microdomains and because resistance to solubilization in non-ionic detergents at +4 °C is considered indicative of an association of a given protein with DRMs, various detergents were tested. Detergents are recognized to vary in their ability of solubilizing membrane proteins and of enriching cholesterol and glycerophospholipids (43). Extracted with TX-100, caveolin-1 and the noncaveosomal Src kinase Lyn, both used as raft markers (10), floated in the low density fractions 2 and 3, showing enrichment of cholesterol and low cellular protein content. However, the association of COLXIII and furin with DRMs did not withstand extraction by cold TX-100 (Figs. 1 and 2B). A flotation difference between COLXIII and Lyn/caveolin-1 was observed when extracted with detergents Brij96 and CHAPS. In CHAPS, a significant amount of Lyn was found in fractions...
of the lowest density, which also contained the highest amount of cholesterol, whereas COLXIII, furin and caveolin-1 were found to be present in the intermediate density fractions 3 and 4 (Figs. 1 and 2C). Also, when extracted with Brij96, Lyn and caveolin-1 subfractionated exclusively in the low density fractions with a peak concentration of cholesterol (Figs. 1 and 2D), whereas COLXIII and furin were present in the intermediate density fractions 3–5. In less selective Brij35, COLXIII, furin, caveolin-1, and Lyn all resided in the same fractions 3 and 4, containing the highest cholesterol content (Figs. 1 and 2E). In all of the extractions, transferrin receptor (CD71), used as a nonraft marker, was correctly subfractionated in the low cholesterol/high protein membrane fractions of the highest density (25, 28, 44).

Cholesterol is required for the stabilization of the structural integrity of membrane microdomains. Depletion of cellular cholesterol leads to disintegration of these domains and subsequent loss of associated proteins. Hence, sensitivity to cholesterol deprivation leading to solubilization from the light density fractions suggests association of a given protein with cholesterol-enriched microdomains (25). We first depleted HT-1080 cells of the cellular cholesterol with MCD, followed by extraction of the membrane fractions in either detergent-free conditions or with CHAPS, Brij96, or Brij35. In all cases, the association of COLXIII with the DRMs was lost or greatly reduced. Most prominent with Brij96 and CHAPS, cholesterol depletion caused COLXIII to disappear from the light density fractions totally, and especially with Brij96, furin was also solubilized from the light density fractions. In Brij35, only remnants of COLXIII remained in the light density fractions (Fig. 1). As a control, replenishment of the membrane cholesterol with the MCD-cholesterol complex restored COLXIII in the light density fractions (data not shown).

Sensitivity of COLXIII Ectodomain Shedding to Cholesterol Deprivation—Next we studied whether the ectodomain shedding of COLXIII is dependent on the partial localization of COLXIII and furin in the DRMs. This was determined in a whole cell shedding assay following disruption of the raft structures by manipulation of the cellular cholesterol level. The methods most frequently used to achieve this include lowering of the cholesterol content by sequestration with 

FIGURE 2. Determination of cholesterol and total protein contents of density gradient fractions. Equal numbers of HT-1080 cells were taken for the determination of the cholesterol/total protein ratios of fractions from the isopycnic density centrifugation following various detergent treatments. A, detergent-free (high pH/carbonate); B, 1% TX-100; C, 1% CHAPS; D, 1% Brij96; E, 1% Brij35. A high cholesterol/protein ratio was shown for the light/intermediate density fractions (fractions 1–4), whereas cellular proteins were concentrated in the high density fractions of the gradient (fractions 7–10). The protein content is shown as absorbance value A$540_{nm}$. Cholesterol is shown as percentage values where the fraction with the highest amount of cholesterol is marked as 100%.
HT-1080 cells (Fig. 3C, right panel), but it did reduce COLXIII ectodomain shedding by up to 50% in a dose-dependent manner (Fig. 3C, left panel). These two mechanistically dissimilar treatments resulting in compromised availability of membrane cholesterol for raft formation caused the same eventual effect on COLXIII ectodomain shedding. To achieve a more robust decrease in the cellular cholesterol level, HT-1080 cells were treated with a combination of MCD, Lovastatin, and mevalonate. Lovastatin inhibits the de novo biosynthesis of cholesterol whereas mevalonate shunts the synthesis to a nonsterol pathway (14, 33, 34). Using 4 μM Lovastatin and 0.25 mM mevalonate with increasing concentrations of MCD, the cellular cholesterol level was reduced by up to 80% (Fig. 3D, right panel), and this treatment also reduced COLXIII ectodomain shedding by up to about 75% in a dose-dependent manner (Fig. 3D, left panel). However, because the cells with the lowest cholesterol level obtained with this treatment exhibited initial signs of reduced viability, the experiment was repeated with a milder treatment to ascertain that the observed effect did not result from the reduced cell viability as such. With 2 μM Lovastatin, 0.25 mM mevalonate, and somewhat decreased MCD concentrations, a clear reduction in the cholesterol level was achieved (Fig. 3E, right panel), as also seen in the reduced filipin staining intensity (Fig. 4D). Concomitantly, COLXIII ectodomain shedding was reduced (Fig. 3E, left panel), only this time without any signs of adverse cellular effects.

The results thus far suggest that the ectodomain shedding of COLXIII is dependent on the partial localization of COLXIII in the DMVs. Earlier we have shown that PMA can up-regulate COLXIII ectodomain shedding (22). To test whether this
induction by PMA is related to the partial localization of COLXIII in the DRMs, the cellular cholesterol levels of PMA-stimulated HT-1080 cells were manipulated with either MCD or filipin. Resulting from either of the deprivation schemes, the ectodomain shedding was clearly decreased (Fig. 5). We considered the possibility that upon PMA stimulation, COLXIII and/or furin might be stimulated to move laterally on the plasma membrane from the nonraft regions preferentially to the raft regions where the shedding takes place. However, no such lateral redistribution was found in the sucrose gradients following PMA stimulation, regardless of the detergent used (data not shown). Obviously, the clarification of the detailed mechanism of PMA stimulation warrants further studies.

Part of COLXIII Ectodomain Shedding Occurs via the Action of Cell Surface-bound Furin—Furin is mainly localized in the Golgi apparatus, but because of intracellular reshuffling, it is also known to exist at the cell surface (46). To verify the presence of furin on the plasma membrane of HT-1080 cells, we performed immunopatching and cell surface biotinylation assays (Fig. 6, B and C). Immunopatching with furin antibody revealed a granular staining pattern at the cell surface, whereas no cell surface staining was seen if primary (Fig. 6B) or secondary antibodies (data not shown) were omitted. Furthermore, cell surface biotinylation/pull-down assay resulted in the recovery of biotinylated furin (Fig. 6C). Thus, both experiments confirm the presence of furin at the cell surface of HT-1080 cells. Consistent with this, cell-impermeable furin inhibitor α1-PDX inhibited shedding of biotinylated COLXIII ectodomain (Fig. 6D).

However, the cell surface cleavage seems to make only a fraction of the total ectodomain shedding. Namely, the effect of α1-PDX on the ectodomain shedding of unbiotinylated HT-1080 cells was negligible when compared with that of the cell permeable CMK (Fig. 6E). It thus seems very likely that the cleavage event may take place at two locations. This hypothesis was further supported by the experiment where the membrane proteins at the cell surface were biotinylated, treated with either MCD or PMA, and subsequently recovered by streptavidin pull-down. Again, PMA up-regulated the ectodomain shedding at the cell surface as shown by the increased amount of biotinylated ectodomain in the medium (Fig. 6A, upper panel), in line with our earlier results (22). Cholesterol depletion by MCD reduced ectodomain shedding at the cell surface, as indicated by the decrease in the amount of biotinylated ectodomain in the pull-down medium. Interestingly, however, the residual medium after the streptavidin pull-down following the MCD treatment contained unbiotinylated ectodomain (Fig. 6A, lower panel). Because this fraction had not been bound to streptavidin, it obviously did not contain biotin, and hence it could be interpreted as being of intracellular origin.

Golgi-derived Vesicles Contain Cleaved Ectodomain—The origin of the residual unbiotinylated ectodomain was explored by ultracentrifugation of the culture media, all of which contained material that sedimented at 100,000 × g. COLXIII was found to be present in the sedimented material in the cleaved form, as indicated by its smaller size compared with the full-length COLXIII detected in the cell lysate (Fig. 7A). The size difference between the COLXIII in the lysates and that in either the media or the sedimented material was about 10 kDa, coinciding well with our earlier findings and with the consensus RRRR cleavage site for proprotein convertases on the ectodomain (22). Incidentally, this material also contained furin (Fig. 7A). Because MCD treatment is known to induce vesicle secretion from the Golgi (37, 45, 47), we wondered whether this pellet material in fact consisted of Golgi-derived vesicles. Various marker proteins were used to ascertain its origin, and it was found to be immunopositive for the Golgi marker GM130 but immunonegative for calnexin and Rab-5 (Fig. 7A), which serve as markers of the ER and endosomal vesicles, respectively. This immunoassay thus enabled us to deduce that the pellet material did indeed consist of Golgi-derived vesicles. Cell surface biotinylation was used again, this time to verify the intracellular origin of the vesicles. The sedimented vesicles were GM130-immunopositive, indicating Golgi origin, and contained cleaved ectodomain, as seen by direct immunodetection with anti-COLXIII antibody. Because the ectodomain found in the vesicles was not biotinylated, it could not have originated from the cell surface but must instead have come from an intracellular source (Fig. 7B).

The presence or absence of the transmembrane domain creates a hydrophobicity difference between the uncleaved COLXIII and the released ectodomain. Cleavage at the RRRR site on the ectodomain renders the ectodomain more hydrophilic than intact COLXIII, because of the lack of the transmembrane domain. The culture media were therefore subjected to TX-114 phase separation, in which all the cleaved ectodomain was found to segregate into the water phase with
no intact COLXIII seen in the detergent-rich phase (Fig. 7C). This confirmed the hydrophilic nature of the ectodomain found in the vesicles, \textit{i.e.} its cleaved form. The medium-derived

shedding of biotinylated COLXIII ectodomain (D), whereas with unbiotinylated HT-1080 cells, its effect on the ectodomain shedding was negligible when compared with that of 100 ng/ml CMK (E). The media were precipitated with ice-cold methanol, followed by analysis for COLXIII ectodomain content by Western blotting under reducing conditions on 8% gels.
ectodomain showed the same size difference relative to the full-length COLXIII as before.

Cell Fractionation Shows COLXIII to Be of Full Length in the Golgi—The site of intracellular cleavage was further analyzed by fractionation of the HT-1080 cells, whereupon full-length COLXIII was detected in the microsomal fraction representing the rough ER and, although very faintly, in the Golgi membrane fraction (Fig. 8A). The microsomal fraction was correctly immunopositive for calnexin (Fig. 8A). Next, the cell constituents were fractionated based on their buoyancy differences in density gradient sucrose ultracentrifugation. COLXIII was enriched in the same fractions as the Golgi marker GM130, but not in Rab-5-immunopositive fractions representing endosomes, nor could we detect any COLXIII in the high density calnexin-immunopositive ER fractions, most likely because of low amounts (Fig. 8B). HT-1080 cells were then treated with BFA to assess the effect of intracellular transport blockage on the putative intracellular ectodomain cleavage. BFA inhibits anterograde vesicle transport from the Golgi to the plasma membrane by fusing the Golgi membranes to the ER, while leaving the trans-Golgi network unaffected (48–50). As expected as a result of the BFA treatment, the segregation of COLXIII and the Golgi marker protein GM130 had shifted to the heavy fractions, which also contained the bulk of the calnexin, because of Golgi-ER fusion (Fig. 8C). Despite the cessation of intracellular transport to the cell surface, only full-length COLXIII was found in the Golgi fraction, as ascertained by size comparison with the variant in the cell lysate (Fig. 8D).

Lack of Ectodomain in Golgi-derived Vesicles after CMK, but Not after α1-PDX Treatment—CMKs are potent inhibitors of the proprotein convertases (51, 52), and we had previously found no ectodomain in the medium after CMK treatment (22). Here the vesicles released by the HT-1080 cells after treatment with the cell-permeable CMK were totally devoid of cleaved COLXIII, despite the fact that the vesicle formation was not affected (Fig. 9A). Treatment of the HT-1080 cells with CMK did not affect cellular COLXIII expression (Fig. 9A). In complete contrast to CMK, vesicles contained cleaved COLXIII following treatment with cell-impermeable furin inhibitor α1-PDX (Fig. 9B).

DISCUSSION

The ectodomain shedding of an increasing number of transmembrane proteins has recently been found to be associated with localization of these proteins in the DRMs (13–17). In this study we demonstrate, using detergent extraction and isopycnic density gradient centrifugation, that some of COLXIII and its cleaving enzyme furin subfractionate into light density fractions representing DRMs, which were resistant to solubilization with detergents Brij96, Brij35, and CHAPS. The rest of COLXIII and furin resided in high density detergent-soluble fractions. In addition, detergent-specific differences in the distribution of COLXIII were also detected. This differential solubilizing effect of detergents has been observed also with other proteins and lipids, because DRMs obtained with different detergents vary considerably in their composition. Similarly, DRMs extracted from different cell lines vary from each other (43). The fact that COLXIII and furin were detected in DRMs

FIGURE 8. Cellular fractionation reveals only full-length COLXIII in the rough ER and the Golgi. A, postnuclear supernatant of HT-1080 cell homogenate was centrifuged in a discontinuous sucrose gradient at 100,000 × g for 2.5 h. The smooth ER (sER) fraction was recovered at the 1.1/1.25 M interface. The Golgi fraction from the 0.25/1.1 M interface was used as a size marker for the Golgi membranes. B, postnuclear supernatant of HT-1080 cell homogenate was centrifuged in a discontinuous sucrose gradient at 74,000 × g for 2.5 h. Fractions of 1 ml were collected from the top of the gradient and analyzed for COLXIII and calnexin contents by Western blotting under reducing conditions on 8% gels. C, HT-1080 cells were pre-treated with 30 μM BFA for 6 h prior to similar fractionation and analysis. D, the GM130-immunopositive fractions were analyzed for the size of COLXIII by Western blotting under reducing conditions on an 8% gel. Lanes 1 and 3 include GM130-immunopositive Golgi fractions from untreated cells, lane 4 from BFA-treated cells. Cell lysate (lane 1) was used as a size marker for the full-length COLXIII.
extracted with a number of detergents supports our interpretation of results showing the partial presence of COLXIII and furin in DRMs. Cholesterol removal by MCD resulted in a loss of detergent resistance of COLXIII and furin, indicating that cholesterol is a pivotal and crucial structural component of these DRMs. Interestingly, COLXIII and the raft marker Lyn, a Src kinase known to be enriched in noncaveolar lipid rafts (10), segregated differently in sucrose density gradients. Compared with COLXIII, Lyn was found to float in fractions of slightly lower density, which were also TX-100-resistant. This difference in detergent insolubility and floating properties suggests that the DRMs in which COLXIII localizes are distinct from the TX-100 resistant rafts. Different models have been proposed to explain the differential segregation of proteins in density gradients after detergent extraction. Segregation differences may reflect heterogeneity in composition among the DRMs resulting in varying detergent solubilities or structural hierarchy of a detergent-resistant core-raft and a peripheral raft (9, 10, 25). The results obtained in this study could be explained with both theoretical models. Namely, lack of resistance to TX-100 could point to the possibility that COLXIII resides in the less detergent-resistant peripheral raft area. It is also feasible that COLXIII resides in DRMs with lipid and protein composition as well as detergent solubility different from the TX-100 resistant rafts.

Partial localization in DRMs is important for the ectodomain shedding of COLXIII in HT-1080 cells, because manipulation of the amount or availability of cellular cholesterol effectively hampered ectodomain shedding. For an effective shedding to occur, both furin and COLXIII have to be present in the same membrane compartment. Based on the effects of the cellular cholesterol manipulation and loss of detergent resistance by MCD treatment, it is likely that these membrane compartments are DRMs. We have previously shown that PMA is able to stimulate COLXIII ectodomain shedding (22), so we considered the possibility that the PMA induction might be mediated by lateral translocation of COLXIII and/or furin from the high density membrane compartments to the DRMs. However, in sucrose gradients we did not observe such lateral movement of COLXIII or furin to low density fractions, irrespective of the detergents used. Nevertheless, PMA stimulation of the ectodomain shedding was sensitive to the manipulation of the cellular cholesterol levels by MCD and filipin. This indicates that the mechanism of the PMA stimulus requires an intact cholesterol-enriched membrane microdomain structure. The detailed mechanism of PMA induction obviously merits further analysis to be elucidated. All in all, characterization of the DRM association on the COLXIII ectodomain shedding is a novel feature for COLXIII. Recently, a similar phenomenon was described for the transmembrane COLXVII (53).

In this study we demonstrate the previously anticipated duality of COLXIII ectodomain shedding. Furin is known to reside predominantly in the Golgi apparatus, but also at the plasma membrane as a result of intracellular recycling (46, 54). This was also the case with HT-1080 cells. The role of furin in facilitating the COLXIII ectodomain shedding at the cell surface was supported by the observation that in biotinylated HT-1080 cells, cell-impermeable furin inhibitor α1-PDX dramatically reduced the release of biotinylated ectodomain into the culture medium. However, this study also shows that in HT-1080 cells, COLXIII is actively cleaved intracellularly. Namely, despite the effective inhibition of COLXIII ectodomain shedding at the cell surface by α1-PDX, the amount of total ectodomain shedding remained essentially unaltered. This suggests that only a minor fraction of COLXIII ectodomain shedding in HT-1080 cells occurs at the cell surface, the major cleavage location being inside the cell. The role of intracellular cleavage coincides with our previous findings where Golgi inhibitors were able to down-regulate COLXIII shedding in HT-1080 cells effectively (22).

In the biotinylation experiment MCD increased the amount of unbiotinylated, i.e. intracellularly derived, COLXIII ectodomain in the medium. MCD has been shown to induce vesicle release from the Golgi apparatus of cultured cells in a yet unidentified manner (37, 45, 47). This was also the case with HT-1080 cells because the culture medium contained vesicles that, based on their protein content, were derived from the Golgi. The vesicular COLXIII was always smaller in size than the full-length protein, and its strict separation into the hydrophilic phase in the TX-114 phase separation assay suggests that it did not contain any detergent-soluble hydrophobic domains such as a transmembrane segment. This indicates that COLXIII ectodomain sequestered in vesicles was fully cleaved. Despite the cell surface biotinylation, the vesicular COLXIII ectodomain was always without biotin, further underlining its origin in the Golgi. Because cholesterol depletion clearly reduced overall COLXIII ectodomain shedding, it is more appropriate to address the increased amount of this subfraction of ectodomain in the medium resulting from induced vesicle release rather than up-regulated ectodomain shedding. However, because of the lipid recycling in cells (7), lowering of the cell surface cholesterol level by MCD may also affect intracellular cholesterol level and thus intracellular COLXIII ectodomain.
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cleavage. Because both the plasma membrane and the Golgi contain DRMs (1, 6), it is plausible that they may be essential for the proprotein convertase activity required for COLXIII ectodomain cleavage at both locations.

COLXIII was also detected in the rough ER-derived microsomes and the Golgi fractions. In both preparations it was of the same size as the uncleaved COLXIII in the cell lysates. As the ectodomain in the Golgi-derived vesicles found in the culture medium was cleaved, however, enzymatic ectodomain cleavage must have occurred after its departure from the Golgi. Because no cleaved form of COLXIII was detectable in the Golgi despite the BFA-induced cessation of anterograde vesicular transport, and because CMK, but not α1-PDX, totally abolished the detection of any ectodomain in the vesicles despite unhampered vesicle liberation, cleavage of the ectodomain must have occurred at the exit from the Golgi. Ectodomain cleavage may thus be a gating event, so that only the cleaved COLXIII ectodomain is allowed to enter the vesicles to be secreted.

Vesicular shedding adds a new functional perspective to COLXIII ectodomain cleavage. We also recovered vesicles from the media of untreated control cultures, indicating that vesicle formation can also occur without exogenous MCD stimulation. Such vesicle secretion is characteristic of quickly dividing cells, e.g. cancer cells (55), and it has been speculated that vesicle components may play an important role in the modulation of the cancer tissue stroma (55–58). We have observed up-regulated COLXIII expression in the cancer stroma and in various cancer cell lines (23). Because the soluble COLXIII ectodomain can affect adhesion-related cell functions, the up-regulated expression and ectodomain shedding of COLXIII may influence the behavior of cancer cells (22). At the moment, however, the exact roles of cell surface ectodomain shedding and secretion via vesicle release related to the functions of COLXIII and its soluble ectodomain remain to be elucidated.

We present here new data concerning the membrane localization and the processing of the ectodomain of COLXIII. On one hand, partial localization in the lipid-rich membrane microdomains seems to be relevant to the shedding of the ectodomain, which can play a role in the matrix remodelling by interactions with other components of the matrix (22, 24, 59). Also, the soluble variant has exhibited matrix-dependent anti-adhesive effects (22). On the other hand, COLXIII is known to reside in focal adhesions (19, 20), where it is likely to contribute to cell adhesion. This is supported by studies with a knock-in mouse model synthesizing COLXIII without the cytosolic and transmembrane domains. Fibroblasts derived from these mice showed reduced cell adhesion (60). The dualistic nature of COLXIII as a membrane bound and a soluble variant with opposite properties confers the cell an economical way to modulate the composition of its external surface and the neighboring microenvironment. We also show that COLXIII ectodomain is cleaved in the Golgi to be secreted in Golgi-derived vesicles. These data provide a new insight into the processing of COLXIII and other transmembrane collagens.

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