Dimerization of the Human MUC2 Mucin in the Endoplasmic Reticulum Is Followed by a N-Glycosylation-dependent Transfer of the Mono- and Dimers to the Golgi Apparatus

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Pulse-chase experiments in the colon cell line LS 174T combined with subcellular fractionation by sucrose density gradient centrifugation showed that the initial dimerization of the MUC2 apomucin started directly after translocation of the apomucin into the rough endoplasmic reticulum as detected by calnexin reactivity. As the mono- and dimers were chased, O-glycosylated MUC2 mono- and dimers were precipitated using an O-glycosylation-insensitive antiserum against the N-terminal domain of the MUC2 mucin. These O-glycosylated species were precipitated from the fractions that were collected with the galactosyltransferase activity during the subcellular fractionation, indicating that not only MUC2 dimers but also a significant amount of monomers are transferred into the Golgi apparatus. Inhibition of N-glycosylation with tunicamycin treatment slowed down the rate of dimerization and introduced further oligomerization of the MUC2 apomucin in the endoplasmic reticulum. Results of two-dimensional gel electrophoresis demonstrated that these oligomers (putative tri- and tetramers) were stabilized by disulfide bonds. The non-N-glycosylated species of the MUC2 mucin were retained in the endoplasmic reticulum because no O-glycosylated species were precipitated after inhibition by tunicamycin. This suggests that N-glycans of MUC2 are necessary for the correct folding and dimerization of the MUC2 mucin.

The mucus layer on the epithelial surface of the mucous membrane is mainly made up of water and the gel-forming components, the mucus glycoproteins, or mucins, consisting of more than 50% O-linked oligosaccharides (1, 2). The peptide chain of mucins has domains with a high abundance of Ser, Thr, and Pro, usually in repetitive sequences (tandem repeats). The oligosaccharide chains are O-linked to Ser and Thr, thereby forming highly glycosylated domains or mucin domains.

The apoprotein of the human intestinal MUC2 mucin, which is fully sequenced, contains two mucin domains with large amounts of the amino acids Thr, Pro, and Ser (3, 4); the larger of these domains consists of well conserved 23-amino acid repeated sequences. The mucin domains are flanked by Cys-rich domains; one C-terminal, one N-terminal, and one central domain. The carboxyl and amino termini of the human MUC2 mucin and the blood coagulation factor, the von Willebrand factor (vWF),1 show sequence similarities in the positions of the cysteines. The vWF forms disulfide-bonded dimers between two C termini, and the N termini mediate further oligomerization (5). We have earlier shown that the human MUC2 apomucin forms dimers before being O-glycosylated (6). To study the initial assembly of the human MUC2 mucin in more detail, pulse-chase labeling and subcellular fractionation has been performed on LS 174T cells. An early dimerization was observed in the endoplasmic reticulum; there was no further oligomerization, and the dimerization was followed by O-glycosylation of the mono- and dimer in the Golgi apparatus. Tunicamycin treatment slowed down the dimerization rate, introduced formation of putative tri- and tetramers, and prevented transfer of the apomucin into the Golgi apparatus.

MATERIALS AND METHODS

Antibodies—A rabbit antiserum α-MUC2TR (PH1900), against a synthetic peptide based on the tandem repeat region of the human MUC2 apoprotein, was raised (6). The rabbit antiserum, PH1491 (later referred to as α-MUC2N3), prepared against a synthetic peptide (CPKDRPIYEDLKK) based on amino acids 1167–1180 on the N terminus of the human MUC2 apomucin was prepared as follows. A New Zealand White rabbit was immunized once with 500 μg of peptide conjugated to 400 μg of keyhole limpet hemocyanin in Freund’s complete adjuvant and then twice with 250 μg of peptide conjugated to 200 μg of keyhole limpet hemocyanin in Freund’s incomplete adjuvant.

The specificity of the α-MUC2N3 antiserum was tested by inhibition with the immunizing peptide. First, the optimum concentration for inhibition was tested by inhibiting binding to the immobilized peptide as described previously (6). One mg of specific and nonrelevant peptide/25 μl of antiserum was used for inhibiting immunoprecipitation, performed as described below.

Tissue Culture—The colon adenocarcinoma cell line LS 174T (ATCC CL 188) was cultured as described (6).

Metabolic Labeling—Cells were seeded at a concentration of about 50 × 106 cell/28-cm² Petri dish the day before labeling. Cells were preincubated in methionine-free medium for 1 h followed by radiolabeling with 150 μCi 35S methionine (Redivue Promix [35S]) labeling mix, Amersham Pharmacia Biotech)/dish. When cells were pulse-labeled for 2 min, 500 μCi of labeling mix/dish was added. In pulse-chase experiments cells were chased with culture medium supplemented by 15 μg of Met/ml of medium and 25 μg of Cys/ml of medium. Cells were washed and lysed as described (6), with the addition of 5 mM N-ethylmaleimide (NEM) in the lysis buffer. Inhibition of N-glycosylation was performed by incubating cells in 20 μg of tunicamycin (Calbiochem)/ml of methionine-free medium 1 h prior to labeling, as well as during pulse and chase.

Subcellular Fractionation—Cells were washed twice with 5 ml of 250 mM sucrose and twice in 5 ml 50 mM sucrose. Cells were harvested in 500 μl of 50 mM sucrose including protease inhibitors (110 μg/ml phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, 60 μg/ml leupeptin, 3.8 mM ethylene glycol bis(β-aminoethyl ether)-N,N,N′,N′-tetrasulfonic acid disodium salt) and lysed as described (6). One mg of specific and nonrelevant peptide/25 μl of antiserum was used for inhibiting immunoprecipitation, performed as described below.

1 The abbreviations used are: vWF, von Willebrand factor; NEM, N-ethylmaleimide; PAGE, polyacrylamide gel electrophoresis.
μg/ml calpain inhibitor I) using a cell scraper. The cell suspension was homogenized with 15 strokes in a Dounce homogenizer using a tight pestle, and the sucrrose concentration was adjusted to 250 mm by an additional 5 homogenization strokes. Washing and homogenization were performed on ice. The homogenized suspension was centrifuged at 4000 rpm at +4 °C for 10 min, and the supernatant was recovered. The cell pellet was washed twice in 250 μl of 250 mm sucrose, and all supernatants were pooled and carefully placed on a sucrose gradient in a centrifuge tube containing a 4-ml gradient of 35–50% sucrose on top of a 400-μl 65% sucrose cushion. All sucrose solutions used contained 3 mM imidazole and were at pH 7.4. Ultracentrifugation was performed 50,000 rpm at 12 °C for 3–5 h in a Beckmann vertical rotor (Vti, 65.2). Fractions were collected from the bottom. Phosphate-buffered saline was added to a final volume of 1.2 ml, and 300 μl of lysate buffer (250 mM Tris-HCl, pH 7.4, 750 mM NaCl, 25 mM EDTA, 5% (v/v) Triton X-100), including protease inhibitors (as above) and 5 mM NEM, was added. Samples were sonicated three times for 2 s each (intensity 15) on a MSE Soniprep 100. The fractions collected were analyzed for NADPH cytochrome c reductase activity and galactosyltransferase activity (7, 8). Fractions were also analyzed by 12% SDS-PAGE (9) followed by Western blot, and calnexin was visualized using a monoclonal α-calnexin antibody (Transduction Laboratories, Lexington, KY) and ECL (Amersham Pharmacia Biotech), followed by video densitometry.

**Immunoprecipitation—**Immunoprecipitated samples were dissolved in nonreducing or reducing sample buffer at 95 °C for 5 min as described (6). Samples were analyzed on SDS-agarose gels using 0.8% stacking gel of SeaKem Gold (FMC) and a separation gel of 1.3% agarose (FMC) or 2% Ultrapure agarose (Life Technologies, Inc.). Electrolyte solutions used contained 0.8% sucrose, and the dimer banded at 31.5% sucrose, giving estimated sedimentation coefficients of about 8.3 and 10.2 Svedberg units, respectively, using the method of McEwen (12). The ratio of the sedimentation coefficients for the dimer and monomer was estimated to be just above 1.2. Analysis of different apoB species with known masses by the same method showed a sedimentation coefficient of 6.0 S for apoB-100 (512 kDa) and apoB-72 (369 kDa), and apoB-53 (271 kDa) were metabolically labeled with an 0.1% SDS and 10 mM dithiothreitol for 5 min at 95 °C. Samples were centrifuged, and the SDS concentration in the supernatant was adjusted to 0.1% with 0.2 M phosphate buffer. N-Octylglycoside was added at an amount 10-fold greater than the amount of SDS followed by the addition of 2 units of PNGase F (Boehringer Mannheim). Samples were incubated at 37 °C for 20 h, and iodoacetamide was added at an amount 2.5-fold greater than the amount of dithiothreitol, followed by incubation for 30 min in the dark. Samples were then immunoprecipitated with the α-MUC2TR antisera as described above.

**O-Glycosidase Digestion—**Immunoprecipitated samples were washed and incubated in 100 μl of 0.2 M phosphate buffer, pH 8.0, containing 1% SDS and 10 mM dithiothreitol for 5 min at 95 °C. Samples were centrifuged, and the SDS concentration in the supernatant was adjusted to 0.1% with 0.2 M phosphate buffer. N-Octylglycoside was added at an amount 10-fold greater than the amount of SDS followed by the addition of 2 units of PNGase F (Boehringer Mannheim). Samples were incubated at 37 °C for 20 h, and iodoacetamide was added at an amount 2.5-fold greater than the amount of dithiothreitol, followed by incubation for 30 min in the dark. Samples were then immunoprecipitated with the α-MUC2TR antisera as described above.

**SDS-Agarose and Autoradiography—**Immunoprecipitated samples were dissolved in nonreducing or reducing sample buffer at 95 °C for 5 min as described (6). Samples were analyzed on SDS-agarose gels using 0.8% stacking gel of SeaKem Gold (FMC) and a separation gel of 1.3% Ultrapure agarose (Life Technologies, Inc.) and 1.3% Sea Plaque agarose (Biorad). Two-dimensional gel electrophoresis was performed at 10 mA for 18 h using a discontinuous buffer system (6, 9). Gels were fixed in 30% ethanol and 10% acetic acid, incubated in Amplify (Amersham Pharmacia Biotech), dried, and exposed to film at ~70 °C as described (6). Two-dimensional gel electrophoresis was performed by analyzing the first dimension under nonreducing conditions. The lane was cut out, and the strip was incubated for 30 min in 0.5 M Tris-HCl, 0.1% SDS, pH 6.8, containing 50 mM dithiothreitol and then placed on top of another gel. The strip was overlaid with 0.8% (w/v) agarose gel containing 10 mM dithiothreitol.

**Rate Zonal Ultracentrifugation—**Metabolically labeled LS 174T cells were immunoprecipitated with the α-MUC2TR antisera, and samples were dissolved in phosphate-buffered saline containing 2% SDS at 95 °C for 5 min. Samples were layered on top of a 5-ml linear 10–40% (w/v) sucrose gradient containing 0.1% SDS. Ultracentrifugation was performed at 50,000 rpm at 20 °C for 14 h in a Beckmann swing-out rotor (SW 55 Ti). Fractions were collected from the bottom of the tube and analyzed by 3–5% SDS-PAGE. McA-RH7777 cells expressing apoB-100 (512 kDa) and apoB-48 (246 kDa) or the recombinant apoB-80 (410 kDa), apoB-72 (369 kDa), and apoB-53 (271 kDa) were metabolically labeled, and the cell supernatants were collected, immunoprecipitated with an α-apoB antisera, subjected to rate zonal ultracentrifugation as described above, and analyzed by 3–15% PAGE (10, 11). The sedimentation coefficients for the MUC2 mono- and dimer and apoB-variants were estimated according to the method of McEwen (12).

**RESULTS**

**Sedimentation of the Non-O-glycosylated Mono- and Dimer by Rate Zonal Ultracentrifugation—**By immunoprecipitation of LS 174T cells using the α-MUC2TR antisera directed against the large tandem repeat of the MUC2 mucin, we have shown that the human MUC2 apomucin forms a disulfide-stabilized oligomer that is interpreted as a dimer (6). To confirm that the oligomeric species was a dimer, sedimentation of immunoprecipitated mono- and dimers were analyzed by rate zonal ultracentrifugation. The gradient was recovered form the bottom of the tube into 34 fractions, and the sucrose concentration was measured, and each fraction was analyzed by SDS-PAGE. The intensity of the mono- and dimer bands was estimated by densitometry and plotted in Fig. 1. The monomer banded at 29% sucrose, and the dimer banded at 31.5% sucrose, giving estimated sedimentation coefficients of about 8.3 and 10.2 Svedberg units, respectively, using the method of McEwen (12). The ratio of the sedimentation coefficients for the dimer and monomer was estimated to be just above 1.2. Analysis of different apoB species with known masses by the same method showed a sedimentation coefficient of 6.0 S for apoB-100 (512 kDa) and a ratio of S values of about 1.2 between apoB-100 and apoB-48/53. That this ratio is similar to the one for the two MUC2 species suggests that the MUC2 monomer forms a dimer and not a trimer or larger oligomeric species at the initial biosynthetic step.

**O-Glycosylated Mucins**—An antiserum (α-MUC2N3), raised against a peptide from the non-O-glycosylation N terminus parts of MUC2, has now been used to study the MUC2 biosynthesis also after initiation of O-glycosylation. Immunoprecipitation of metabolically labeled LS 174T cells using this antiserum followed by analysis with agarose (2% Ultrapure, Life Technologies, Inc.) and ECL (Amersham Pharmacia Biotech), followed by video densitometry and plotted in Fig. 1. The monomer banded at 29% sucrose, and the dimer banded at 31.5% sucrose, giving estimated sedimentation coefficients of about 8.3 and 10.2 Svedberg units, respectively, using the method of McEwen (12). The ratio of the sedimentation coefficients for the dimer and monomer was estimated to be just above 1.2. Analysis of different apoB species with known masses by the same method showed a sedimentation coefficient of 6.0 S for apoB-100 (512 kDa) and a ratio of S values of about 1.2 between apoB-100 and apoB-48/53. That this ratio is similar to the one for the two MUC2 species suggests that the MUC2 monomer forms a dimer and not a trimer or larger oligomeric species at the initial biosynthetic step.

**O-Glycosylated Monomers and Dimers of the Human MUC2 Mucin—**An antisera (α-MUC2TR), described in an accompanying article (13), gave similar results to the α-MUC2N3 antisera. Precipitation of the α-MUC2N3 antisera with the peptide used for immunization inhibited the precipitation of these two MUC2 species (results not shown). Bands precipitating to the same positions were also found when the N-acetylgalactosamine binding lectin from H. pomatia was used for precipitation. That these bands were not precipitated with the O-glycosylation-sensitive antisera, α-MUC2TR, but rather with the H. pomatia lectin suggested that the two MUC2 bands were O-glycosylated. The upper and weaker of the two α-MUC2N3-precipitated bands disappeared upon reduction of
the disulfide bonds, whereas the lower band remained at the same position. A probable explanation for the disappearance of the larger band upon reduction was that the upper band was a disulfide-stabilized dimer of the lower band. To further illustrate this, a two-dimensional gel electrophoresis was performed analyzing the sample under nonreducing conditions in the first dimension and under reducing conditions in the second (Fig. 2B). The upper band migrated to the same position as the lower band after reduction, supporting the interpretation that the upper band was a dimer of the lower band. The bands were diagonally oriented on the two-dimensional gel, reflecting a nonreducible size polydispersity, probably due to O-glycosylation heterogeneity.

**biosynthesis of the MUC2 Apomucin as Studied by Subcellular Fractionation**—To determine the intracellular localization of the non-O-glycosylated and O-glycosylated MUC2 species, subcellular fractionation by sucrose density gradient centrifugation was performed on LS 174T cells. The gradient was recovered from the bottom in 14 fractions, which were analyzed for calnexin (marker for the rough endoplasmic reticulum), NADPH cytochrome c reductase activity (marker for the smooth endoplasmic reticulum), and galactosyltransferase activity (marker for the Golgi complex) (Fig. 3). The sucrose gradient was adjusted to obtain endoplasmic reticulum fractions with lower sucrose density, and the second smallest showed a corresponding relative migration of the O-glycosylated dimer, suggesting that it was the non-O-glycosylated dimer (non- O-D).

** oligomerization of Non-N-glycosylated MUC2 Apomucins**—The importance of N-glycans in dimerization and further processing of the MUC2 mucin in LS 174T cells was studied. Cells were treated with increasing amounts of tunicamycin, and an optimal concentration completely inhibiting N-glycosylation (20 μg of tunicamycin/ml of culture medium) was chosen for the subsequent experiments (results not shown). Metabolically labeled LS 174T cells were treated with (Fig. 6A, lanes 2 and 4) and without (lanes 1 and 3) tunicamycin, followed by immunoprecipitation using the α-MUC2TR antiserum. Two major and two minor bands were observed when analyzing samples from tunicamycin-treated cells under nonreducing conditions (Fig. 6A, lane 2). When the sample from tunicamycin-treated cells was analyzed under reducing conditions, only one band was shown (lane 4), migrating as the lowest band observed under nonreducing conditions (lane 2). The reduced band migrated as the monomer treated with PNGase F, an enzyme eliminating most of the N-glycans from the protein core (lane 5). This also made it likely that the inhibition of N-glycosylation by tunicamycin was complete. The lowest band upon tunicamycin treatment (lane 2) was thus the non-N-glycosylated MUC2 monomer (Fig. 6A, non-N-M), and the second smallest showed a corresponding relative migration to the O-glycosylated dimer, suggesting that it was the non-O-glycosylated dimer (non-O-D).

To determine the oligomeric state of the two minor bands and to verify the interpretation of the two major ones found in Fig. 6A, lane 2, two-dimensional gel electrophoresis was performed. Metabolically labeled LS 174T cells were immunoprecipitated with the α-MUC2TR antiserum and analyzed under nonreducing conditions in the first dimension and under reducing conditions in the second dimension (Fig. 6B). All four non-reduced bands migrated to the same position on the gel after reduction, determining the nature of the non-N-glycosylated mono- and dimer and suggesting the formation not only of
dimers but also of aggregates corresponding to putative trimers (non-\(\text{N}-\text{A1}\)) and tetramers (non-\(\text{N}-\text{A2}\)) in tunicamycin-treated cells.

The Rate of Dimerization and O-Glycosylation in Tunicamycin-treated Cells—To follow the rate of dimerization, further oligomerization, and O-glycosylation of the MUC2 apomucin in normal and tunicamycin-treated cells, LS 174T cells were metabolically labeled for 30 min followed by a chase for 0–5 h. Serial immunoprecipitations were performed on the cell lysates starting with the \(\alpha\)-MUC2TR antiserum (Fig. 7A), followed by precipitation with the \(\alpha\)-MUC2N3 antiserum (Fig. 7B) and analysis under nonreducing conditions by SDS-agarose gel electrophoresis. In the nontreated cells, the formation of N-glycosylated mono- and dimers during the first hour was fol-

FIG. 3. Subcellular fractionation of the human colon carcinoma cell line LS 174T. Cells were homogenized in sucrose and subcellularly fractionated by sucrose density gradient centrifugation. The centrifugation tubes were unloaded into 14 fractions with 12 drops in each. All fractions were analyzed for calnexin (●), NADPH-cytochrome c reductase activity (○), galactosyltransferease activity (□), and the sucrose (w/w) concentration (+). The enzyme activities and amount of calnexin are expressed in arbitrary units.

FIG. 4. Distribution of MUC2 apoproteins in the endoplasmic reticulum and the Golgi complex. Metabolic labeling of LS 174T cells was performed for 2 h, followed by sucrose density gradient centrifugation. The centrifugation tubes were unloaded into a lysis buffer containing NEM (14 fractions, as described in Fig. 3). Serial immunoprecipitations were performed on the fractions starting with the \(\alpha\)-MUC2TR antiserum (A) followed by the \(\alpha\)-MUC2N3 antiserum (B). Samples were analyzed under nonreducing conditions by SDS-agarose (1.3% Ultrapure, Life Technologies, Inc., and 1.3% Sea Plaque, FMC). The designation \(M\) refers to monomer, \(D\) to dimer, \(O-M\) to O-glycosylated monomer, \(O-D\) to O-glycosylated dimer, and \(IF\) to stacking-separation gel interface.

FIG. 5. Intracellular localization of the dimerization of the human MUC2 apomucin. LS 174T cells were pulse-labeled for 2 min, followed by chase for 0, 10, and 20 min (A, B, and C, respectively). Subcellular fractionation by sucrose density gradient centrifugation was performed on the cell lysates, and 14 fractions were collected and pooled in pairs in lysis buffer containing NEM, followed by immunoprecipitation using the \(\alpha\)-MUC2TR antiserum. Precipitated samples were analyzed under nonreducing conditions by SDS-agarose (1.3% Ultrapure, Life Technologies, Inc., and 1.3% Sea Plaque, FMC). Designations are as in Fig. 2.
followed by a decrease in the amount during the next hours. As the non-O-glycosylated mono- and dimers of MUC2 started to disappear after about 3 h, the α-MUC2N3 antisera started to precipitate O-glycosylated MUC2 mono- and dimers. In tunicamycin-treated cells, formation of non-O-glycosylated mono- and dimers was also observed, but the rate was considerably slower than in the nontreated cells. With increased chase times, also, further oligomerization of the non-O-glycosylated MUC2 was observed. No O-glycosylated bands were observed on the autoradiograms when immunoprecipitation was performed using the α-MUC2N3 antisera. These results suggest that no non-O-glycosylated MUC2 apomucins were transported into the Golgi apparatus. Chase times up to 10 h were tested and did not show any O-glycosylated species precipitated with the α-MUC2N3 antisera (results not shown).

**Tunicamycin-dependent Oligomerization in the Endoplasmic Reticulum**—To determine the intracellular localization of the non-O-glycosylated MUC2 oligomeric forms, subcellular fractionation was performed (Fig. 8). Tunicamycin-treated cells were labeled for 30 min and chased for 2 h, followed by subcellular fractionation according to Fig. 3. After centrifugation, the tube was unloaded into 14 fractions, pooled in pairs, followed by immunoprecipitation using the α-MUC2TR antisera and analysis under nonreducing conditions by SDS-agarose gel electrophoresis. Already, the heaviest endoplasmic reticulum fraction revealed not only the non-O-glycosylated monomer and dimers, but also the aggregates corresponding to putative trimers (non-N-A1) and tetramers (non-N-A2). The tunicamycin-dependent oligomerization thus occurs early in the endoplasmic reticulum. The intensity of the four non-O-glycosylated bands decreased and was very weak over the fractions corresponding to the Golgi fractions, suggesting that these were not transported to the Golgi apparatus. This was also shown by the inability to precipitate any O-glycosylated homologues from these fractions by using the α-MUC2N3 antisera (not shown).

**DISCUSSION**

In earlier studies on the biosynthesis of human MUC2 apomucin in the colon carcinoma cell line LS 174T (6), we showed that the apomucin forms a non-O-glycosylated oligomer, as determined by two-dimensional gel electrophoresis. This oligomeric form was interpreted as a dimer based on the size estimated by SDS-PAGE. Sedimentation of the non-O-
glycosylated mono- and dimer by rate zonal ultracentrifugation has now been performed on SDS-treated samples. The estimated sedimentation coefficients of the mono- and dimer were 8.3 and 10.2 S, respectively. These sedimentation coefficients cannot be compared with sedimentation coefficients of globular proteins and can thus only be used to compare the mass between the two species. Because the sedimentation coefficient of a particle is proportional to the mass, assuming a similar diffusion constant of the di- and monomer, the relationship between their sizes is estimated to be just about 1.2. The ratio of the S values for SDS-treated apoB-100 and apoB-48/52, having a relation in mass of 1.2, was also determined and found to be about 1.2, suggesting a similar ratio for a doubled mass for other proteins also. These results, together with the migration in SDS-PAGE and SDS-agarose gel electrophoresis, strongly suggest that the monomer of the human MUC2 mucin forms a dimer in the initial step of its biosynthesis.

To study the initial biosynthesis of the human MUC2 mucin in more detail, subcellular fractionation by sucrose gradient centrifugation on LS 174T cells has now been performed. Fractions containing the rough endoplasmic reticulum were well separated from the Golgi apparatus as judged from the presence of calnexin and the absence of galactosyltransferase activity (Fig. 3). On the other hand, less dense parts of the endoplasmic reticulum, containing NADPH cytochrome c reductase activity, were not completely separated from the fractions containing galactosyltransferase activity. Within the first minutes of biosynthesis, both the MUC2 mono- and dimers were observed in the endoplasmic reticulum. This showed that the dimerization occurs directly after translation of the apoprotein. Both the monomer and dimer of the MUC2 apomucin are found in fractions containing lighter parts of endoplasmic reticulum, proposing that the process of folding and dimerization might continue when MUC2 is moved within the reticulum.

Studies on the biosynthesis of the non-O-glycosylated mono- and dimers of the MUC2 apomucin showed that the amounts of these had their maximum 30 min to 1 h after labeling and that both declined simultaneously as the chase time increased. This is in accord with the biosynthesis of the vWF, in which dimers of the MUC2 apomucin showed that the amounts of the mono- and dimers might continue when MUC2 is moved within the reticulum, proposing that the process of folding and dimerization continues in fractions containing lighter parts of endoplasmic reticulum. It was also verified by two-dimensional gel electrophoresis that both O-glycosylated mono- and dimers were found in fractions containing lighter parts of endoplasmic reticulum. It was also verified by two-dimensional gel electrophoresis that both O-glycosylated monomers and dimers from the rough endoplasmic reticulum because no glycosylated MUC2 mono- and dimers were found in tunicamycin-treated cells, proposing that the process of folding and dimerization might continue when MUC2 is moved within the reticulum.

A comparison of the amount of non-O-glycosylated mono- and dimer being transferred from the endoplasmic reticulum to the Golgi apparatus is difficult to make because the α-MUC2N3 antiserum is less efficient in precipitating MUC2 compared with the α-MUC2TR antiserum. This means that these immunoprecipitations could not be used to compare the amount of MUC2 apomucin transported into the Golgi apparatus. However, further studies of the MUC2 apomucin assembly show the formation of larger and more complex forms of the O-glycosylated MUC2 apomucins in the Golgi apparatus (13). The relatively low intensity of the bands representing the O-glycosylated forms of the MUC2 apomucin precipitated by the α-MUC2N3 antiserum could then not only depend on this antiserum but also on the transformation of O-glycosylated species into larger, insoluble MUC2 species not found using the present approach.

By treating LS 174T cells with tunicamycin, the importance of addition of N-glycans to the growing MUC2 apomucin was analyzed. By the mobility shift observed on the gel between the apomucins precipitated from tunicamycin-treated and nontreated cells (Fig. 6A), it was evident that the N-glycosylation of the MUC2 apomucin was inhibited. Studies on the biosynthesis of the human MUC2 apomucin after treatment with tunicamycin have been reported earlier (15, 16), but none of them showed any difference in mobility between the N-glycosylated and the non-N-glycosylated MUC2 apomucins. Despite the lack of N-glycans, the human MUC2 apomucin formed dimers in the endoplasmic reticulum. Pulse-chase experiments showed that this dimerization was delayed compared with dimerization in the nontreated cells. The lower rate of dimerization in tunicamycin-treated cells could be due to misfolding of the protein, thus allowing for the formation of incorrect disulfide bonds. As observed in Fig. 6A, large amounts of non-N-glycosylated MUC2 mono- and dimers still appear after a 5-h chase as compared with a rapid decrease of mono- and dimer after a 2-h chase in nontreated cells. The human non-N-glycosylated MUC2 apomucins were not transported out of the endoplasmic reticulum because no O-glycosylated MUC2 monomers and dimers were found in tunicamycin-treated cells, proposing the idea that the mono- and dimers formed were not correctly folded and trapped in the endoplasmic reticulum control machinery. In a pulse-chase experiment with a 10-h chase, the amount of mono- and dimers was lower than it was after a 5-h chase, but still no MUC2 species were precipitated with the α-MUC2N3 antiserum (results not shown). These observations suggest that non-N-glycosylated MUC2 apomucins slowly de-
graded in the endoplasmic reticulum and that they were never transported to the Golgi apparatus.

The similarities in the cysteine position in the C-terminal of the vWF with several mucins, including the human MUC2 and the porcine submaxillary mucin, suggests similarities in the dimer formation of these proteins. Expression of the C-terminal domain of the porcine submaxillary mucin in COS cells (17) proposed that the dimerization of the C-terminal domain occurred irrespective of N-glycosylation inhibition. The inhibition of N-glycan formation did not inhibit further transport of the porcine submaxillary mucin dimers into the Golgi apparatus.

Inhibition of N-glycosylation of the human vWF in human umbilical vascular endothelial cells inhibited dimerization and further transport into the Golgi complex (18). The different biosynthetic patterns for the non-N-glycosylated human MUC2 apomucin, the vWF, and the porcine submaxillary mucin could be the result of studies performed in different cell lines. However, different mechanisms could be involved in controlling the folding and the transport of the different proteins through the cell, despite similarities in the localization of the disulfide bonds. Alignment of the C-terminal amino acid sequences of the MUC2 apomucin, the porcine submaxillary mucin, and the vWF according to the positions of the cysteine domains found in the vWF showed no similarity in the possible N-glycosylation sites of the far C-terminal end postulated to be involved in dimerization. Some similarity in the possible N-glycosylation sites was observed upstream of the C-terminal domain but never involving all three sequences. This suggests that inhibition of N-glycans could have different effects during the biosynthesis of the three proteins.

Not only dimers, but also two fainter bands larger in size than the dimers, were observed in the endoplasmic reticulum when tunicamycin-treated cells were precipitated using the α-MUC2TR antiserum. These two faint bands were interpreted as disulfide bond-stabilized putative tri- and tetramers of the non-N-glycosylated MUC2 apomucin as seen by studies done with two-dimensional gel electrophoresis (Fig. 6B). In the first dimension, the mono- and dimers and the two oligomeric bands were separated form each other under nonreducing conditions. In the second dimension, under reducing conditions, all bands migrated to the same position. Because the lysis buffer contained NEM, inhibiting in vitro disulfide bond formation, the disulfide-stabilized oligomers must be formed in the cells. The bands migrating as putative tri- and tetramers could also be interpreted as the non-N-glycosylated mono- and dimers destined for degradation and covered with chaperones causing the apparent larger size. This is unlikely, however, because the same large amount of chaperones must then be bound. Also, the interaction between chaperones and proteins is not known to be resistant to boiling in 5% SDS. Two faint bands larger in size than the dimer could also be shown without tunicamycin treatment, but in much smaller amounts than in treated cells because these could be observed only after weeks of exposing the gel to x-ray film.

Comparing the biosynthesis of the human MUC2 mucin with studies on other mucins would indicate if there is a common biosynthetic pathway for all secreting mucins. The biosynthetic studies on rat gastric mucin by Dekker et al. (19, 20) showed the formation of not only dimers but also trimers and possibly tetramers before O-glycosylation of the apomucin. Studies on human gastric mucin and human gallbladder mucin by Klomp et al. (21, 22) showed that both apomucins form only one oligomer species in the endoplasmic reticulum, interpreted as a tri- or tetramer based on the size determined by SDS-PAGE. Our finding of glycosylated monomers is also in contrast to the biosynthesis of oligomeric mucins, as described for the rat gastric mucin (19, 20). Oligomerization was interpreted as a prerequisite for the transport of this rat gastric mucin into the Golgi apparatus. However, for the human MUC2 mucin, the N-glycans are interpreted as required for the correct folding of MUC2 monomer and dimer, and only these are allowed to pass the endoplasmic reticulum control machinery. The finding of Perez-Vilar et al. (17) of the transport of a recombinant truncated C-terminus of the porcine submaxillary mucin through the whole secretory machinery of COS cells could propose another biosynthetic pathway or cell type specific control processes for this mucin compared with others. One may then speculate that there is not one common biosynthetic pathway for all gel-forming mucins, but instead biosynthetic differences between different mucins, as well as between mucin-producing cells.

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