O-Glycosylated MUC2 Monomer and Dimer from LS 174T Cells Are Water-soluble, whereas Larger MUC2 Species Formed Early during Biosynthesis Are Insoluble and Contain Nonreducible Intermolecular Bonds*

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The MUC2 mucin is the major gel-forming mucin in the small and large intestine. Due to its sequence similarities with the von Willebrand factor, it has been suggested to dimerize in the endoplasmic reticulum and polymerize in the trans-Golgi network. Using an O-glycosylation-sensitive MUC2 antiserum, a dimerization has been shown to occur in the endoplasmic reticulum of LS 174T cells (Asker, N., Axelsson, M. A. B., Olofsson, S.-O., and Hansson, G. C. (1998) J. Biol. Chem. 273, 18857–18863). Using an antiserum immunoprecipitating O-glycosylated MUC2 mucin, monomers and dimers were shown to occur in soluble form in the lystate of LS 174T cells. The amount of O-glycosylated dimer was small, and no larger species were found even after long chase periods. However, most of the labeled MUC2 mucin was found in pelleted debris of the cell lysate. This insoluble MUC2 mucin was recovered by immunoprecipitation after reduction of disulfide bonds. Analysis by agarose gel electrophoresis revealed two bands, of which the smaller migrated as the O-glycosylated monomer and the larger migrated as the O-glycosylated dimer of the cell lysis supernatant. Mucins insoluble in 6 M guanidinium chloride could also be obtained from LS 174T cells. Such mucins have earlier been found in the small intestine (Carlstedt, I., Herrmann, A., Karlsson, H., Sheehan, J., Fransson, L.-Å., and Hansson, G. C. (1993) J. Biol. Chem. 268, 18771–18781). Reduction of the mucins followed by purification by isopycnic density gradient ultracentrifugation and analysis by agarose gel electrophoresis revealed two bands reacting with an anti-MUC2 tandem repeat antibody after deglycosylation. These bands migrated identically to the bands shown by metabolic labeling, and they could also be separated by rate zonal ultracentrifugation. These results suggest that the MUC2 mucin is forming nonreducible intermolecular bonds early in biosynthesis, but after initial O-glycosylation.

The mucosal surfaces comprise a 1000-fold larger interface between the external and internal milieu than the skin. At the same time, the chemical and microbiological challenges are more demanding. The most important protective component of the mucosal surfaces is the mucus layer, the gel properties of which are due to macromolecules called mucins. A majority of the mucins known today belong to this classical gel-forming type, although a few glycoproteins defined as mucins are membrane-bound with yet unknown physiological functions (1). The present definition of mucin includes all glycoproteins that consist of more than 50% O-linked oligosaccharides and that have a majority of these oligosaccharides localized to mucin domains. These domains have a high number of O-glycosylated Ser and Thr amino acids, often appearing in tandem repeat sequences. Gel-forming mucins are probably altered in several diseases. Thus, alterations in the mucus barrier are probably essential in the pathogenesis of, for example, infections, peptic ulcers, and inflammatory bowel disease. Diseases such as cystic fibrosis and chronic bronchitis, and also trivial infections, are characterized by increased mucus viscosity. Despite its medical interest, the biochemical nature of these altered mucin properties is still poorly understood, largely due to the difficulties associated with the large size of these molecules.

The gel-forming mucins are proposed to be disulfide bond-stabilized linear polymers of highly glycosylated proteins (2), although other models are discussed (3). A typical example of such a mucin is encoded by the MUC2 gene, one of the few mucin genes fully sequenced (4). The MUC2 mucin occurs in small and large intestine (5, 6) and probably also in the airways.

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cellular fractionation (11). By the help of an antiserum directed against protein epitopes unaffected by O-glycosylation, the monomer and dimer were identified in O-glycosylated form. However, the amount of especially O-glycosylated dimers was small, suggesting that these disappeared somewhere. The dimers were found to form insoluble MUC2 mucin occurring in cell debris pelleted from cell lysate. The oligomeric stage of this insoluble MUC2 mucin could not be analyzed, as reduction was necessary for solubilization. When analyzing the reduced insoluble MUC2 mucin, not only the monomeric forms expected from the von Willebrand factor analogy were found. Up to half the amount of monomers were linked together with nonreducible bonds and migrated as O-glycosylated dimers on agarose gels.

Mucins insoluble in chaotropic solutions, such as 6 M guanidinium chloride, have earlier been reported in the small intestine (12, 13), and in the gall bladder bile of some patients (14). Here we report that the cell line LS 174T can also form mucins insoluble in 6 M guanidinium chloride. These were found to contain the MUC2 mucin with nonreducible bonds, migrating similarly to the metabolically labeled insoluble MUC2 mucin from cell debris pellets.

MATERIALS AND METHODS

Antibodies and Cell Lines—The polyclonal sera α-MUC2TTR and α-MUC2N3 have been described (10, 11). An antiserum called α-MUC2C2 was raised in a rabbit against a synthetic peptide, CIKRP-DNQHVILKPGDFK, based on amino acids 4995–5013 on the C terminus of the human MUC2 apoprotein (4). A New Zealand White rabbit was immunized once with 500 μg of peptide conjugated via Cys to 400 μg of keyhole limpet hemocyanin in Freund's complete adjuvant, and twice with 250 μg of peptide conjugated to 200 μg of keyhole limpet hemocyanin in Freund's incomplete adjuvant. The interval between the immunizations was 2 weeks. The specificity of the α-MUC2C2 antiserum was tested as described for the α-MUC2N3 antiserum (11) using 1 μg of immunizing peptide/25 μl of antiserum for inhibiting immunoprecipitation. The colon adenocarcinoma cell line LS 174T (ATCC CL 188), producing MUC2 mucin, was cultivated as described before (10).

Pulse-Chase, Immunoprecipitation, and Electrophoresis Procedures—Newly confluent LS 174T cells were starved for 1 h in 2 ml of methionine-free minimum essential medium (Life Technologies, Inc.) with 10% fetal bovine serum and 2 mM glutamine per 27-cm² Petri dish. Brefeldin A (Epicentre Technologies), 0.7 μM (16). The membrane was then blocked for 3 h in 30% ethanol and 10% acetic acid, soaked in Amplify (Life Technologies, Inc.) and 1% Sea Plaque low gelling temperature agarose (FMC) or 2% Ultrapure (Life Technologies, Inc.) and 1% Sea Plaque high gelling temperature agarose (FMC) or 2% Ultrapure (Life Technologies, Inc.). The buffer contained 0.378 M Tris-HCl, pH 8.8, and 0.1% SDS. The stacking gel was made of 0.8% agarose (Seakem Gold, FMC) in 0.126 M Tris-HCl, pH 6.8, and 0.1% SDS. The electrode buffer at the cathode was 0.05 M Tris, 0.384 mM NaCl, and 0.1% SDS, and at the anode 0.025 M Tris, 0.192 mM glycine, and 0.05% SDS. The current for electrophoresis was about 10 mA. Gels were fixed for 3 h in 30% ethanol and 10% acetic acid, soaked in Amplify fluorographic reagent (Amersham Pharmacia Biotech) with 5% glycerol for 30 min, dried on slab gel dryer for 2 h at 50 °C, and exposed at −80 °C to a Biomax MS film (Kodak).

Purification of Insoluble Mucins Followed by Western Blot—LS 174T cells were cultured in roller bottles for 10 days with daily medium changes and washed twice in cold PBS. The purification was performed as described before (13). In short, the attached cells were extracted with guanidinium chloride (6 μM guanidinium chloride, 5 mM EDTA, 10 mM NaH₂PO₄, pH 6.5, 5 mM N-ethylmaleimide, and 0.5 mM phenylmethanesulfonyl fluoride (Sigma), homogenized in a Dounce homogenizer (loose pestle), and incubated for 1 h at room temperature under agitation. Insoluble material was pelleted by centrifugation for 25 min at 40,000 × g, and the pellet was washed and centrifuged six times in similar guanidinium chloride buffer. The pellets were brought into solution by reduction of disulfide bonds in reduction guanidinium chloride (6 μM guanidinium chloride, 100 mM Tris, 5 mM EDTA, 10 mM freshly added dithiothreitol, pH 8.0) for 5 h at 37 °C under gentle agitation. Cysteine groups were alkylated by the addition of 150 mM iodoacetamide (powder) followed by incubation overnight at room temperature under agitation in the dark. The mucins were then purified by three rounds of isopycnic density gradient ultracentrifugation (15), with two with 4 μM guanidinium chloride and one with 0.2 μM guanidinium chloride. After unloading the gradient into fractions, the mucin peaks were identified by periodic acid-Schiff blot slot, dialyzed against water, lyophilized, redisolved, and boiled for 3 min in 0.8% agarose (SeaKem Gold, FMC) in 0.126 M Tris-HCl, pH 6.8, 20% glycerol, and 0.1% SDS. After electrophoresis performed as above (without Gel Bond Film and without stacking gel), the proteins were electrophoretically transferred (1 mA/cm² for 3 h at +4 °C, Sartoblot II-S) to a nylon blotting membrane (Immobilon-P, Millipore) using 48 mM Tris, 39 mM glycine, 0.0375% SDS, and 10% methanol (pH 8.3) as transfer buffer. The membrane was washed several times in water after blotting and dried before deglycosylation with gaseous hydrogen fluoride at room temperature overnight (16). The membrane was then blocked in saturated casein (Sigma) in 150 mM NaCl, 20 mM Tris-HCl, pH 7.5, 0.1% Tween 20 for 1 h at room temperature and stained with 1% α-MUC2TTR antiserum for 1 h at room temperature, followed by incubation for 1 h at room temperature with peroxidase-conjugated anti-rabbit antibody (DAKO) diluted 1:1000. Both antibody incubations were in 10% of saturated casein in 150 mM NaCl, 20 mM Tris-HCl, pH 7.5, 0.01% Tween 20. The assay was developed by the ECL reagent (Amersham Pharmacia Biotech), according to the manufacturers recommendations and using Biomax MS film (Kodak).

Rate Zonal Ultracentrifugation—MUC2 mucin insoluble in 6 μM guanidinium chloride was purified as described above, lyophilized, and redisolved in water (2 μg/μl). 20 μl of this solution was layered on top of a linear 22–43% (w/w) sucrose gradient in 150 mM NaCl, 20 mM Tris-HCl, pH 7.5, 0.02% NaN₃, with a volume of 5 ml in a 13 × 51-mm ultracentrifuge tube (Beckman). The ultracentrifugation was performed in a Beckman swinging bucket rotor (SW55Ti) for 15 at 40,000 rpm at +5 °C. Fractions (200 μl) were recovered from the top, and the rate of sedimentation was determined by a refractometer (Bausch & Lomb, Rochester, NY). The material was eluted in 0.3 M NaCl, 20 mM Tris-HCl, pH 6.8, 20% glycerol, 5% SDS and subjected to SDS-agarose gel electrophoresis, Western blotting, hydrogen fluoride treatment, and assay as described above. The intensity of the bands obtained on the film was measured by video densitometry using a video camera from KAPPA Messtechnik (Gleichen, Germany) and software from Bildanalys (Stockholm, Sweden).
had been assembled. When the cells were cultured for 3 h after radioactive labeling (Fig. 1C), O-glycosylated monomers (O-M) and dimers (O-D) were found by both the α-MUC2N3 and the α-MUC2C2 antisera. The O-glycosylation-sensitive α-MUC2TR antisera, however, did not precipitate these species, but only the remaining non-O-glycosylated monomer and dimer. The O-glycosylated monomer migrated to a position slightly above the non-O-glycosylated dimer on the gel, whereas the O-glycosylated dimer migrated only a short distance from the stacking gel-separation gel interface. No larger bands specifically reacting with the α-MUC2N3 and α-MUC2C2 antisera could be found in the separation or stacking gels. Cells were also labeled for 30 min in the presence of Brefeldin A (Fig. 1B). This drug blocks the traffic of vesicles from the endoplasmic reticulum to the Golgi apparatus but not the traffic in the opposite direction, resulting in a transport of Golgi stack enzymes into the endoplasmic reticulum (17). As a result of this, glycosyltransferases from the proximal parts of the Golgi occur in the endoplasmic reticulum O-glycosylating proteins prematurely. Such O-glycosylation, however, is incomplete, giving smaller sized mucins than the native O-glycosylated molecule. Monomers (Fig. 1, o-M) and dimers (o-D) glycosylated in this way were precipitated by all of the antisera. This included the α-MUC2TR antisera, reacting with a sequence repeated about 100 times in MUC2, whereas the Brefeldin A treatment probably gave a glycosylation of only some of the tandem repeats, allowing the antisera to react with remaining non-O-glycosylated repeats. The partially O-glycosylated bands (Fig. 1, o-M and o-D), but not the non-O-glycosylated monomer and dimer bands (M and D), reacted with the Helix pomatia lectin (not shown). The monomers and dimers observed after Brefeldin A treatment migrated to a position between the non-O-glycosylated and the native O-glycosylated species. This shows that O-glycans decrease the electrophoretic mobility of the MUC2 mucin on SDS-agarose electrophoresis dependent on the amount of glycosylation and that migration on agarose gels could be used for interpreting the glycosylation status of mucins.

Larger MUC2 Species Than O-Glycosylated Dimer Are Insoluble and Contain Nonreducible Intermolecular Bonds—No MUC2 species larger than O-glycosylated dimer were observed on the agarose gels irrespective of the labeling and chase times used. To find out where the predicted larger forms of MUC2 were lost, the cell debris pelleted from the metabolically labeled cells, lysed with detergent and ultrasonicated, was reduced by dithiothreitol. The obtained solubilized material was immunoprecipitated with the α-MUC2N3 antisera, followed by agarose gel electrophoresis (Fig. 2C). Two major MUC2 bands were found, of which the larger showed a partial separation into two. As a comparison, nonreduced MUC2 immunoprecipitated in the normal way from the clarified cell lysate is shown (Fig. 2B). This contains O-glycosylated monomer (O-M) and O-glycosylated dimer (O-D). A corresponding reduced lane was also included to demonstrate that the dimer (O-D) is reducible disappearing upon thiol reduction (Fig. 2A). The monomer (O-M) and dimer (O-D) correspond in size to the two main bands of the reduced insoluble portion. It seems therefore reasonable to believe that the upper band from the insoluble portion (Fig. 2C, O-X) consists of two monomers held together with bonds that are nonreducible by mercaptoethanol and dithiothreitol. This nonreducible material (O-X) is different from the in vivo-occurring reducible dimer (O-D) and will therefore be referred to as X-dimer in this paper. Bands migrating to the same positions as monomer and X-dimer could also be precipitated from the debris pellets by the α-MUC2C2 antisera or by the H. pomatia lectin (not shown). No material at all in the reduced debris...
After deglycosylation, the Western blot was assayed using immunoprecipitations with a-MUC2N3 of LS 174T cell lysate (panel A, reduced; panel B, nonreduced) and of the reduced corresponding cell debris pellet (panel C). Cells were labeled for 2 h, lysed, and ultrasonicated. Insoluble material was pelleted by centrifugation. The pellet obtained was washed in PBS, and the mucin content was solubilized by reduction in 50 mM dithiothreitol and alkylation in 150 mM iodoacetamide. Both the cell lysate and the reduced pellet were then subjected to preimmune immunoprecipitation followed by specific immunoprecipitation with a-MUC2N3. Shown is also a a-MUC2TR-stained Western blot of SDS-agarose gel electrophoresis on mucins insoluble in 6 M guanidinium chloride obtained from LS 174T cells (panel D). These mucins were solubilized by reduction in 10 mM dithiothreitol and alkylation in 30 mM iodoacetamide, prior to purification by three rounds of isopycnic CsCl density gradient ultracentrifugation, in which the fractions were assayed by periodic acid-Schiff (pAS) slot blot. The result from the third centrifugation in 0.5 M guanidinium chloride is shown in panel E. Marked are the fractions pooled and subjected to dialysis, lyophilization, and SDS-agarose gel electrophoresis followed by Western blot. After deglycosylation, the Western blot was assayed using a-MUC2TR, pereoxidase-conjugated secondary antibody, and the ECL reagent (panel D). For D, the same sample as in C was included, and it migrated identically on the gel (not shown). All gels used were 2% Ultrapure (Life Technologies, Inc.). The designation O-M refers to O-glycosylated monomer, O-D to O-glycosylated dimer (in panels A and B), and O-X to pairs of O-glycosylated monomers linked by nonreducible bonds, i.e. X-dimers (in panels C and D).

pellets could be precipitated using the a-MUC2TR antiserum (not shown). This shows that non-O-glycosylated MUC2 species are soluble and not incorporated into insoluble material. It should also be noted that no signs of nonreducible bonds have ever been observed in non-O-glycosylated MUC2 species (10, 11).

MUC2 Mucin, Which Was Insoluble in 6 M Guanidinium Chloride, Also Contained Nonreducible Bonds—Mucins insoluble in 6 M guanidinium chloride have earlier been reported from small intestine (12, 13), where the major part in the rat has been shown to be due to Muc2 (6, 8). Cultivated LS 174T cells were extracted with 6 M guanidinium chloride, and the insoluble mucin complex was pelleted by centrifugation and washed six times. The material was solubilized by dithiothreitol reduction and purified by three rounds of isopycnic density gradient ultracentrifugation in guanidinium chloride (15). The periodic acid-Schiff slot blot intensity of the fractions from the third preparative ultracentrifugation step, in 0.5 M guanidinium chloride, is shown in Fig. 2E, having a peak within the density interval between 1.53 and 1.49 g/ml, as expected for mucins (15). The material from these fractions was pooled and analyzed by SDS-agarose gel electrophoresis to compare the electrophoresis pattern to that obtained by immunoprecipitation. The mucins were Western blotted onto nylon membranes and deglycosylated by gaseous hydrogen fluoride before staining with the tandem repeat antisemur a-MUC2TR. Two bands were found, migrating to the same positions on the gel as monomer and X-dimer, respectively (Fig. 2D). No staining at all was found with a-MUC2TR on a non-deglycosylated control membrane (not shown). Because this antisemur does not bind O-glycosylated MUC2, this finding is further evidence that the species were O-glycosylated. This procedure also confirms that the bands at monomer and X-dimer position on the gels are indeed the MUC2 mucin, because the deglycosylation exposed the epitopes detected by the a-MUC2TR antisemur.

Separation of Monomer and X-dimer by Rate Zonal Ultracentrifugation—As an alternative molecular size separation method to SDS-agarose gel electrophoresis, not dependent on electrical charge, the monomer and X-dimer were also subjected to rate zonal ultracentrifugation. MUC2 mucin insoluble in 6 M guanidinium chloride solubilized by reduction was layered on top of a sucrose gradient, which, after the centrifugation, was recovered as 25 fractions run on agarose gel and assayed by Western blot (Fig. 3A). The intensity of the obtained monomer and X-dimer bands in every fraction was measured by video densitometry (Fig. 3B). As expected for heterogeneously glycosylated molecules, both monomer and X-dimer showed a broad distribution over the gradient. Some of the material migrated to the bottom of the ultracentrifuge tube, probably due to aggregation, but not by covalent linkages because it could be dissolved by boiling in SDS prior to electrophoresis. Such aggregation might also have contributed to the broad distribution over the gradient. Both species, however, showed fairly distinct intensity maxima, probably representing similar glycosylation stages of nonaggregated molecules. These maxima were localized at 29.25% sucrose for the monomer and 31.5% sucrose for the X-dimer, giving sedimentation values of 14 S and 19 S, respectively, as calculated by the method of McEwen (18). As shown in Fig. 2E, both species had the same density, about 1.51 g/ml, as expected for O-glycosylated mucins (15). If the diffusion constant of the O-glycosylated MUC2 monomer is similar to that of monomer units from human cervical mucins in water (19), 4.7 × 10^-6 cm^2/s, our results would give a mass of the MUC2 monomer of about 2.7 × 10^6.

The diffusion constant of the X-dimer is not known, making a calculation of its absolute mass impossible. However, together with the SDS-agarose gel electrophoresis results, the rate zonal ultracentrifugation makes it likely that the X-dimer indeed is a dimer. It also shows that the separation observed on the agarose gels is due to size differences and not, for example, charge.

**Pulse-Chase Analysis of the Appearance of Soluble and Insoluble MUC2 Mucin**—In order to study the appearance of non-O-glycosylated and O-glycosylated soluble MUC2 species, immunoprecipitation with the a-MUC2N3 antisemur (Fig. 4A) was performed by immunoprecipitation with the a-MUC2TR.

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**FIG. 2. Comparison of nonreduced and reduced soluble and insoluble MUC2 mucin on SDS-agarose gel electrophoresis.** Fluororadiography of SDS-agarose gel electrophoresis on immunoprecipitations with a-MUC2N3 of LS 174T cell lysate (panel A, reduced; panel B, nonreduced) and of the reduced corresponding cell debris pellet (panel C). Cells were labeled for 2 h, lysed, and ultrasonicated. Insoluble material was pelleted by centrifugation. The pellet obtained was washed in PBS, and the mucin content was solubilized by reduction in 50 mM dithiothreitol and alkylation in 150 mM iodoacetamide. Both the cell lysate and the reduced pellet were then subjected to preimmune immunoprecipitation followed by specific immunoprecipitation with a-MUC2N3. Shown is also a a-MUC2TR-stained Western blot of SDS-agarose gel electrophoresis on mucins insoluble in 6 M guanidinium chloride obtained from LS 174T cells (panel D). These mucins were solubilized by reduction in 10 mM dithiothreitol and alkylation in 30 mM iodoacetamide, prior to purification by three rounds of isopycnic CsCl density gradient ultracentrifugation, in which the fractions were assayed by periodic acid-Schiff (pAS) slot blot. The result from the third centrifugation in 0.5 M guanidinium chloride is shown in panel E. Marked are the fractions pooled and subjected to dialysis, lyophilization, and SDS-agarose gel electrophoresis followed by Western blot. After deglycosylation, the Western blot was assayed using a-MUC2TR, pereoxidase-conjugated secondary antibody, and the ECL reagent (panel D). For D, the same sample as in C was included, and it migrated identically on the gel (not shown). All gels used were 2% Ultrapure (Life Technologies, Inc.). The designation O-M refers to O-glycosylated monomer, O-D to O-glycosylated dimer (in panels A and B), and O-X to pairs of O-glycosylated monomers linked by nonreducible bonds, i.e. X-dimers (in panels C and D).
antiserum (Fig. 4B) was performed on the soluble parts of the lysates from LS 174T cells that had been pulse-chased for up to 3 days. The O-glycosylated molecules appeared after about 1 h and reached maximum radiolabeling after about 2 h (Fig. 4A). The amounts of both species then quickly decreased to levels that still remained constant in the cells after three days. As shown previously (10, 11), the non-O-glycosylated precursors appeared within 0.5 h and showed maximum intensity after 1 h, as is also shown in Fig. 4B. The non-O-glycosylated monomer decreased to low levels after only a few hours, whereas the dimer, formed from the monomer, was still present in reasonable amounts after 24 h.

The appearance of the insoluble MUC2 mucin was studied by immunoprecipitation with the α-MUC2N3 antiserum on reduced debris pellets from the experiment in Fig. 4 (panel A) and from LS 174T cells metabolically labeled for 2 h and chased for 24 h in the presence of 25 mM ammonium chloride (panel B). The pellets were washed in PBS, the mucin content solubilized by reduction in 50 mM dithiothreitol and alkylated in 150 mM iodoacetamide prior to preimmune immunoprecipitation followed by specific immunoprecipitation with α-MUC2N3. The gel was 2% Ultrapure (Life Technologies, Inc.). O-X and O-M are as in Fig. 2.

8 h is, to our experience, not real but rather a randomly occurring artifact, probably caused by incomplete reduction (incomplete solubilization) or destruction of the antibodies by the reducing or alkylating agents.

Subcellular Fractionation—In an attempt to localize the cellular location for the formation of the insoluble MUC2 molecules, subcellular fractionation was performed, using two different types of homogenization. However, all of the insoluble material after homogenization was recovered in the pellet ob-
tained by centrifugation at 1,400 × g prior to subcellular fractionation (not shown). No nonreducible X-dimers were found in the subcellular fractions. This means that the appearance of insoluble MUC2 could not be studied by subcellular fractionation.

**Nonreducible Bond Formation Does Not Require Low pH**—The N terminus of the MUC2 mucin shows similarities in the localizations of the Cys residues with the von Willebrand factor. Because the von Willebrand factor dimers are known to form polymers stabilized by N-terminal disulfide bonds by a process that requires an acidic pH (9), a similar mechanism has been proposed for the MUC2 mucin. No disulfide-stabilized MUC2 species larger than dimers were detected in this study. To study whether a lowered pH could influence the formation of the nonreducible bonds in MUC2, LS 174T cells were cultured in the presence of ammonium chloride. This neutralizes the acidic pH in the distal Golgi apparatus and secretory vesicles (20). The ratio between monomer and X-dimer was constant after ammonia treatment (Fig. 5B), although the amount of both species was decreased, which we have noticed before. This indicates that the formation of the nonreducible bonds in MUC2 is not pH-sensitive.

**DISCUSSION**

The striking similarities in the localizations of the Cys residues between the N and C termini of MUC2 and the von Willebrand factor made it likely that the assembly followed similar pathways. The present results show that this is not the case, and instead an insoluble form of MUC2 is formed early via intermolecular bonding that cannot be reduced. The present results are dependent on the specificity of the anti-MUC2 antisera available. The antisera a-MUC2N3 and a-MUC2C2 were able to precipitate glycosylated MUC2 monomers and reducible dimers from the soluble cell lysate portion and monomers and nonreducible X-dimers from reduced insoluble cell debris pellet. The conclusion that the immunoprecipitated species were O-glycosylated was based on the observations that they (i) were larger than the non-O-glycosylated species and the incompletely glycosylated species produced upon Brefeldin A treatment (Fig. 1); (ii) appeared later in time during pulse-chase than the non-O-glycosylated species (Fig. 4A); (iii) showed nonreducible size polydispersity on the gels, most probably due to heterogeneous glycosylation, (iv) were localized to the Golgi apparatus according to subcellular fractionation (11), (v) reacted with the H. pomatia lectin (11), and (vi) were not immunoprecipitated by a-MUC2TR (Fig. 1). In addition, monomers and X-dimers from the guanidinium chloride insoluble preparation were regarded as O-glycosylated because they (vii) reacted with the a-MUC2TR antiserum on Western blot only after deglycosylation (Fig. 2D), (viii) had a density of 1,51 g/ml (Fig. 2E), as expected for O-glycosylated mucins (15), and (ix) had higher sedimentation coefficients on ultracentrifugation than (SDS-treated) non-O-glycosylated species, as for monomers 14 S (Fig. 3B) and 8 S (11), respectively. It should be noted that the a-MUC2TR antiserum is much more efficient in immunoprecipitating MUC2 than the O-glycosylation-insensitive antisera, as shown in Fig. 1. This makes immediate kinetic comparisons between the amounts of O-glycosylated and non-O-glycosylated MUC2 mucin, immunoprecipitated with the different types of antisera, impossible. The reason for the difference in effectiveness is not totally clear, but it might include different antibody titers and a large consumption of O-glycosylation-insensitive antibodies by epitopes on nonlabeled MUC2 mucin stored in the cells. The O-glycosylation-insensitive antisera are directed against nonrepeated protein epitopes, whereas the epitopes of α-MUC2TR are tandemly repeated about 100-fold in every molecule. This might also contribute to the differences wherein the MUC2TR antisera should be less sensitive for the peptide conformation as caused by, for example, the high number of probable Cys-linkages in the non-tandem repeat regions. Because the MUC2 apomucin is very large, repeated binding sites might also minimize mechanical dissociation from the immunoprecipitin during the washing steps.

The two bands observed on the agarose gels of reduced insoluble MUC2 mucin were regarded as a monomer and a dimer (called X-dimer), because they migrated similarly on the gel to the monomer and reducible dimer of the soluble MUC2 mucin. Because mobility comparisons are uncertain when dealing with broad bands caused by heterogeneous O-glycosylation, and because separation could be due also to charge, rate zonal ultracentrifugation was performed to verify that the monomer and X-dimer were indeed of different size. This analysis suggested a mass of 2.7 MDa for the monomer using a previously published diffusion coefficient for human cervical mucins (19). With a predicted size for the non-O-glycosylated MUC2 monomer of 600 kDa, this suggests that the mass is made up of about 80% O-glycans, which is within the usual range of mucin glycosylation (13, 21). The lack of information on the diffusion constant for the X-dimer makes it impossible to calculate its mass and state that it is indeed a dimer. For human cervical mucins, which have MUC5AC, MUC5B, MUC2, and MUC6 as known gel-forming components (22, 23), it has been shown that oligomers, with the average size of four monomer units, have a sedimentation coefficient about twice that of the monomers (40.4 for oligomers and 19.2 S for monomers in water) (19, 21). The sedimentation coefficient of the X-dimer was only 1.3–1.4 times larger than that of monomer, and if oligomerization does not affect the diffusion properties of MUC2 and human cervical mucins in very different ways, these figures may suggest that the X-dimer consists of two or, less likely, three monomer units. The ratio between sedimentation coefficients for non-O-glycosylated, SDS-treated MUC2 dimer and monomer was just above 1.2, as was the ratio between apoB100 and apoB553/48 (11). Dimerization of O-glycosylated and non-O-glycosylated MUC2 apomucins may affect the diffusion constants in proportional ways, if O-glycans and bound SDS, respectively, cause similar stretching of the tandem repeat regions. If this is the case, one should thus expect identical ratios. The small differences found for the ratios, together with the results from the SDS agarose gel electrophoresis support the interpretation that the X-dimer is indeed a dimer.

The finding of nonreducible bonds associated with MUC2 mucin insolubility was an unexpected phenomenon. Because the insoluble MUC2 portion had to be solubilized by reduction prior to analysis, the rate of disulfide bond-stabilized oligomerization could not be investigated. It is therefore impossible to say whether nonreducible links between disulfide bond-stabilized dimers are enough to give the insolubility or whether the mucins are further oligomerized by disulfide bonds before the nonreducible links are assembled. If the von Willebrand factor analogy is correct, no further oligomerization than dimerization should occur until the trans-Golgi network, because such oligomerization requires low pH. It seems possible that the nonreducible bonds are assembled earlier because their formation does not require low pH, as shown by ammonium chloride treatment (Fig. 5B). As already mentioned, no nonreducible bonds could be seen using α-MUC2TR either in supernatant (10, 11) or pellet (not shown), showing that no non-O-glycosylated species were linked with such bonds. This indicates a localization of the nonreducible bond formation in the Golgi rather than in the endoplasmic reticulum. Attempts to verify

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2 M. A. B. Axelsson, N. Askar, and G. C. Hansson, unpublished data.
this theory by two different subcellular fractionation protocols failed, as the insoluble MUC2 mucin was found already in the cell debris routinely pelleted after homogenization, and thus was excluded from the ultracentrifugation step. It is not clear why the insoluble mucins were pelleted in these experiments when they should have been encapsulated into membrane vesicles. One possibility for this could be that they gave the whole vesicle a density high enough for pelleting; another possibility is that in some way caused the vesicles to rupture. However, vesicles containing Golgi markers were obtained at subcellular fractionation and contained soluble O-glycosylated mono- and dimers (11). This might suggest that there could exist different vesicles containing soluble and insoluble MUC2 mucins.

The nature of the nonreducible bonds is presently unknown. The fact that the linkages are nonreducible and survive 6 m guanidinium chloride or boiling in 5% SDS suggests an uncomplicated covalent nature. This linkage could be between amino acids within the primary MUC2 sequence. Another possibility is that the bonding is via smaller linking proteins or peptides. Mucins have previously been proposed to be polymerized via a linking peptide, but an isolated linking peptide has been shown to exist. One possibility for this could be that they gave the whole cell debris routinely pelleted after homogenization, and thus failed, as the insoluble MUC2 mucin was found already in the cellular fraction and contained soluble O-glycosylated mono- and dimers (11). This might suggest that there could exist different vesicles containing soluble and insoluble MUC2 mucins.

Nonreducible Bonds of Insoluble MUC2 Mucin

The X-dimers, obtained by reduction, are obviously soluble because they can be immunoprecipitated. Our results contradict that such soluble particles exist in vivo in substantial amounts (Fig. 2A). Thus, nonreducible bonds between monomers seem to be absent or rare in vivo. If monomers are not forming nonreducible links, this suggests a conformational change of the MUC2 molecule upon disulfide-stabilized dimer formation, allowing the formation of the nonreducible bond. This would imply that the sites for the two different kinds of bonds are localized near each other on the molecule, in the C terminus if the von Willebrand factor analogy is correct. If the nonreducible bonds are C-terminal and if they are formed in the Golgi, one may speculate on the presence of a specific enzyme system catalyzing their formation. Attempts were made to move an eventual bond formation catalyzing enzyme system from the Golgi to the endoplasmic reticulum by the help of Brefeldin A. This did not, however, give rise to any nonreducible bonds after 30 min either in the soluble or insoluble portion of the cell lysate (not shown). If the formation of the nonreducible bonds takes place in the early Golgi, the C terminus heparin binding domain on the MUC2 mucin could participate in some way, because this domain is cleaved off already in the cis-Golgi apparatus (26). Such a function of this domain might be to bind heparan sulfate and thus orient the MUC2 dimers in a way facilitating nonreducible bond formation.

In Fig. 2B, the X-dimer band consists of two equally strong adjacent bands. The presence and intensity of the upper of these is, in our experience, highly variable, and often only one X-dimer band can be found. However, the intensity of this additional band had a tendency to increase upon prolonged pulse-chase times (Fig. 5A). The variable additional bands indicate that some MUC2 monomer residues might be involved in more than one nonreducible bond and that the formation of the second bond could be delayed. The presence of nonreducible bonds within MUC2 from the intestine has been proposed by Carlstedt et al. (27). They have shown that reduced MUC2 from intestinal tissue can form a ladder of bands upon agarose gel electrophoresis, suggesting that more than two nonreducible bonds can be formed in vivo.

Mucins that are insoluble in 6 m guanidinium chloride are consistently found in the small intestine (12, 13) and, to a more variable extent, in for example the gall bladder (14). The physiological function of mucin insolubility could be to improve the mucus barrier in water-filled lumen organs. The finding that both guanidinium chloride insoluble MUC2 mucins and the metabolically labeled water-insoluble MUC2 mucins contain the unexpected X-dimers suggests that water and guanidinium chloride insolubilities are reflecting an identical phenomenon. This could propose that one of the key events in the formation of the very resistant and insoluble MUC2 is the formation of nonreducible bonds. How the bonds give rise to insolubility is not clear. It could also be the case, of course, that they are not vital for insolubility but are only synthesized with disulfide bond-stabilized polymerization and/or oligosaccharide elongation, yielding the insolubility. An attractive hypothesis, however, could be that the nonreducible bonds serve as cross-links between linear MUC2 oligomers.

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