Calcium and pH-dependent packing and release of the gel-forming MUC2 mucin

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MUC2, the major colonic mucin, forms large polymers by N-terminal trimerization and C-terminal dimerization. Although the assembly process for MUC2 is established, it is not known how MUC2 is packed in the regulated secretory granulae of the goblet cell. When the N-terminal VWD1-D2-D'D3 domains (MUC2-N) were expressed in a goblet-like cell line, the protein was stored together with full-length MUC2. By mimicking the pH and calcium conditions of the secretory pathway we analyzed purified MUC2-N by gel filtration, density gradient centrifugation, and transmission electron microscopy. At pH 7.4 the MUC2-N trimer eluted as a single peak by gel filtration. At pH 6.2 with Ca²⁺ it formed large aggregates that did not enter the gel filtration column but were made visible after density gradient centrifugation. Electron microscopy studies revealed that the aggregates were composed of rings also observed in secretory granulae of colon tissue sections. The MUC2-N aggregates were dissolved by removing Ca²⁺ and raising pH. After release from goblet cells, the unfolded full-length MUC2 formed stratified layers. These findings suggest a model for mucin packing in the granulae and the mechanism for mucin release, unfolding, and expansion.

mucin | bicarbonate | cystic fibrosis | unpacking

Mucins are large glycoproteins that coat the surface of cells in the respiratory, digestive, and urogenital tracts (1, 2). Their main function is protection of epithelial cells from infection and physical injury. Mucins are characterized by mucin domains that are heavily *O*-glycosylated on the protein sequence rich in proline, threonine, and serine, therefore called PTS domains (3). These domains have little interspecies sequence conservation but often have tandemly repeated amino acid sequences that vary in number and length (3). There are several mucin types; the gel-forming mucins are the only ones that form large polymers. In humans there are four gel-forming mucin genes that are known to be expressed, MUC2 in the intestine (4), MUC5AC in lungs and stomach, MUC5B in lungs and saliva, and MUC6 in stomach (1).

MUC2 mucin is the major component of the mucus (mixture of mucins and other associated proteins) in the small and large intestine (2). In colon this is organized into two layers: an inner, densely packed layer that is attached to the epithelium that is impermeable to bacteria, and an outer, easily removable loose layer that is the habitat for the commensal bacteria (5). Human MUC2 mucin has 5,179 amino acids and contains multiple domains arranged in the following order (Fig. 1A): von Willebrand D1 domain (VWD1), VWD2, VWD'D3, (VWD1-D2-D'D3), first CysD, small PTS, second CysD, large PTS (tandemly repeated), C-terminal VWD4 followed by VWB, VWC, and a cystine-knot domain (CK) (4). The primary translational product of full-length MUC2 is quickly dimerized in the endoplasmic reticulum (ER) via disulfide bonds in the CK domain (6). The dimers pass into the Golgi apparatus, where the two PTS domains become *O*-glycosylated to form the two mucin domains. In the trans-Golgi network the glycosylated dimers are then trimerized by disulfide bonding in the VWD3 (7). Thus, MUC2 forms large net-like structures that are densely packed in the secretory granulae of the goblet cell. The pH along the secretory pathway shifts gradually,

from 7.2 in the ER to 6.0 in the trans-Golgi network and to 5.2 in the secretory granulae, at the same time as the intragranular Ca²⁺ concentration increases, suggesting that the packing of MUC2 may be pH and Ca²⁺ dependent.

Von Willebrand factor (VWF), with N- and C-terminal regions homologous to MUC2 (8), has originated from the mucins during evolution (3) but has been studied more intensively than the mucins. VWF has N-terminal VWD1-D2-D'D3 and C-terminal VWD4-B1-B2-B3-C1-C2 and CK domains. The middle part of VWF, corresponding to the CysD and mucin domains in MUC2, has been replaced by the VWA1-A2-A3 domains (8). Just as for MUC2, VWF assembly involves dimerization via the CK domains (9, 10). In contrast to MUC2 mucin, VWF is cleaved in the VWD' domain by furin and multimerized by dimerization in its N termini (9). VWF multimers condense into tubules and form cigar-shaped Weibel-Palade bodies in the endothelial cell secretory granulae. Expression of VWF in cell types with a regulated secretory pathway generated organelles that were indistinguishable from Weibel-Palade bodies (9). Weibel-Palade body-like tubules are assembled in vitro from the N-terminal part of VWF, where the VWD1-D2 propeptides and VWD'D3 dimers form a helical tubule at pH 6.2 with high calcium, but not at pH 7.4 (11). 3D reconstructions based on transmission electron microscopy (TEM) images showed that the N-terminal VWF helix consists of 4.2 repeating units per turn (11). This pH-dependent packing required protonation of conserved histidine residues (12). Because the packing of VWF tubules depends solely on the N-terminal part of VWF, it was suggested that the rest of the VWF extended radially from the helix axis (11). More recently it was shown using TEM that the C-terminal part of VWF with the VWA2-A3-D4-B1-B2-B3-C1-C2 and CK domains zips up into an elongated dimeric bouquet, where the VWB1-C2 and CK domains form a stem and the VWA2-A3-D4 a raceme with three pairs of flower-like domains at pH 6.2, but not pH 7.4 (13). These dimeric C-terminal bouquets and the N-terminal helical tubules are dissolved at pH 7.4, enabling unfolding and release of a long extended "rope" that follows the blood stream (14, 15). Hence, the molecular details for the pH- and Ca²⁺-dependent packing of the VWF multimer have been largely elucidated.

The packing and secretion of MUC2 has not yet been studied in molecular terms. Here we show that MUC2 mucin is packed in secretory granulae of goblet cells owing to its N-terminal VWD domains (MUC2-N). Purified MUC2-N protein was analyzed by TEM, suggesting how MUC2-N is packed in these granulae. This suggests a model of how the full-length MUC2

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Fig. 1. Domain structure of MUC2 and N-terminal protein and storage of MUC2 N terminus in goblet cell vesicles. (A) MUC2 monomer consists of multiple domains: VWD [D1 (orange), D2 (yellow), D'D3 (blue) and D4 (light gray)], CysD (red), PTS (green), VWD (gray), VWC (dark gray), and cystine-knot CK (black). The MUC2-N plasmid contains the N-terminal VWD (D1-D2-D'D3) domains, CysD1, Myc-tag (black), and GFP (light green). The α -MUC2-N3 antibody recognizes the VWD/D3 domain. (B) Differentiated LS174TdnTCF4 cells expressing MUC2-N (green) were immunostained with anti-MUC2Cm1 MAb (red) detecting endogenous MUC2 showing colocalization. (Scale bars, 5 μ m.) (C) LS174TdnTCF4 cells expressing the MUC2-N protein (green) were induced to differentiate into goblet-like cells. Increasing accumulation of MUC2-N in larger vesicles was observed in the more differentiated cells. Nucleus, DAPI (blue). (D and E) Induced LS174TdnTCF4 cells with MUC2-N (green) were immunostained for ER with anti-calnexin (red) (D) or for Golgi with anti-GalNAcT2 (red) (E). No colocalization was apparent. (Scale bars, 5 μ m.) (F) Lysate from a radioactive pulse-chase experiment (30 min, 3 h, 20 h) of LS174TdnTCF4 (noninduced) expressing the MUC2-N protein that was immunoprecipitated using anti-myc MAb and analyzed by nonreduced SDS/PAGE. MUC2-N is first detected as a monomer, and during processing when passing the secretory pathway it forms trimers that were stored within the cells for more than 20 h.

muin can expand >1,000-fold upon secretion and form a stratified inner mucus layer as revealed by electron and confocal microscopy studies of tissue sections from human and mouse colon.

Results

N-Terminal Part of MUC2 Mucin Contains the Goblet Cell Storage Information. The N-terminal 1,397 amino acids of MUC2 tagged with Myc-tag and EGFP, MUC2-N (Fig. 1A) (7), was stably expressed in the human colonic cell line LS174T expressing an inducible dominant negative form of the transcription factor TCF4 (16). Induction of this factor by doxycycline stopped cell division and differentiated the cells into typical goblet cells, accumulating MUC2-N in the same granulae as the full-length MUC2 mucin (Fig. 1B). The fluorescent MUC2-N was observed in intracellular vesicles that with time formed aggregates with increasing size, as found in the theca of typical goblet cells (Fig. 1C). The aggregated MUC2-N vesicles did not colocalize with the ER stained with calnexin (Fig. 1D), nor the Golgi apparatus stained for the peptidylGalNAcT-2 glycosyltransferase (Fig. 1E). When these cells were pulse-chase labeled, MUC2-N was observed as a monomer after 0.5 h (Fig. 1F, marked "M") and transformed into a trimer (marked "T") after 3 and 20 h. Together this suggests that the MUC2-N trimer is accumulated in the goblet cell storage granulae.

N-Terminal VWD Domains of MUC2 Form Ring Structures. Because the sorting information resided in the N terminus, we mimicked the packing of MUC2 in the mucin granulae by exposing purified disulfide-bonded MUC2-N trimers to the pH and Ca^{2+} conditions of these vesicles. The pSNMUC2-MG vector was stably expressed in CHO cells that lack a regulated secretory pathway, which may affect MUC2 processing and folding and give a different glycosylation compared with the intestine (7). The MUC2-N protein was purified from spent culture medium and analyzed by gel filtration, density gradient centrifugation, and TEM using negative staining.

MUC2-N eluted as a single peak on gel filtration at pH 7.4, with an apparent molecular mass of 1.2 MDa (Fig. 2A). By TEM of MUC2-N at pH 7.4 in the absence of Ca^{2+} the MUC2-N protein adopted an outstretched conformation, with three globular structures extending from a central core showing the trimeric MUC2 N terminus (Fig. 2D). Each globular structure and the

Fig. 2. MUC2-N forms pH- and calcium-dependent rings. (A) Gel filtration of MUC2-N at pH 7.4, pH 6.2 with EDTA or Ca^{2+} , and pH 5 with Ca^{2+} . The MUC2-N trimer formed larger aggregates at lower pH that did not enter the gel beads. (B and C) Density gradient centrifugation of MUC2-N protein incubated at pH 8 with EDTA (B) or pH 6.2 with Ca^{2+} (C). As analyzed by reducing SDS/PAGE and silver staining, the MUC2-N trimer was found in fractions 14–18 in high and low pH conditions, whereas larger aggregates were detected in fractions 20–22 only at pH 6.2 with Ca^{2+} . (D) EM (negative stain) showing that MUC2-N trimer adopts an outstretched appearance at pH 7.4, with three globular structures extending out of a trefoil core fragment, suggesting that the VWD1-D2 domains are connected via a flexible linker to the disulfide-bonded (indicated by white bars) VWD3 trimeric core (E) (same color code as in Fig. 1). (F) EM showing that MUC2-N forms five-, six-, and seven-sided ring structures at pH 6.2 with Ca^{2+} composed of multiple MUC2-N trimers. (G) Glutaraldehyde cross-linking at pH 6.2 with Ca^{2+} before density gradient centrifugation (fraction 22 from C) allowed for isolating confluent 2D sheets of concatenated MUC2-N rings as seen by EM (negative stain). (Scale bars, 50 nm in D, 100 nm in F and G.)

Fig. 4. Dissolution of MUC2-N aggregates by HEPES-EDTA or NaHCO_3 -treatment. (A) MUC2-N aggregates formed at pH 5 with 50 mM CaCl_2 were analyzed by gel filtration with or without addition of 100 mM sodium bicarbonate (pH 8.3). Addition of sodium bicarbonate partially dissolved the MUC2-N aggregates. (B and C) MUC2-N aggregates formed at pH 6.2 with Ca^{2+} were dialyzed against 20 mM HEPES (pH 8), 10 mM EDTA (B) or 50 mM NaHCO_3 (pH 8.3) (C) and then analyzed by density gradient centrifugation and reducing SDS/PAGE and silver staining. Higher pH conditions or sodium bicarbonate treatment both partially dissolved the MUC2-N aggregates that were formed at low pH with Ca^{2+} in fractions 20–22.

densely packed ring-like structures were ≈ 35 nm in diameter and might therefore be the N-terminal VWD domains of MUC2. In addition, less densely packed areas were also observed with diameters of up to ≈ 100 nm, suggesting that in these areas the MUC2 mucin was partially unfolded. Because the C-terminal part of VWF extends out of the N-terminal tubules, we assumed that the C-terminal part and the mucin domains of MUC2 stand perpendicular to the N-terminal concatenated ring platform, as suggested in the model of Fig. 5B. The two PTS domains are heavily *O*-glycosylated, and the polypeptide backbone of the formed mucin domain adopts an outstretched rigid rod-like conformation with ≈ 0.2 nm per amino acid (18, 19). This information and the length of MUC2 glycans (19) allowed us to model the mucin domains as rods with 4-nm diameter (green in Fig. 5B). The total length of the two mucin domains is then more than $0.5 \mu\text{m}$, accounting for ≈ 76 nm (381 amino acids) for the small and ≈ 488 nm (2,439 amino acids) for the large mucin domain (4). For VWF it was recently demonstrated that the C-terminal VWB1-C2 and CK domains arrange pairwise in a stem-like structure at pH 6.2 (13). A similar arrangement of the C-terminal VWB-C and CK domains of MUC2 should further stabilize the C-terminal ends and allow the rest of MUC2 mucin to stand perpendicular to the N-terminal ring platform (Figs. 1A and 5B). In such a model the MUC2 mucin is tightly packed at one end by the N-terminal VWD domains by concatenated rings and at the other end by the C-terminal VWB-C and CK domains forming stem-like structures.

Because full-length MUC2 is dimeric C-terminally and trimeric N-terminally, it can be assumed that the unfolded MUC2 mucin adopts an outstretched net-like structure after release from the goblet cells. During unpacking the MUC2-N ring structure is disrupted, and the mucin domains begin to separate (Fig. 5C). As shown in Fig. 5D, the net is formed by N-terminal VWD3 disulfide-linked trimers and C-terminal disulfide-linked dimers of the CK domains. Such a net should be easily formed without entanglement problems from the packed mucin structure in Fig. 5B. Support for such a flat, net-like molecular architecture of the fully unfolded and secreted stacked stratified sheets was revealed

Fig. 5. Packing and unfolding of MUC2. (A and B) Packing of MUC2 at pH 6.2 in presence of Ca^{2+} in secretory granules of goblet cell. (A) EM of a cross-section of secretory granules from a human goblet cell showing ring-like structures. (Scale bars, 200 nm at left, 100 nm at top right, 40 nm at bottom right.) (B) MUC2 is organized at one end around the MUC2-N concatenated rings stabilized by noncovalent interactions of N-terminal VWD (orange, yellow, and blue) domains and at the C-terminal end by CK (black) dimers. The extended mucin domains drawn to scale (green) connect neighboring VWD/D3 trimers (blue) of the MUC2-N ring platform (Lower) with the C-terminal ends (Upper). (C and D) Unfolding of MUC2 at high pH and low Ca^{2+} after release from goblet cell secretory granules. (C) During unpacking the MUC2-N rings fall apart, and the mucin domains begin to stretch out. (D) The released MUC2 is assumed to adopt an outstretched net-like flat sheet composed of trimeric disulfide-linked MUC2-N (in the corners) and dimeric MUC2-C (in the middle of the sides). The expanded net is $>1,000$ -fold larger than the granular rings. (E and F) The inner mucus layer of the colon show stratified sheets (s) as predicted from the unfolded MUC2 model. (E) EM of the inner mucus layer organized as stratified sheets (s) on top of an enterocyte with its glycocalyx from a colon tissue section. (Scale bar, $2 \mu\text{m}$.) (F) Mouse colon tissue section fixed in Carnoy and stained with the anti-MUC2C3 antiserum (green) and FISH (red) as revealed by fluorescence microscopy of the stratified MUC2 inner mucus layer (s) devoid of bacteria and the outer mucus layer (o) with bacteria. (Scale bar, $25 \mu\text{m}$.)

by TEM of the inner mucus layer in human colon tissue (Fig. 5E). A stratified MUC2 mucin stained with anti-MUC2C3 antiserum was also observed by fluorescence microscopy for the inner stratified firmly attached mucus layer (s, green in Fig. 5F). This inner mucus layer has been shown to be impermeable to bacteria and to separate bacteria (red) in the outer mucus layer (o) from the epithelium (e) (5).

Discussion

The molecular details on the packing of VWF have recently revealed that all information necessary for proper assembly and packing of VWF is built into the molecule itself and is regulated by pH- and Ca^{2+} -dependent interactions of the different types of structural domains (11, 13, 15). Because VWF and MUC2 share the same type of structural domains in their C-terminal parts, and both form disulfide-bonded dimers, it may be assumed that the C-terminal ends undergo similar pH-dependent conformational changes during packing and release. The C-terminal VWB1-B2-B3-C1-C2 and CK domains of VWF adopt a stem-like structure containing a paired dimeric arrangement of each domain at pH 6.2 and then unfolds into an extended, linear structure at pH 7.4 (13). It may thus be speculated that the C-terminal VWB-C and CK domains of MUC2 can also form paired stem-like structures and stabilize the C-terminal ends at pH 6.2 inside the secretory granulae during packing.

Although both VWF and MUC2 form disulfide-linked dimers in the C-terminal part, the N-terminal parts are assembled differently: the VWF forms disulfide-linked dimers, whereas MUC2 forms disulfide-linked trimers, both within the N-terminal VWD3 domains (7, 9). The pH-dependent control of VWF multimerization in the N-terminal VWD1-D2-D'D3 domains depends on two conserved histidine residues of the VWD2 domain, His³⁹⁵ and His⁴⁶⁰, respectively (12). Although the histidine residue His³⁹⁵ is present in the VWD2 domain of MUC2, the second histidine residue His⁴⁶⁰ is absent and may influence the different oligomeric state of these two structurally related proteins. Furthermore, the formation of disulfide-linked trimers by the VWD3 domains of MUC2 has structural implications on the arrangement of the N-terminal VWD1-D2-D'D3 domains during pH- and Ca^{2+} -dependent packing inside the secretory granulae. Instead of forming helical rods as for VWF, we found that the MUC2-N protein formed concatenated rings with varying sizes. By TEM 2D reconstruction of five- and six-sided rings, we suggested that each ring was made up of five or six repeating units, respectively, and each repeating unit of three VWD1-D2 peptides and one disulfide-linked VWD3 trimer. A majority of the single rings were six-sided, and packing of these favors a planar array, but five- and seven-sided rings, although not observed in the pictures of the concatenated rings, could if present cause deviation from a planar platform.

The middle part of VWF contains the VWA1-A2-A3 domains that are replaced by two CysD domains flanking the two mucin domains in MUC2 (Fig. 1A). The length of this part in MUC2 was estimated to be more than 0.5 μm . Because the smallest ring-like structures observed in the secretory vesicles of goblet cells from colon tissue sections were ≈ 35 nm in diameter, they were probably similar to the MUC2-N concatenated rings. Because the mucin domains are poorly visible on TEM, we can only speculate on their orientation, but most likely these stand perpendicular to the MUC2-N rings for three reasons: (i) MUC2-N rings form a planar ring platform, and it is not possible for the mucin domains to protrude into the MUC2-N rings owing to steric hindrance; (ii) the two mucin monomers connected via the C-terminal dimer must be on the same side; and (iii) to allow expansion, each corner of the MUC2-N rings with three VWD3 domains are connected to neighboring trimers via paired mucin domains. Thus, each mucin pair must be located on the same side. However, alternatively to the model in Fig. 5, the different mucin pairs

could also protrude out perpendicular on both sides of the planar ring platform. Upon release the MUC2 N- and C-terminal ends are unfolded, and potential Ca^{2+} -stabilized interactions between the glycans are lost. The loss of the N-terminal packing, mucin domain interactions, and perhaps C-terminal packing will allow expansion of the full-length MUC2, something that can explain the stacked, stratified layers that we observed in the secreted inner mucus layer. Because the radius of the unfolded, full-length MUC2 rings is ≈ 1 μm (two times the length of the mucin domains) and ≈ 17.5 nm of the tightly packed MUC2-N concatenated rings (in 2D reconstruction of six-sided rings), the theoretical total area expansion upon release could be up to 3,000-fold, although one can assume that this is an upper purely theoretical number sufficiently close to the previously estimated 1,000-fold expansion (17).

The presence of trimers instead of dimers in the VWD3 probably explains the difference in the packing between VWF and MUC2. Full-length VWF is packed as helical tubules in the Weibel-Palade bodies, whereas full-length MUC2 is packed in the secretory granulae of goblet cells as concatenated rings. This is probably important for the different modes of secretion. VWF unfolds slowly into linear strings that follow the blood stream (14), whereas MUC2 mucin should be secreted fast, including a large volume expansion. Until now, mucins have been proposed to be packed inside the goblet cell granulae by Ca^{2+} -dependent cross-linking of the negatively charged glycans present on the mucin domains (17). This model predicted a random packing and is not compatible with some mucins having only neutral glycans (20) and the fast expansion without causing serious random entanglement. However, our model with parallel mucin domains is probably further stabilized by glycan interactions in which Ca^{2+} is important to shield potential negatively charged glycans. In support for our model of mucins, we also found that the secreted and unfolded full-length MUC2 formed layers of stratified sheets that build the inner mucus layer of colon. This arrangement is probably very important for this layer being impermeable to bacteria, an important property for maintaining a separation of the commensal bacteria from the epithelium necessary for colon homeostasis (5). An understanding of the packing and release of mucins probably also has other important medical implications. Because a correct expansion requires a fast pH increase and Ca^{2+} removal, HCO_3^- -containing natural buffers are probably very important, as this can fulfill both these demands. A key example is the cystic fibrosis conductance regulator channel, which in addition to Cl^- also secretes HCO_3^- and is required for intestinal mucus release (21–23). Lower amounts of HCO_3^- present during mucus secretion will probably slow down and stop expansion as the MUC2 N-terminal rings are still intact. This may cause the viscous mucus phenotype of the disease cystic fibrosis.

Materials and Methods

Animals. All mice were C57BL/6 and experimental procedures approved by the Animal Ethical Committee in Gothenburg. Immunostaining of distal colon after fixation in methanol-Carnoy's fixative was performed on 10- to 14-wk-old mice (5).

Stable Goblet-Like Cells Expressing the MUC2 N Terminus. The plasmid pSNMUC2-MG (encoding amino acids 21–1397 of MUC2 fused with Myc-Tag and EGFP) (7) was transfected (Lipofectamine 2000; Invitrogen) into LS174T cells expressing an inducible dominant negative TCF4 (16). Paraformaldehyde [4% (vol/vol), 20 min] fixed cells were permeabilized [0.1% (vol/vol) Triton X-100, 5 min] and the primary antibodies were added: anti-MUC2C2^{m1}, a MAb against the C-terminal peptide, CIKRPDQNHVILKPGDFK (1/50), anti-GalNAc-T2 (UH4, 1/200) (24), and anti-calnexin (1/200) (BD). The secondary antibody, anti-rabbit-TRITC (1/100) (Jackson Labs) or anti-mouse-Cy3 (1/3000) (Invitrogen), was added (1 h), and the samples were examined using an LSM 510 confocal microscope (Zeiss) after mounting in ProLong Anti-fade (Invitrogen). Confluent 6-cm dishes were starved in cysteine/methionine free medium (90 min), pulsed with 200 μCi ³⁵S-Met/Cys (PROMIX; Amersham) 45

min, medium exchanged, and supplemented with 15 $\mu\text{g}/\text{mL}$ methionine and 25 $\mu\text{g}/\text{mL}$ cysteine and chased for 30 min, 3 h, and 20 h. The cells were lysed in Triton X-100 and immunoprecipitated using anti-myc MAb and anti-mouse Dynabeads, followed by analysis on 3–6% SDS/PAGE (7).

Analytical Gel Filtration. MUC2-N expressed in CHO-K1 (7) was purified (20 μg) and applied to a Superose 6 PC 3.2/30 column (GE) and eluted at 0.02 mL/min using an Ettan HPLC (GE). The column void volume was 0.843 mL and the standards thyroglobulin, ferritin, aldolase, and ovalbumin eluted at 1.315 mL, 1.494 mL, 1.629 mL, and 1.753 mL, respectively. The MUC2 N-terminal protein was injected onto the column equilibrated with 10 mM sodium phosphate (pH 7.4), 140 mM NaCl; 20 mM Mes (pH 6.2), 150 mM NaCl, 5 mM EDTA or 10 mM CaCl_2 ; 20 mM HAc (pH 5); 150 mM NaCl and 50 mM CaCl_2 . N-terminal aggregates formed at pH 5 (20 mM HAc, 50 mM CaCl_2) were dissolved by addition of 100 mM NaHCO_3 (pH 8.3).

Density Gradient Centrifugation. One hundred micrograms of purified MUC2-N (7) was dialyzed (Spectra/Por; M_r 12–14,000 cutoff, Spectrum) against 20 mM Mes (pH 6.2), 50 mM CaCl_2 or 20 mM Hepes (pH 8), 10 mM EDTA. N-terminal aggregates formed at pH 6.2 (20 mM Mes pH 6.2, 50 mM CaCl_2) were dissolved by dialyzing (Slide-A-Lyzer, M_r 10,000 cutoff; Thermo Scientific) against 20 mM Hepes (pH 8), 10 mM EDTA, or 50 mM NaHCO_3 (pH 8.3). The protein was then cross-linked with 0.25% (wt/vol) glutaraldehyde for 10 min at 37 °C and the reaction stopped by adding 100 mM Tris-HCl (pH 9). Samples were layered on top of a 10-mL linear 5–40% (wt/vol) Nycodenz gradient containing 20 mM Hepes (pH 8) and 10 mM EDTA, with 660 μL of 40% (wt/vol) Nycodenz bottom cushion. Density gradient centrifugation was performed at 274,000 $\times g$ for 16 h at 20 °C in a Beckman SW41Ti rotor. Twenty-two fractions (0.5 mL) were collected from the top and analyzed by reducing SDS/PAGE, silver staining, and TEM.

Gold Labeling of Anti-MUC2-N3 Antiserum. Rabbit anti-MUC2-N3 antiserum, raised against the synthetic peptide CPKDRPIYEEDLKK of the VWD3 domain, was purified by thiopropyl Sepharose 6 B (Amersham) affinity chromatography (7). Twelve micrograms of affinity-purified anti-MUC2-N3 antibody was diluted (1:20) in 5 mM NaHCO_3 (pH 9) and dialyzed (Slide-A-Lyzer MINI, 10,000 cutoff; Thermo Scientific) against the same buffer overnight at 4 °C. The dialyzed antibody was added in small aliquots to pH-adjusted colloidal gold (pH 9, 10 μL 0.2 M $\text{K}_2\text{CO}_3/\text{mL}$, 6-nm particle size; Aurion) under constant

stirring. After 10 min, the gold-labeled antibody was ultracentrifuged at 45,000 $\times g$ for 45 min at 4 °C in a Beckman TLA-55 rotor. The soft pellet was resuspended in 20 mM Mes (pH 6.2), 150 mM NaCl, and 10 mM CaCl_2 . The program system used for image processing in 2D was EMAN1 (25). Top views of symmetric five- and six-sided rings were selected using the 2D-refine program in EMAN1. The number of collected particles was 461 for the six-sided rings.

Transmission EM. MUC2-N was analyzed by negative stain TEM at ≈ 20 or 40 $\mu\text{g}/\text{mL}$ in 20 mM Mes (pH 6.2), 150 mM NaCl, 10 mM CaCl_2 or in 20 mM Tris (pH 7.4), and 150 mM NaCl; centrifuged at 17,000 $\times g$ for 20 min at 4 °C; and incubated for 24 h at 37 °C. Aliquots (4 μL) were adsorbed onto glow-discharged carbon-coated copper grids (400 mesh; Analytical Standards) for 60 s, washed twice with water, and stained with 2% (wt/vol) uranyl acetate for 30 s, air-dried, and observed in a Jeol JEM 2100F or LEO 912AB (200 kV or 120 kV, respectively). Images were recorded with a 4K $\times$ 4K CCD camera (Tietz Video) at a magnification of 83,400 $\times$ with a sampling of ≈ 1.8 Å per pixel (cross-linked sample); 55,600 $\times$, sampling of ≈ 2.7 Å per pixel (non-cross-linked sample). The defocus values were in the range of 1.7–2.5 μm .

Colon tissue from human biopsies was prepared for TEM as previously described (26). The specimens were fixed in Karnovsky fixative [2% (vol/vol) paraformaldehyde and 2.5% (wt/vol) glutaraldehyde in 50 mM sodium cacodylate buffer, pH 7.2] for 24 h, followed by sequential staining using 1% (wt/vol) OsO_4 for 4 h, 1% (vol/vol) tannic acid for 3 h, and 1% (wt/vol) uranyl acetate overnight. Samples were dehydrated and embedded in Epoxy resin (Agar 100). Ultrathin sections (50 nm; Ultracut E; Reichert, Depew, NY) were collected on mesh copper support grids. The sections were contrasted using lead citrate and tannic acid and examined in a Zeiss 902 electron microscope.

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