Characterization of mucins from cultured normal human tracheobronchial epithelial cells

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Thornton, David J., Thomas Gray, Paul Nettesheim, Marj Howard, J a Seok Koo, and John K. Sheehan. Characterization of mucins from cultured normal human tracheobronchial epithelial cells. Am J Physiol Lung Cell Mol Physiol 278: L1118–L1128, 2000.—Early-passage normal human tracheobronchial epithelial (NHTBE) cells grown in air-liquid interface cultures in medium containing retinoids differentiate into a mucociliary epithelium over a 2- to 3-wk period and express increasing mRNA levels of the airway mucin genes MUC5AC and MUC5B as the cultures age; the levels of MUC2 mRNA were very low throughout the study. Using specific antibodies to MUC5AC and MUC5B mucins, we noted a gradual increase in these two mucins in the intracellular and apically secreted pools as a function of time. A low level of MUC2 mucin was detected, which did not change with time. The intracellular and apically secreted mucins isolated from day 14 and day 21 cultures by density gradient centrifugation were similar in density to those previously isolated from human respiratory mucus secretions. The sedimentation rate of the apically secreted mucins indicated that they were highly oligomerized, polydisperse macromolecules similar to those previously documented from in vivo secretions. In contrast, the cell-associated mucins from the cultured NHTBE cells were much smaller, possibly only monomers and dimers. Anion-exchange chromatography detected no differences in charge density between the reduced and carboxymethylated cell-associated and secreted forms of the MUC5AC and MUC5B mucins. The MUC5AC mucin was of similar charge density to its in vivo counterpart; however, MUC5B was more homogeneous than that found in vivo. Finally, evidence is presented for an intracellular NH2-terminal cleavage of the MUC5B mucins. These studies indicate that the mucins produced by cultured NHTBE cells are similar to those found in human airways, suggesting that this cell culture model is suited for studies of respiratory mucin biosynthesis, processing, and assembly.

MUC2; mucin oligomerization; amino-terminal cleavage of MUC5B

MUCUS IS ESSENTIAL IN PROTECTING internal body surfaces from injury. Respiratory tract mucus has the added function of trapping inhaled particles, which can then be moved out of the airways by the beating action of the cilia. However, overproduction of mucus, which occurs in diseases of the respiratory tract involving inflammation such as chronic bronchitis, asthma, and cystic fibrosis, causes airway narrowing or obstruction, thus impeding airflow. The rheological properties of mucus are dictated by the large gel-forming mucins (26). To understand how mucin synthesis and secretion are regulated under physiological and pathological conditions and how they can be controlled pharmacologically is therefore an important goal in pulmonary medicine.

To date, nine mucin genes have been identified and (at least partially) cloned and sequenced. Of these, eight mRNAs (MUC1, -2, -3, -4, -5AC, -5B, -7, and -8) are expressed in human airways (see Ref. 25 for a review), but it is not clear whether all of these messages are being translated and whether the products are secreted. Three mucins, MUC2, MUC5AC, and MUC5B, have been reported to be expressed by airway epithelium; however, only MUC5AC and MUC5B have been convincingly demonstrated to be major components of human airway secretions (17, 18, 21, 27, 29, 34, 39). The former appears to be produced by the goblet cells in the tracheobronchial surface epithelium, whereas the latter is secreted primarily by the submucosal glands. However, it has been recently demonstrated that MUC5B mucins are also synthesized in goblet cells (39).

It is difficult to study the production of human airway mucins in vivo, and, therefore, we have developed a system to culture early-passage normal human tracheobronchial epithelial (NHTBE) cells under conditions that support growth and differentiation into a mucociliary epithelium. Mucociliary differentiation, which is dependent on the presence of retinol or retinoic acid in the culture medium, was shown to occur over a period of 2–3 wk (2, 12, 14, 20, 40). The cells, which are cultured on a porous membrane at the interface between medium and air, form a confluent polarized epithelial cell sheet between day 10 and day 14 and progressively differentiate into a mucociliary phenotype, secreting mucin onto the apical surface (11, 12). These apical secretions can be readily collected and analyzed qualitatively and quantitatively (12). This cell culture system is well suited for detailed studies on...
the biosynthesis of human airway secretions and the factors and mechanisms that regulate them (28).

The purposes of the studies presented here were to determine whether the gel-forming mucins expressed in human airways, namely MUC2, MUC5AC, and MUC5B, are present in the cell layer and apical secretions of these cultures and to determine the physicochemical properties compared with those of their counterparts from human airway secretions produced in vivo.

EXPERIMENTAL PROCEDURES

Air-liquid interface cell culture, collection of mucins, and histology. Passage 2 NHTBE cells were seeded at 10^5 cells onto uncoated, 25-mm, semipermeable membranes (Transwell Clear, Costar). The day of seeding was considered to be time 0 of the experiment. The cells were cultured in serum-free, hormone-supplemented medium containing retinoic acid (5 x 10^{-8} M) as previously described (12). The cultures were maintained at 37°C in 5% CO₂ in air. At indicated times, apical mucin secreted during a 24-h period was collected by washing the surfaces of the cultures with PBS (termed apical washings; 20 ml for 30 cultures). The wash was then diluted 1:1 with 8 M guanidinium hydrochloride (GuHCl), and the cell layer was solubilized in 6 M GuHCl (15 ml for 30 cultures).

Histological specimens were prepared from intact day 21 cultures that were fixed in 10% neutral-buffered Formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. A complete description of the methods used for transmission electron microscopy has been previously reported (12). Briefly, the cultures were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer, postfixed in 0.1% osmium tetroxide, and stained with uranyl acetate. Samples were dehydrated, embedded in epoxy resin, cut, and viewed.

Competitive RT-PCR. Methods to detect and quantitate MUC2, MUC5AC, and MUC5B mRNAs have been previously reported in detail (14, 20). Briefly, from a separate set of triplicate cultures run concurrently with those used for mucin collection, total RNA was isolated with TRI Reagent (Molecular Research Center, Cincinnati, OH) and reverse transcribed into cDNA with RNA PCR kits and protocols (Perkin-Elmer, Morrisville, NC). Oligonucleotide primers were designed according to the published sequences for human MUC2 (13; GenBank accession no. L21998; 5'-primer, TGCCTGGCGCT-GTCTTTG; 3'-primer, CAGCTCAGCATGCAGTGC), human MUC5AC (21; GenBank accession no. U06711; 5'-primer, TCCGGCCCTATCTCTCTC; 3'-primer, ACTGGGCACTGGTG-GCTG), and human MUC5B (8; GenBank accession no. Y09788; 5'-primer, TGCAATCAGCATGCAGTGCAG; 3'-primer, TTCTCAGGGTCAGGTCTCTTC). PCRs were performed in the presence of internal standards, so-called MIMICs (10⁻¹ amol/reaction for MUC5AC and MUC5B and 10⁻² amol/reaction for MUC2; Clontech PCR MIMIC construction kit, Palo Alto, CA). PCR conditions were similar for all three mucin genes except that both MUC5AC and MUC5B went through 27 cycles of amplification, whereas MUC2 required 35 amplification cycles. Conditions were 1.5 mM MgCl₂, denaturation at 95°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min. MUC2 oligonucleotides generated, as predicted, a 438-bp cDNA product and a 360-bp MIMIC product; MUC5AC oligonucleotides generated a 146-bp cDNA fragment and a 340-bp MIMIC fragment, whereas oligonucleotides for MUC5B generated a 348-bp cDNA fragment and a 486-bp MIMIC fragment. Oligonucleotides for β₂-microglobulin, which was used as a control gene for the RT-PCR, were purchased (Clontech) and generated a 335-bp PCR fragment. Specific amplification for MUC2, MUC5AC, and MUC5B was confirmed by sequencing (double-stranded DNA cycle-sequencing system; GIBCO BRL, Life Technologies, Gaithersburg, MD) the PCR fragments. The amplification efficiency for MUC2, MUC5AC, and MUC5B cDNA and MIMICs (14, 19) was verified by determining the efficiencies of cDNA and MIMIC produced after various numbers of PCR cycles, and quantification of the assays was determined by titrating MIMIC against a constant amount of cDNA.

To determine the levels of MUC2, MUC5AC, and MUC5B mRNAs, PCRs were performed in the presence of known amounts of MIMIC. PCR products were separated on 2% agarose gels (containing 50 ng/ml of ethidium bromide), and the resulting bands were analyzed with a digital imaging system (Alpha Innotech, San Elieandro, CA). The ratio of the signal intensity of the target cDNA to that of the MIMIC was determined.

Preparation of mucins. Mucins were purified essentially as previously described (6). In brief, the NHTBE cell layer extracts and apical washings were centrifuged at a starting density of 1.40 g/ml in 4 M GuHCl-CsCl with a Beckman Ti70.1 rotor at 40,000 rpm for 68 h at 15°C. In addition, the mucin-containing fractions from the 4 M GuHCl-CsCl gradient of the cell layer extract were subjected to further purification in a 0.2 M GuHCl-CsCl density gradient. Centrifugation was performed at a starting density of 1.5 g/ml under the conditions described above. After each centrifugation, the tubes were emptied from the top. The density of each fraction was determined with a Hamilton syringe as a pycnometer.

Preparation of reduced and carboxymethylated mucin subunits. Reduced and carboxymethylated mucin subunits were obtained by treatment of the purified mucins in 6 M guanidinium chloride-0.1 M Tris, pH 8.0 (reduction buffer), with 10 mM dithiothreitol (DTT) for 5 h at 37°C. Iodoacetamide was then added to a final concentration of 25 mM, and the mixture was left in the dark overnight at room temperature (5). Alternatively, the mucins were reduced and carboxymethylated on nitrocellulose membranes after slot or Western blotting. Briefly, the blotted membrane was washed in distilled water for a few minutes and incubated in reduction buffer containing 10 mM DTT at room temperature for 15 min. After the DTT solution was removed, the membrane was incubated in the same buffer containing 25 mM iodoacetamide at room temperature for 10 min and then washed twice (5 min) with distilled water (1).

Anion-exchange chromatography. Reduced and carboxymethylated mucins were chromatographed on a Pharmacia Mono Q HR 5/5 column eluted with a linear gradient of 0–0.4 M lithium perchlorate-10 mM piperazine, pH 5.0, in 6 M urea containing 0.02% 3-[(cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) (29).

Agarose gel electrophoresis. Agarose gel electrophoresis was performed as previously described (1, 33). After electrophoresis, the molecules were Western blotted onto nitrocellulose membranes before detection of mucins with antibodies. For analysis of the intact unreduced molecules, the gels were washed for 10 min in transfer buffer at pH 8.0 and then treated with 10 mM DTT for 15 min before vacuum transfer (1).

Polyclonal antisera. Polyclonal antisera were raised in rabbits against synthetic peptides coupled to keyhole limpet hemocyanin from specific sequences within the mucins MUC2, MUC5AC, and MUC5B. The antisera used were MAN-5ACI, which was raised against the same peptide sequence as LUM5-1 (RNDQEQGPFKM; 29), MAN-5BI (ELGQVVEC-SLDGFGLVCR), which has been previously described (34),

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MAN-2I, and MAN-5BIII. MAN-2I is similar to the antiserum LUM2-3 (4) and was raised against a synthetic peptide, NGLQPVRVEDPDGC, in the nontandem repeat region of MUC2 toward the COOH terminus. MAN-2I shows a behavior similar to that reported for LUM2-3 with the precursor and mature forms of MUC2 produced by the PC/AA cell line in culture (1). MAN-5BIII was raised against a synthetic peptide, CSWYNGHRPEPGLG, found in Cys1 domain at the NH2 terminus of the region encoded by the large central 10.7-kb exon of MUC5B (10). The antisera were used at the following dilutions: 1:10,000 for MAN-2I and MAN-5ACI and 1:2,000 for MAN-5BI and MAN-5BIII.

Determination of the levels of MUC2, MUC5AC, and MUC5B mucins with time in culture. Aliquots (10 µl) of the cell lysate extracts (15 ml) and apical washings (30 ml) on days 10, 14, and 21 of culture were reduced and carboxymethylated, slot blotted onto nitrocellulose, and probed with mucin-specific antisera (for details of antisera, see Polyclonal antisera). The antisera were used at the following dilutions: 1:10,000 for MAN-2I and MAN-5ACI and 1:2,000 for MAN-5BI and MAN-5BIII.

RESULTS

Mucin gene expression and secretion by cultured NHTBE cells. Early-passage NHTBE cells were cultured at the air-liquid interface in serum-free retinoic acid-containing medium. The cells formed a confluent monolayer within 7–8 days after being seeded and progressively differentiated, forming a polarized mucociliary epithelium between days 14 and 21 (Fig. 1A). Ultrastructural examination of the day 21 cultures (Fig. 1B) revealed cells with numerous small secretory granules of varying electron density, some appearing to be exocytosed. Large confluent granules typical of goblet cells in vivo were rare. The surface of these cells was covered with microvilli. On days 10, 14, and 21, apical washings and cell lysates were collected to measure secreted and intracellular mucins with MUC2, MUC5AC, and MUC5B mucin-specific antibodies. Total RNA was obtained from separate sets of simultaneously grown cultures, and the levels of MUC2, MUC5AC, and MUC5B mRNA expression were determined by competitive RT-PCR. As shown in Fig. 2A,
MUC5AC and MUC5B mRNAs were low on day 10 but were clearly increased on days 14 and 21. Low levels of MUC2 mRNA were expressed throughout the course of the study; they peaked on day 14 and decreased by day 21 to the levels observed on day 10. It should be noted that the level of MIMIC used in the competitive PCR for MUC5AC and MUC5B message levels, and thus the levels of MUC2 are very low compared with the other two mucin genes.

The levels of MUC2, MUC5AC, and MUC5B mucins in the apical washings and cell lysates were determined by immunoreactivity with mucin-specific antisera, and the data are shown in Fig. 2, B and C. Over the 21 days of the experiment, there was an ~10-fold increase in the amount of secreted and cell-associated MUC5AC and MUC5B mucins. It should be noted that the MAN-5ACI antiserum is ~20 times more sensitive than the MAN-5BIII antiserum (Kirkham S, Sheehan JK, and Thornton DJ, unpublished observations), suggesting that the MUC5B mucin is more abundant than the MUC5AC mucin. A low level of the MUC2 mucin (close to the background signal) was detected throughout the culture period, both in the apical-washing and cell-associated mucins. This correlates with the low levels of MUC2 mRNA.

Mucin purification. The cell-associated and secreted mucins from day 21 cultures were purified by isopycnic density gradient centrifugation. The cell lysate was subjected to 4 M GuHCl-CsCl density gradient centrifugation (Fig. 3A), and a carbohydrate-rich peak was observed at a density of 1.35–1.45 g/ml, the density range expected for mucins extracted from in vivo respiratory secretions (18, 27, 31, 39). This material was separated from lower-buoyant density proteins, as assessed by absorbance at 280 nm measurements, toward the top of the gradient (Fig. 3A, fractions 1–6). An aliquot from each fraction was reduced and carboxymethylated and subjected to agarose gel electrophoresis, and Western blots of the gels were probed with the anti-mucin antibodies MAN-5ACI (Fig. 3B) and MAN-5BIII (Fig. 3C). The data show a clear separation of the “mature” glycosylated MUC5AC and MUC5B mucins (fractions 10–16) from their “putative” precursors found at lower-buoyant density (fractions 1–5). A similar observation was made recently for the cell-associated mature and precursor forms of the MUC2 mucin synthesized by the intestinal cell line PC/AA in culture (1).

Fig. 2. Time course of MUC2, MUC5AC, and MUC5B mRNA and mucin protein levels in NHTBE cultures. Apical-washing, cell lysate, and total cellular RNAs were extracted on days 10, 14, and 21 of culture. A: relative levels of mucin gene mRNAs were determined by competitive RT-PCR. For MUC2 PCR, 10⁻⁴ amol of MIMIC (internal standard) and for MUC5AC and MUC5B PCR, 10⁻¹ amol of MIMIC were used (see EXPERIMENTAL PROCEDURES). cDNA-to-MIMIC ratios, which are a measure of relative mRNA levels, were 0.5, 0.7, and 0.5 for MUC2; 0.5, 1.0, and 1.3 for MUC5AC, and 0.6, 1.0, and 1.1 for MUC5B for days 10, 14, and 21, respectively. β2M, β₂-Microglobin used as a control gene. Levels of secreted (B) and cell-associated (C) mucins were determined with mucin-specific antisera (see EXPERIMENTAL PROCEDURES for details).
Absorbance measurements at 280 nm suggested that DNA was present in the mucin peak. Therefore, mucin-containing fractions were pooled and subjected to 0.2 M GuHCl-CsCl isopycnic density gradient centrifugation (Fig. 3D) to separate the mucins (1.45–1.55 g/ml) from the DNA. Reactivity with the MAN-5ACI and MAN-5BIII antisera coincided with the PAS-reactive material (corresponding to the mucins), and fractions 4–12 were pooled for further analysis.

The secreted mucins were also purified by 4 M GuHCl-CsCl density gradient centrifugation (Fig. 4). A PAS-rich peak was present (1.35–1.45 g/ml) that was well separated from the lower-buoyant density proteins. Unlike for the cell-associated mucin extract, agarose electrophoresis did not identify any putative precursor bands (data not shown), indicating that only mature glycosylated forms of the mucins were secreted. No evidence of DNA contamination was found, and, therefore, this preparation was not subjected to further density gradient purification. The mucin-containing fractions were reactive with the MAN-5ACI and MAN-5BIII antisera, and fractions 11–16 were pooled for further analysis.

MUC5AC and MUC5B mucin characterization. The pooled apical-washing and cell-associated mucin fractions before and after reduction were separated by
agarose gel electrophoresis, transferred to nitrocellulose membranes, and probed with the anti-mucin antibodies MAN-5ACI (Fig. 5A) and MAN-5BIII (Fig. 5B). Also shown for comparison is an agarose gel electrophoretic separation of the MUC5AC and MUC5B mucins present in an in vivo respiratory secretion (Fig. 5C). The intact high-molecular-mass MUC5AC and MUC5B mucins (average molecular mass for total unfractionated preparation ~15 MDa) from the in vivo secretion barely entered the gel, whereas their reduced and carboxymethylated mucin subunits (average molecular mass 2.5 MDa) had a much greater electrophoretic migration (Fig. 5C). After reduction and carboxymethylation, the MUC5AC and MUC5B mucins isolated from the cell lysates and the apical washings (Fig. 5, A and B) exhibited an electrophoretic migration similar to that of their in vivo counterparts. The intact secreted MUC5AC and MUC5B mucins (Fig. 5, A and B) barely entered the gel, suggesting that like the MUC5AC and MUC5B mucins in the in vivo secretion, these mucins were high-molecular-mass, multimeric glycoproteins. In contrast, the intact mucins from the cell lysate (Fig. 5, A and B) exhibited a similar migration rate to the reduced and carboxymethylated mucin monomers, suggesting that the unreduced cell-associated mucins are not highly oligomerized and are possibly mainly monomers and dimers. It is noteworthy that after reduction of the MUC5B mucins, two bands were detected (Fig. 5B).

To analyze the size distribution of the isolated mucins in more detail, the pooled, purified intact and reduced and carboxymethylated mucins were subjected to rate zonal centrifugation on preformed 6–8 M GuHCl gradients. Previously, it has been shown that this technique can separate respiratory mucins with molecular masses between ~2.5 and 30 MDa (31, 35). The unreduced MUC5AC and MUC5B mucins isolated from the secretions are characterized by a broad range of sedimentation rates consistent with polydisperse, high-molecular-mass macromolecules, which, after reduction, are found as a single less rapidly sedimenting species (Fig. 6, A and C). The unreduced cell-associated MUC5AC and MUC5B mucins are less polydisperse...
and sediment only slightly faster than the reduced monomeric mucins, indicating that they are much smaller than the secreted forms (Fig. 6, B and D), in agreement with the electrophoretic data (Fig. 5).

The dramatic difference in apparent size between the cell-associated mucins and those apically secreted on day 21 of culture prompted us to investigate the size distribution of the secreted mucins at earlier days of culture. There was a marked increase in the average sedimentation rate of the secreted MUC5AC mucins with increasing time in culture (Fig. 7). However, this was not the case for the MUC5B mucins. On day 10, the MUC5B antiserum response was too low to measure, whereas on days 14 and 21, the mucins appeared as similar broad dispersions across the gradient (Fig. 7).

Our laboratory (27, 29) and others (39) have analyzed the reduced and carboxymethylated MUC5AC and MUC5B mucins isolated from respiratory secretions using anion-exchange chromatography on Mono Q column. These studies have indicated that the charge density for the MUC5AC mucin is distinct from two charged variants of the MUC5B mucin as illustrated in Fig. 8A. This contrasts with the situation with the cultured cells where, unlike the in vivo secretions, the reduced and carboxymethylated mucins, whether cell associated or secreted (day 14 or 21), were similar (Fig. 8, B–E). Although the charge density of the MUC5AC mucin was similar to that observed in vivo, there was no evidence for the different charged variants of the MUC5B mucin.

As noted above (Fig. 5B), two populations of MAN-5BIII-reactive molecules were observed after agarose gel electrophoresis of the reduced and carboxymethylated cell-associated and secreted MUC5B mucins: one with an electrophoretic migration expected for the reduced and carboxymethylated MUC5B subunit and the other more rapidly migrating. This suggested that a small fragment of the MUC5B mucin that was released after reduction might have accounted for the faster migrating band. To further investigate this, we performed rate zonal centrifugation of the reduced and carboxymethylated mucin preparation (Fig. 9). Again, two populations of MAN-5BIII-reactive molecules were observed. The slow sedimenting species had little PAS reactivity, suggesting that it had a low level of glycosylation (Fig. 9), and did not react with the MAN-5BI antiserum, consistent with it being a protein-rich fragment from the NH2 terminus. The more rapidly sedimenting population, which was reactive with MAN-5BI, was associated with the bulk of the PAS reactivity (Fig. 9), as would be expected for the reduced and carboxymethylated MUC5B subunit.

**DISCUSSION**

The MUC5AC and MUC5B mucins are the two major gel-forming glycoproteins secreted in the airways (16–18, 27, 29, 34). Although MUC2 mRNA expression has been demonstrated in the respiratory tract (3, 28, 37), there is little biochemical evidence for any significant amount of this mucin in airway mucus secretions (18, 29). This pattern of mucin synthesis and secretion is mimicked in the NHTBE cell cultures studied here. In vivo studies (17, 30) have demonstrated that the MUC5AC mucin is produced mainly by mucous cells of the surface epithelium and that MUC5B is mainly the product of cells in the submucosal glands. However, it has been shown that MUC5B mucins are also synthesized in the surface epithelium (39). Although NHTBE cells in air-liquid interface culture do not form glandular structures, these cells do make both serous and mucous products (11, 12). This is not surprising because the surface epithelium and submucosal glandular epithelium have a common developmental origin (36). The maturation of the cells from an undifferentiated phenotype to a complex phenotype embodying
different cell types is associated with a major increase in MUC5AC and MUC5B mucin storage and secretion (~10-fold), whereas MUC5AC and MUC5B mRNAs are only 2- to 3-fold more, suggesting that they might be regulated at the translational or posttranslational level. The low levels of MUC2 mRNA and protein detected remained unchanged during the time of culture. It should be stressed that we cannot say on the basis of our data that MUC5AC and MUC5B mucins are the only two mucins present in the secretions.

The NHTBE cells in culture appear to secrete only the mature forms of the MUC5AC and MUC5B mucins. We can find no difference in density between the mature MUC5AC and MUC5B mucins in the cell lysate and those secreted. Furthermore, there is no apparent difference in the charge density and electrophoretic migration of their reduced subunits. We assume, therefore, that the cell mucins are the direct precursor of those in the apical washings. The distinction between cell-associated (either intracellular or cell-bound) and secreted mucins is not easy to make because some secreted mucin may remain attached to the cell sheet. However, it is quite clear in this case that the secreted mucins are much more highly oligomerized than the mature cell-associated mucins, which are smaller and appear to be primarily but not exclusively dimeric. This conclusion can be drawn from both the agarose electrophoresis and rate zonal centrifugation data and suggests that in NHTBE cultures, mucin oligomerization is directly coupled to the secretion process itself. Further investigations are underway to determine if this is so. The increase in the size of the secreted mucins with time in culture suggests a maturation phenomenon in

![Graphs showing rate zonal centrifugation of MUC5AC and MUC5B mucins from apical washings of 10-day (A), 14-day (B), and 21-day (C) NHTBE cultures.](image)

![Graphs showing anion-exchange chromatography of reduced and carboxymethylated MUC5AC and MUC5B mucins from a human respiratory secretion (A) and from apical washings (B and C) and cell layers (D and E) from NHTBE cultures on days 14 (B and D) and 21 (C and E).](image)
the process of mucin assembly that is not understood. If oligomerization is coupled to secretion, then it is possible that the extent of oligomerization is dependent on the buildup of stored mature molecules within the cell or the rate of their production.

The differential reactivity of a proportion of the intracellular and secreted MUC5B mucins with the two different polypeptide-directed antisera, MAN-5BI and MAN-5BIII, indicate that some of the MUC5B mucins may undergo NH₂-terminal cleavage. MAN-5BIII is directed against an epitope in the Cys¹ domain toward the NH₂-terminus of the central portion of the MUC5B apomucin, whereas MAN-5BI is against a repeated sequence in the R end domains in the central portion of the polypeptide (9, 34). After reduction of the intact mucins, a fragment is generated that is reactive with MAN-5BIII but not with MAN-5BI and has a slower sedimentation rate than the reduced MUC5B subunit, which is reactive with both antisera. Furthermore, this material has a higher electrophoretic mobility and lower PAS reactivity than the reduced subunit. These findings are consistent with a protein-rich fragment generated by reduction and suggest that a proportion of the MUC5B mucins have undergone an NH₂-terminal cleavage. There is evidence for COOH-terminal cleavage of the MUC2 and MUC5B mucins isolated from in vivo mucus secretions (15, 39, 41), but whether this is an intracellular event is not clear. However, the data presented here indicate that NH₂-terminal processing of the MUC5B mucins is an intracellular event, and, furthermore, the cleaved fragment remains associated with the unreduced glycoprotein. The significance of this finding is unknown; however, it should be noted that an NH₂-terminal cleavage is part of the processing of the multimeric glycoprotein von Willebrand factor (vWF). The cleavage of vWF takes place in a post-Golgi compartment, and the cleaved fragment, as appears to be the case here, remains associated with the vWF polymer (37). vWF contains cysteine-rich domains (D domains) that are homologous with regions of the NH₂ and COOH termini of the MUC5AC and MUC5B mucins (25). Some of these cysteine residues are believed to form the intermolecular disulfide bonds by which the mucins oligomerize. Indeed, cysteine residues present within homologous regions of porcine submaxillary mucins have been shown to be involved in the macromolecular assembly of this oligomeric mucin (22–24).

Overall, the macromolecular properties of the MUC5AC and MUC5B mucins produced by NHTBE cultures are very similar to those we find for these mucins isolated from in vivo respiratory secretions. However, agarose gel electrophoresis and anion-exchange chromatography of the reduced and carboxymethylated mucins produced by the NHTBE cells in culture suggest that the glycosylation of the MUC5B mucins is not as complex. The reduced and carboxymethylated subunits of the MUC5AC and MUC5B mucins from the cultures elute almost identically from the anion-exchange column, which suggests that the two populations of molecules may be similarly glycosylated. However, in vivo, the MUC5B mucin can occur in at least two distinct glycoforms (27, 34, 39). The low-charge glycoform of this mucin appears to emanate from the glands, and this form is not found in these cultures.

In summary, the cultured NHTBE cells make and secrete MUC5AC and MUC5B mucins in significant quantities as the cultures mature and differentiate, with the MUC5B mucin appearing to be more abundant. The secreted mucins are polydisperse in size distribution and extend to very highly oligomerized macromolecules, whereas their mature fully glycosylated precursors stored in the cell are smaller and are possibly dimers and monomers. The presence of smaller MUC5B fragment(s) detected after reduction with an antiserum to the NH₂ terminus but not with an antiserum to other parts of the mucin polypeptide suggests that processing occurs as part of the biosynthesis and/or oligomerization process. The pattern of glycosyl-
ation is similar for the two mucins but not identical to that seen in vivo.

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