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Expression and Characterization of a Novel Recombinant Version of the Secreted Human Mucin MUC5AC in Airway Cell Lines

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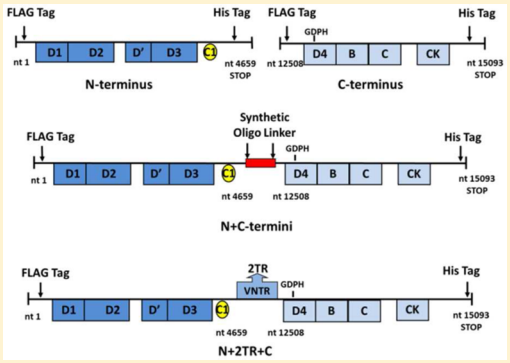
Supporting Information

ABSTRACT: Molecular manipulation and expression of mucins, large glycoproteins that provide the structural framework of mucus, are challenging due to mucins' size and numerous domains, including variable number tandem repeat (VNTRs) regions that are sites of O-glycosylation. Only individual human mucin domains have been expressed in mammalian cells. We produced recombinant versions of MUC5AC, a major secreted mucin in the respiratory tract, encoding the N-terminus, C-terminus, N- and C-termini together, and N- and C-termini interspersed with two native tandem repeat sequences (N+2TR+C) in both tracheal and bronchial cell lines. The latter protein contains all of the functional domains required for the biosynthesis and secretion of glycosylated mucin. The N-terminus protein was found in monomeric and higher molecular mass forms suggesting that secreted MUC5AC may form a branched netlike structure analogous to that described for MUC2. At the C-terminus, proteins underwent cleavage, polymerization, and glycosylation. Thus, they appear to undergo pivotal processing steps as predicted for native MUC5AC, which is analogous to that for other individual recombinant mucin domains. Secretion occurred when cells were grown on transwell filter inserts but not on plastic, indicating that the extracellular environment likely plays a role in mucin processing. The secreted N+2TR+C protein differed in molecular mass from the intracellular form, indicating that additional processing occurred. These recombinant proteins, expressed in different backgrounds, can potentially address the role of different mucin domains on MUC5AC processing and function as well as the role of MUC5AC in health and disease.



Mucins, a family of high molecular mass glycoproteins that can be membrane bound or secreted, form the structural scaffolding for viscoelastic mucus gels at the apical surface of mucosal epithelia. They consist of an apomucin protein core containing proline-, threonine-, and serine-rich sequences, which are frequently organized in repeats or variable number tandem repeat (VNTR) domains that are sites of extensive O-glycosylation.[1] Their heavy O-linked glycosylation along with further modifications, including sulfation, imparts mucus with unique rheological, structural, and chemical properties.[2] Secreted gel-forming mucins that have been identified in humans are MUC2, MUC5AC, MUC5B, MUC6, and MUC19.[2,3] MUC5B and MUC5AC are the major secreted gel-forming mucins in airways.[4−6] There is major interest in the role of these secreted mucins in respiratory diseases such as cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD), both of which are characterized by hypersecretion of mucus, pulmonary obstruction, reduced mucocillary clearance, and chronic bacterial infection.[7]

Recombinant mucins have been an important tool in understanding the structure and biological functioning of mucins.[8−11] The N- and C-termini of the human secreted mucin MUC2 and the C-terminus of MUC5AC have been expressed in a variety of cell lines, including CHO-K1 hamster ovary cells and the human colonic cell line LS174T.[8−11] More recently, the N-termini of MUC5B and MUC6 have also been cloned and expressed.[12,13] Results with MUC5AC and MUC2 recombinant mucins have shown that dimerization of mucins occurs at the C-terminus in an N-glycosylation-dependent manner,[14] and both mucins have been shown to be posttranslationally cleaved in the C-terminal cysteine-rich region of the peptide.[9−11] This autocatalytic cleavage occurs at low pH (<6.0), and the cleavage site is located in the von Willebrand factor (vWF) D4 domain of the mucins.[9−11] Expression of a recombinant cysteine knot (CK) domain of MUC5AC was utilized to determine that it was involved in the formation of disulfide-linked dimers.[15] Recombinant technology has also been used to show that a CysD domain located between two highly glycosylated regions in MUC2 acts as a noncovalent cross-linker and thereby likely plays a role in determining properties of MUC2-containing mucus, including its pore size.[16] Numerous CysD domains have been found in

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both MUC5AC[17] and MUC5B,[18] but their exact function has not yet been elucidated.

More recently, recombinant mucins have been used to assess the folding, packing, release, and assembly of secreted mucins.[13,19,20] The N-terminus of MUC2 is involved in the formation of protease resistant trimers,[8] and ordered packing of mucin N-terminals into concatenated polygons occurs at low pH in the presence of high calcium. MUC2 N-terminal aggregates are dissolved upon release of mucin from the mucin granule when the pH is raised and the calcium concentration is lowered.[19] Further work on the structure of the D3 domain of the MUC2 N-terminus has confirmed that MUC2 mucin forms branched netlike structures.[20] In contrast, studies with MUC5B recombinant N-terminus proteins suggest that MUC5B multimerizes by disulfide linkage between D3 domains to form linear polymer chains.[13]

Molecular manipulation of mucins is challenging, and biochemical reagents and assays available to study these molecules are limited. The complexity of mucins resides in their size and the numerous domains they possess, including vWF domains, cysteine-rich regions, and the VNTR domains. A number of VNTR domains of MUC2, MUC4, MUC5AC, and MUC5B, contained in a MUC1 backbone, have been expressed in cell lines and demonstrated to be extensively O-glycosylated.[21] Extended VNTR regions have been shown to result in higher molecular mass glycans but not to affect the specificity of O-glycosylation.[22] However, MUC1 is a membrane-bound mucin, and no complete gel-forming mucin with both the N- and C-terminal cysteine-rich domains and an intervening VNTR domain has been recombinantly produced to date.

We have designed a suite of MUC5AC mucin constructs and expressed them in airway mammalian cell lines. These include an intact N-terminus (N), intact C-terminus (C), N- and C-termini joined together (N+C), and N- and C-termini interspersed with two native tandem repeat (TR) sequences (N+2TR+C). The latter construct results in a version of MUC5AC in which the size of the glycosylated VNTR domains and the molecular mass are both greatly reduced compared to those of native MUC5AC. Although this limits overall glycosylation and therefore impacts the normal function of the glycoprotein, the N+2TR+C construct contains all of the functional domains required for the biosynthesis and secretion of a glycosylated mucin. These constructs have been expressed in matched pairs of tracheal and bronchial cell lines with mutated and corrected (wild-type) cystic fibrosis transmembrane conductance regulator (CFTR) protein. While murine *Muc5ac* has been cloned in its entirety, and a murine model of Muc5ac overexpression has been established, cloning of only the C-terminus of human *MUC5AC* has been reported to date.[9] Therefore, these constructs offer the potential to address not only the biological function of MUC5AC in health and disease but also the role of different mucin domains in mucin processing and function.

MATERIALS AND METHODS

Cloning Strategy for N, C, N+C, and N+2TR+C Constructs. The overall strategy to clone the suite of MUC5AC constructs was as follows. Overlapping fragments from the MUC5AC N-terminus or C-terminus were amplified from human poly-A tracheal DNA by PCR and cloned into pBluescript pSK or pKS (Stratagene). These fragments were assembled into sequences encoding most of the N- and C-termini by exploiting appropriate restriction sites. N- and C-terminal constructs were completed by inserting appropriate synthetic linkers encoding the required 5′ sequences, including promoter, start codon, signal sequence, and an epitope FLAG tag, along with the 3′ sequences, including an epitope histidine (His) tag and stop codon, and subcloned into PCDNA3.1, which contains a human cytomegalovirus (CMV) immediate-early promoter for high-level stable and transient expression in mammalian hosts. The complete N- and C-terminal clones were then joined using linkers with and without TR sequences to form the N+C construct with no TR sequences and the N+2TR+C construct with two TR sequences (Figure 1). Most of

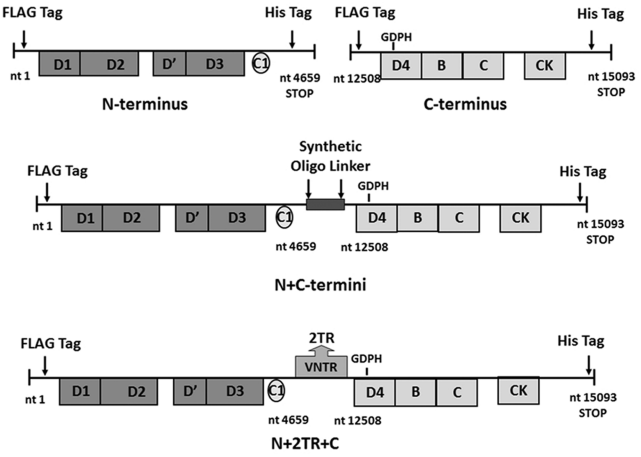

Figure 1. Diagrammatic representation of the N, C, N+C, and N+2TR+C MUC5AC constructs used in this study (not to scale). The individual domains encoded for and the location of the FLAG and His tags for each construct are indicated: vWF-like domains (D1, D2, D′, D4, B, and C), Cys domain 1 (C1), variable number tandem repeat (VNTR), and cysteine knot (CK). The GDPH sequence found in the vWFD4 domain, where cleavage at an Asp−Pro bond occurs, is indicated.

the N-terminus and C-terminus constructs were assembled using the primer sets detailed in Tables 1 and 2. Three primer pairs were employed to amplify the overlapping N-terminal regions 93−1591, 1296−3886, and 3637−5271, and three

Table 1. N-Terminus Primer Sequences

primer name	primer sequence
MUC5AC F93 forward	5′-CTCCGAATCCAGCTACAAGC-3′
MUC5AC R1591 reverse	5′-AGGGTCTGAAGATGGTGACG-3′
MUC5AC F1296 forward	5′-TACCTGCTCTGTGCTTGGAG-3′
MUC5AC R3886 reverse	5′-CATCCGTCGTGTGGTAGATG-3′
MUC5AC F3627 forward	5′-GAGGCTCCCATCTTTGATGA-3′
MUC5AC R5271 reverse	5′-GAAGTCCACGTCGAACCACT-3′

Table 2. C-Terminus Primer Sequences

primer name	primer sequence
MUC5AC F12457 forward	5′-TACTCCACCCAAACCTGCTT-3′
MUC5AC R13925 reverse	5′-CTGGTCGTGAAGAGGGTCAT-3′
MUC5AC F13577 forward	5′-TTCTGAGCAAGGTCTTTGAGC-3′
MUC5AC R14569 reverse	5′-GGTGGGTCACACAGTGGTT-3′
MUC5AC F13975 forward	5′-GGAGAGCCGGTGAAGGTG-3′
MUC5AC R14961 reverse	5′-CTCTTCCACCTCGGTGTAGC-3′

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primers pairs were designed to amplify the overlapping C-terminus regions 12457−13925, 13577−14569, and 13975−14961. Detailed methods for the cloning strategy are supplied in the Supporting Information.

Cell Culture Conditions. IB3-1 cells (ATCC CRL-2777) are a CF bronchial epithelial cell line containing the Phe508del/W1282X CFTR mutation (low level expression of ΔF508-CFTR and no W1282X protein), whereas S9 cells (ATCC CRL-2778) have been corrected for the mutation with wild-type CFTR.[24,25] CFT1-LC3 and CFT1-LCFSN cells, hereafter referred to as LC3 and LCFSN, respectively, are tracheal cell lines (kind gift from James Yankaskas at the University of North Carolina). LC3 cells are homozygous for the Phe508del CFTR mutation, whereas LCFSN cells have been corrected for the mutation with wild-type CFTR.[26,27] Cells were cultured as described previously.[25,27] To detect the secretion of recombinant MUC5AC protein in stably transfected cell clones, cells were cultured on 12 mm transwell filters with a 0.4 μm pore size at a density of 1×10^5 cells/filter.

Transfection Conditions. Cells seeded at a density of 2×10^5 cells/well in 6 well cell culture plates were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Stable clones of IB3-1 and S9 cells expressing MUC5AC constructs were generated by selection with zeocin at a final concentration of 15 μg/mL.

Preparation of Cell Lysates and Collection of Culture Supernate. Conditioned medium from cells grown on transwell filters was collected over a period of 21 days and concentrated ∼20-fold using an Amicon Ultra Centrifugal filter unit. For preparation of cell lysates, cells were washed with ice-cold PBS and lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, and 1% (v/v) Triton X-100) containing a protease inhibitor cocktail (complete, Mini EDTA-free, Roche) and incubated on an orbital shaker table for 15 min at 4 °C. Cell debris was removed by centrifugation (10000*g*, 15 min, 4 °C).

Immunopurification. Recombinant MUC5AC protein was purified using M2 FLAG affinity agarose beads. Forty microliters of anti-FLAG M2 affinity gel (Sigma) was added to 1 mL of cellular lysate, and samples were rotated end over end for 18 h at 4 °C. Bound MUC5AC was eluted from the beads using 100 μL of a 3× FLAG competitive peptide solution (150 ng/μL 3× FLAG peptide in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl) for 30 min at 4 °C. The resin was pelleted by centrifugation at 7500*g* for 30 s, and the supernatant was retained and stored at −80 °C.

Antibodies. FLAG M2 (Sigma) and Penta-His (Qiagen) monoclonal antibodies were used to detect the recombinant mucin immunotags. The MUC5AC antibodies used were monoclonal antibodies 62M1 and 2-11M1 (kind gift from Dr. Jacques Bara, Centre de Recherche Saint-Antoine, Paris, France). Antibody 62M1 reacts with epitopes located in the C- and CK-vWF-like domains located in the C-terminus of MUC5AC,[28] whereas antibody 2-11M1 detects the globular D1/D2 domain located in the N-terminus.[29] A polyclonal anti-calreticulin (A3−900, Pierce) antibody raised against recombinant human calreticulin protein produced in the baculovirus insect cell system and a monoclonal antibody raised against the Golgi fraction of human cells (mAB AE6, Merck/Millipore) were also used to stain cells stably expressing the N+2TR+C construct.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoris (SDS−PAGE) and Western Blotting. Nonreduced and reduced (using 10 mM dithiothreitol) samples were electrophoresed in either 6% SDS−PAGE resolving gels with a 3% stacking gel or 3−8% Tris acetate gels (NuPage). Protein was then transferred to an Immobilon-P polyvinylidene difluoride (PVDF) membrane in transfer buffer (10 mM Bis-Tris, 10 mM bicine, 410 nM EDTA in deionized H_2O (80%, v/v) and methanol (20%, v/v) at pH 7.2) at 15 V and 4 °C for 18 h using a western blotting apparatus (Bio-Rad). Blots were probed with the anti-FLAG (1:1000) antibody in 3% (w/v) nonfat dry milk in PBS containing 0.1% (v/v) Tween 20 and the anti-His (1:1000) antibody in 3% (w/v) BSA in PBS containing 0.1% (v/v) Tween 20. The secondary antibody used was anti-mouse conjugated to horseradish peroxidase (HRP) (1:2000, Sigma) in PBS containing 3% (w/v) nonfat dry milk and 0.1% (v/v) Tween 20. Blots were developed using enhanced chemiluminescence (ECL, Amersham). Blots were also probed with antibodies raised against native MUC5AC.

Immunofluorescent Staining of Expressed Recombinant MUC5AC Protein. Transfected cells grown on either glass coverslips or transwell filters were fixed in 2% (v/v) formaldehyde in PBS for 10 min. Cells were permeabilized with 0.25% (v/v) Triton X-100 in PBS for 10 min and blocked with 10% goat serum in PBS containing 1% bovine serum albumin (BSA). Cells were stained with primary anti-FLAG M2 (1:200), -Penta-His (1:1000), -62M1 (1:200), -2-11M1 (1:200), and -CLH2 (1:200) monoclonal antibodies. Cells were subsequently incubated with an anti-mouse secondary antibody conjugated to Alexa Fluor 594 (1:500) or 488 (1:500). Cell nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI, 1:5000). Cells were mounted in fluorescent mounting medium (Dakocytomation) and viewed using a fluorescence microscope (Olympus BX51). Extracellular mucin was detected by staining cells as described above but omitting the use of Triton X-100 in the permeabilization step. Cells were also stained for both mucin and calreticulin or for mucin and the Golgi apparatus. Cells were stained overnight with either a polyclonal anti-calreticulin or a monoclonal antibody raised against the Golgi fraction of human cells (AE6) and the appropriate secondary antibody conjugated to Alexa Fluor 594. Cells were subsequently stained for mucin using the anti-Penta-His antibody directly conjugated to FITC (Qiagen). Cells were mounted in fluorescent mounting medium (Dakocytomation) and observed using an LSM710 confocal microscope (Zeiss).

Agarose SDS Gel Electrophoresis and Blotting. Agarose SDS gel electrophoresis was performed under reducing and nonreducing conditions as described previously.[30] Reduced and nonreduced mucin protein samples were separated, transferred to nitrocellulose by vacuum blotting, and probed using the anti-Penta-His antibody.

PNGase F Digestion of Recombinant Protein. PNGaseF (PROzyme, EC 3.5.1.52) was used to remove N-glycans from recombinant mucins. Purified recombinant MUC5AC protein was added to reaction buffer (100 mM sodium phosphate, pH 7.5) to yield a final concentration of 20 mM sodium phosphate in a total volume of 45 μL. Protein was denatured by adding 2.5 μL of denaturation solution containing 2.0% (w/v) SDS and 1 M β-mercaptoethanol and incubated at 100 °C for 5 min. To the reaction solution was added 2.5 μL of detergent solution containing 15% (v/v) NP-40 detergent. One microliter of PNGase F enzyme (2.5 units/mL) was used per 100 μL of reaction mix. The reaction mixture was incubated at 37 °C for 18 h.

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Lectin Binding. Binding of digoxigenin (DIG)-labeled *Galanthus nivalis* agglutinin (GNA) lectin (DIG glycan differentiation kit, Roche) was used to characterize glycosylation of expressed recombinant MUC5AC protein according to the manufacturer's instructions. GNA lectin detects terminally linked mannose in high mannose N-glycan carbohydrate chains.

RESULTS

MUC5AC Constructs Used in This Study. The recombinant MUC5AC N-terminus construct comprises the complete wild-type sequence (nucleotides 1−5211) from the start codon and includes the vWF domains D1, D2, D′, and D3 and the first Cys domain (Cys1). The recombinant MUC5AC C-terminus construct extends from nucleotide 12508 of wild-type MUC5AC to the stop codon at nucleotide 15093 and contains the vWF domains D4, B, and C and the cysteine knot (CK) domain. The recombinant MUC5AC N+C construct consists of the N- and C-termini joined by a synthetic DNA linker. The recombinant MUC5AC N+2TR+C construct consists of the N- and C-termini interspersed with two MUC5AC TRs containing the amino acid sequence TTSTTSAP (the most common VNTR sequence within native MUC5AC). Thus, this construct contains all the domains essential for normal mucin processing and serves as a model for native MUC5AC, although with a significant reduction in the potential for normal O-glycosylation. Only one Cys domain is included because we were unable initially to clone more of the VNTR and Cys regions. More Cys-containing domains were generated by PCR, but they could not be stably expressed. A diagrammatic representation of the constructs is shown in Figure 1. Details on the size of the N, C, N+C, and N+2TR+C constructs and the predicted molecular masses of the translated apomucins are given in Table 3.

Table 3. Molecular Sizing of Recombinant MUC5AC Constructs and the Theoretical Molecular Masses of the Translated Apomucins

MUC5AC construct	construct size (kb)	construct + pcDNA3.1 Zeo size (kb)	theoretical molecular mass of the translated apomucin (kDa)
N	4.7	9.7	192
C	2.7	7.7	99
N+C	7.4	12.4	266
N+2TR+C	7.5	12.5	270

Oligomerization of the N-Terminal Domain of MUC5AC Expressed in Normal and CF Tracheal and Bronchial Cell Lines. Recombinant protein could be detected in both tracheal and bronchial cells transiently transfected with the MUC5AC N-terminus construct immunostained with antibodies against FLAG and His epitopes and 2-11M1, which detects an epitope within the D1/D2 domains in the N-terminus of native MUC5AC.[29] Staining with the His antibody was identical to that obtained with the 2-11M1 antibody (Figure 2A). Protein was detected throughout the cytoplasm of the cell.

A single specific immunoreactive band of ∼210 kDa was detected in lysates from both wild-type and CF transfected tracheal cells when run under reducing conditions and probed with anti-FLAG and -His antibodies (Figure 2B). Oligomerization of the N-terminus protein was demonstrated by probing nonreduced samples from both tracheal and bronchial cells with anti-FLAG and -His antibodies (Figure 2B and C, respectively). For the tracheal cells, a monomeric N-terminal band was not detectable under nonreducing conditions, but at least two higher bands with estimated molecular masses of approximately 340 and 710 kDa were observed (Figure 2B).

Probing of transfected bronchial cell lysates suggested that dimeric and possibly trimeric forms of the N-terminus could be detected under nonreducing conditions in addition to the monomeric form of the protein, which was detected at ∼220 kDa. Additional bands with molecular masses of approximately 410 and 690 kDa were also evident (Figure 2C). Interestingly, the monomeric form of the protein detected under reducing conditions in these cells had an apparent molecular mass that was slightly larger than the size of the protein under nonreducing conditions. This suggests that the protein has a more compact conformation in the reduced form. The detection of monomeric protein in nonreduced lysates of these cells also suggests differences in processing between the tracheal and bronchial cell lines.

Transient Expression of N+C and N+2TR+C MUC5AC in Wild-Type and CF Tracheal Cell Lines. Both N+C and N+2TR+C MUC5AC proteins were detected using immunofluorescent staining with antibodies against the FLAG epitope in tracheal LC3 and LCFSN cells (Figure 3A). Analysis of lysates from both cell lines by western immunoblotting with anti-FLAG and -His antibodies resulted in the detection of two bands with molecular masses of >250 kDa (Figure 3B). The predicted molecular masses for the N+C and N+2TR+C apomucins are 266 and 270 kDa, respectively. Additionally, a <250 kDa band was detected with the anti-FLAG antibody, whereas a band between 100 and 150 kDa was detected only with the anti-His antibody (Figure 3B). The detection of bands above 250 kDa suggests that the proteins exceed the predicted molecular mass and may represent different glycoforms. The lower molecular mass band detected with the His antibody is in accordance with the pattern expected for C-terminal cleavage at the GDPH site similar to that observed previously for the C-terminus protein.[9] We also confirmed that recombinant C-terminus protein expressed in tracheal and bronchial cells transfected with the C-terminus construct described in this study was cleaved in an identical manner (results not shown). This indicates that the larger constructs are processed at the C-terminus in a manner similar to that of the C-terminus protein alone. The <250 kDa band detected with the anti-FLAG antibody corresponds to the monomeric form of the N-terminus protein detected in Figure 2C.

Expression of N+C and N+2TR+C MUC5AC in Wild-Type and CF Bronchial Cell Lines. Transient expression of all of the constructs was achieved in both the wild-type and CF bronchial cell lines. Clonal selection of stable cell populations resulted in the generation of three stable clones from IB3-1 cells and one clone from S9 cells expressing the N+C construct. A clone isolated from IB3-1 cells contained the N+2TR+C construct, whereas the production of a stable clone expressing the N+2TR+C construct in S9 cells was unsuccessful. Therefore, for comparison purposes, recombinant N+2TR+C protein from transiently transfected S9 cells was used in all of the experiments. Similar cellular staining patterns were evident for both cell lines with each construct by immunofluorescent staining using anti-FLAG and -His antibodies as well as antibodies against native MUC5AC (Figure 4A). Western blot analysis of cell lysates revealed a similar pattern of bands from

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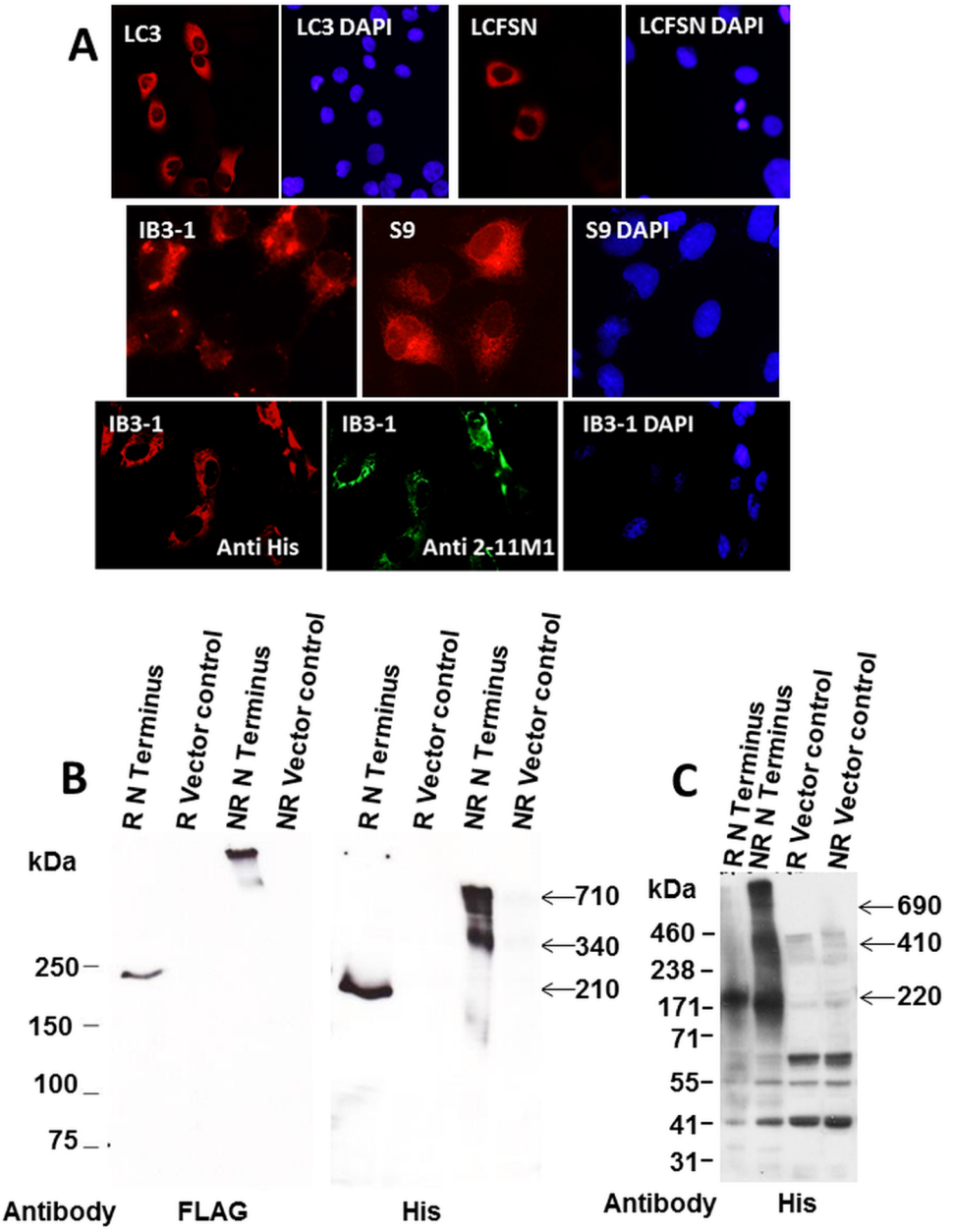

Figure 2. Detection of recombinant MUC5AC N-terminus protein in tracheal and bronchial cell lines transfected with the N construct. (A) Immunofluorescent micrographs demonstrating recombinant MUC5AC N-terminus protein expression in transfected cells using antibodies against FLAG and His epitopes and native MUC5AC (2-11M1). Cells were stained with the anti-FLAG antibody unless otherwise indicated. Corresponding DAPI images are shown to indicate cell nuclei. The same IB3-1 cells were stained with anti-His and -2-11M1 antibodies. Original magnification is 400×. (B) Western blot of cell lysates from tracheal LC3 cells run on a 6% SDS−PAGE gel. Blots were probed with anti-FLAG and -His antibodies. Vector controls contain a cell lysate from LC3 cells transfected with the pcDNA3.1 Zeo vector. Predicted molecular masses of the detected proteins are indicated. C) Western blot of cell lysates from IB3-1 cells run on 3−8% Tris acetate gels and probed with the anti-His antibody. Vector controls contain a cell lysate from IB3-1 cells transfected with the pcDNA3.1 Zeo vector. Estimated molecular masses of the detected proteins are indicated on the right-hand side. All cell lysates were exposed to either reducing (R) or nonreducing (NR) conditions prior to analysis.

stable transfectants probed with the anti-MUC5AC (62M1) and -His antibodies (Figure 4B). Up to four bands were detected, three of which had molecular masses above the predicted apomucin molecular mass of 270 kDa, suggesting different glycoforms of the peptides were present. This profile is more complex than that observed for tracheal cell lines LC3 and LCFSN and may reflect different processing or glycosylation pathways.

Oligomerization of N+C and N+2TR+C Proteins. N+C and N+2TR+C products were purified by immunoprecipitation, separated under nonreducing and reducing conditions using agarose SDS gel electrophoresis, transferred to a membrane, and probed with the anti-His antibody to detect potential high molecular mass products indicative of oligomerization. Several bands were detected with the MUC5AC N+C and N+2TR+C proteins, which in nonreduced samples had molecular masses that were higher than those of reduced samples from both wild-type (S9) and CF (IB3-1) bronchial cells (Figure 5). Results indicate that the VNTR region does not influence oligomerization. Reduced proteins migrated

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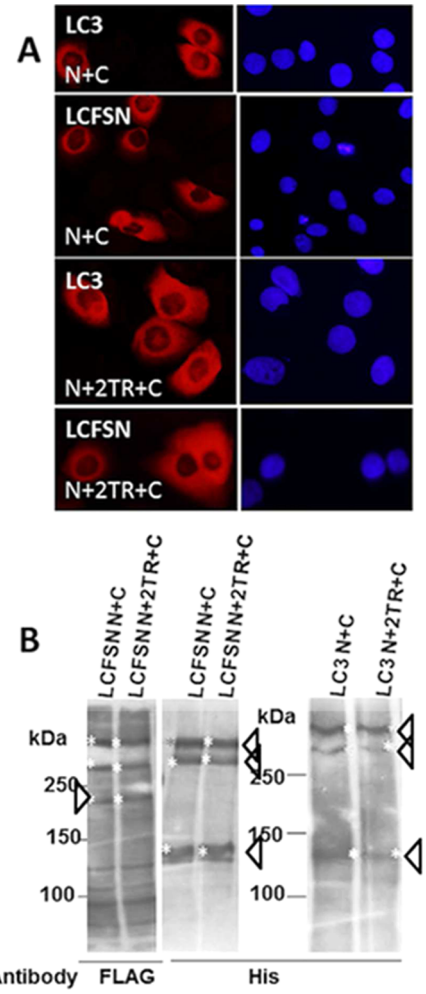

Figure 3. Expression of N+C and N+2TR+C constructs in tracheal cell lines LC3 and LCFSN. (A) LC3 and LCFSN cells transiently transfected with N+C and N+2TR+C constructs, stained with anti-FLAG antibody, and revealed using Alexa Fluor 594 48 h posttransfection. Corresponding DAPI images to visualize nuclei are shown in the right column. Original magnification is 400×. (B) Western blotting was used to detect recombinant proteins present in the lysates of transfected cells. Lysates were run on 6% acrylamide SDS−PAGE gels under reducing conditions, and blots were probed with either anti-FLAG or -His antibodies. Recombinant products detected in transfected cells are indicated by asterisks (*) and arrowheads and correspond to the cleavage products of the C-terminus protein (molecular mass between 100 and 150 kDa present only in blots probed with the anti-His antibody), the N-terminus fragment (molecular mass between 150 and 250 kDa detected only in blots probed with the anti-FLAG antibody), monomeric protein (molecular mass of >250 kDa), and reduced high molecular mass protein (molecular mass of >250 kDa).

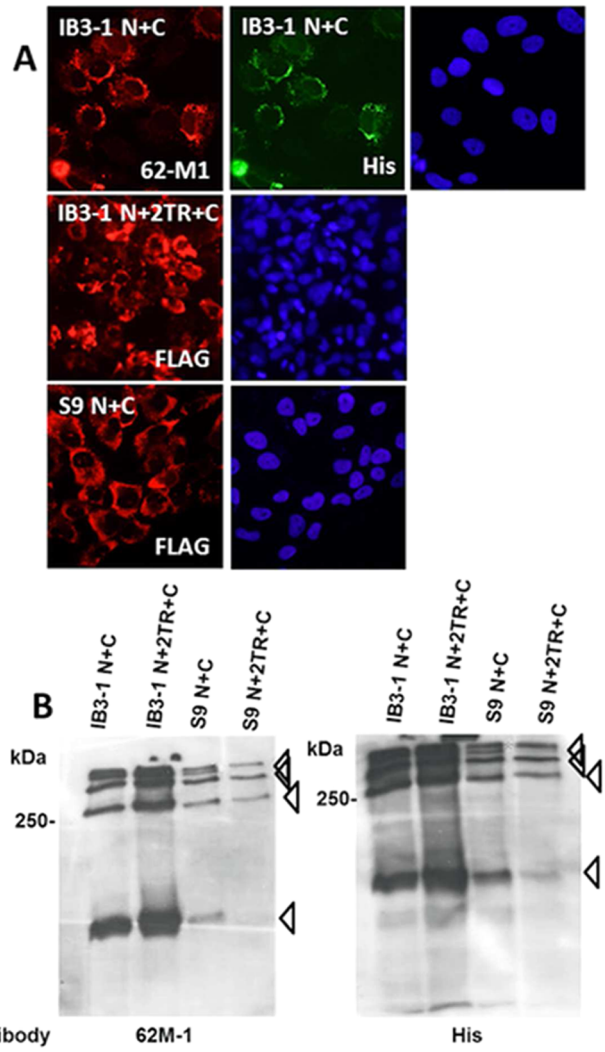

Figure 4. Expression of MUC5AC N+C and N+2TR+C constructs in bronchial cell lines IB3-1 and S9. (A) Expression of recombinant MUC5AC protein in stably transfected cells using antibodies against FLAG, His, and native MUC5AC (62-M1). The same cells were stained with anti-His and -62M1 antibodies. Corresponding DAPI images (blue) illuminating cell nuclei are shown. Original magnification is 400×. (B) Western immunoblots demonstrating expression of recombinant MUC5AC protein in cell lysates of cells transfected with N+C and N+2TR+C constructs. All cells except S9 are stably transfected with the N+2TR+C construct. Samples were run on 6% acrylamide SDS−PAGE gels under reducing conditions, and blots were probed with the native anti-MUC5AC (62-M1) and -His antibodies. Recombinant products detected in transfected cells are indicated with arrowheads and correspond to the cleavage product of the C-terminus protein (molecular mass of <250 kDa), reduced monomeric protein (molecular mass of >250 kDa), and reduced high molecular mass protein (two bands, molecular mass of >250 kDa).

further in the gel than nonreduced proteins, indicating that oligomerization of the N+C and N+2TR+C proteins occurs in both the S9 and IB3-1 cell lines.

The Recombinant MUC5AC Proteins Undergo N-Glycosylation. The molecular masses of all of the detected recombinant proteins appeared to be higher than their predicted molecular masses, suggesting posttranslational modification. GNA lectin, which detects terminally linked mannose in high mannose N-glycan carbohydrate chains, bound to several forms of the N+C and N+2TR+C proteins from both IB3-1 and S9 cells (Figure 6A), suggesting the presence of N-glycosylation. To confirm, FLAG N+C and N +2TR+C immunoprecipitated proteins were digested with PNGase F, an endoglycosidase that removes N-glycans from substrate glycoproteins. Protein digested with PNGase F exhibited a molecular mass that was lower than that of undigested material, indicating the presence of N-glycosylation (Figure 6B and C). The decrease in molecular mass was most obvious in the smaller MUC5AC cleavage products. This was probably due to the limitations in separation of higher molecular mass products by SDS−PAGE. Endoglycosidase H

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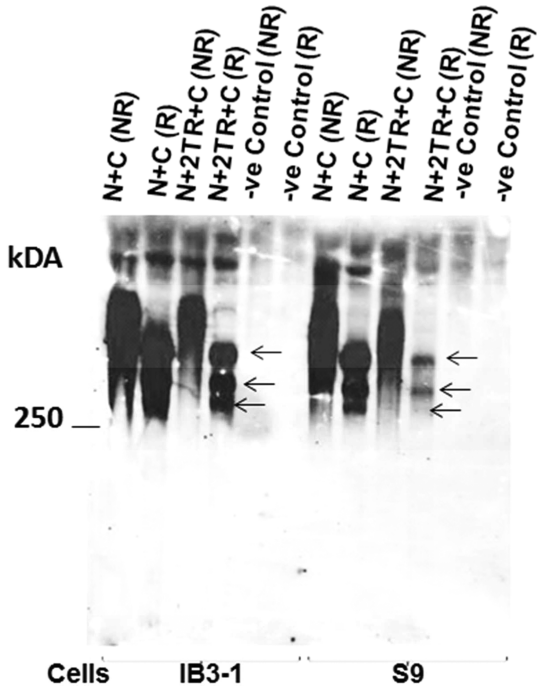

Figure 5. Oligomerization of recombinant MUC5AC N+C and N+2TR+C proteins. Recombinant proteins immunoprecipitated from lysates of IB3-1 or S9 transfected cells using anti-FLAG resin were separated on agarose SDS gels, transferred by vacuum blot to nitrocellulose, and probed with the anti-His antibody. Samples were run under either reducing (R) or nonreducing (NR) conditions. Three bands, indicated by arrows, were detected under reducing conditions in both S9 and IB3-1 cells. The smallest of these bands corresponds to reduced monomeric protein, and the other two are predicted to correspond to dimeric and reduced high molecular mass protein, respectively. The monomeric form of protein was not detected in S9 cells transiently transfected with the N+2TR+C construct, but this is likely due to small amounts of protein expressed in these cells. Negative controls are R or NR immunoprecipitates from IB3-1 and S9 cells transfected with the pcDNA3.1 Zeo vector alone.

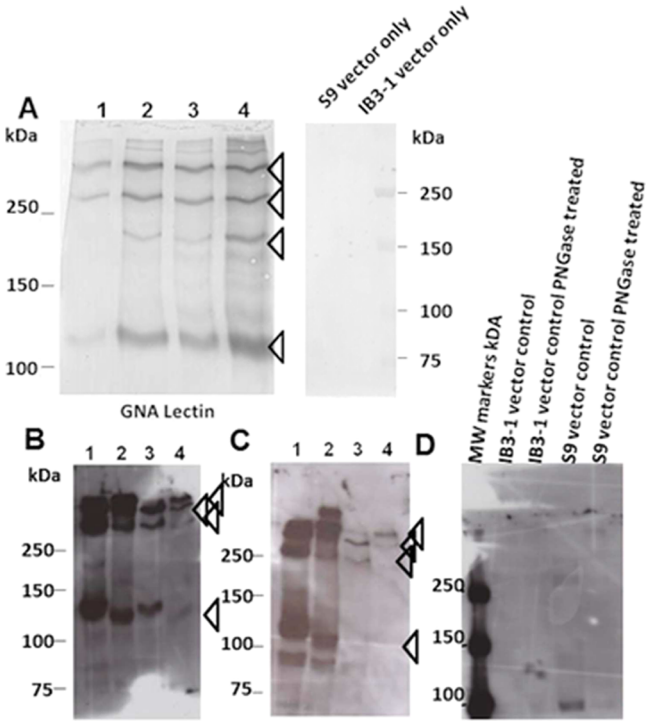

Figure 6. N-glycosylation of recombinant proteins. (A) Lectin blot analysis of recombinant N+C and N+2TR+C MUC5AC proteins. Protein purified from cell lysates by FLAG immunoprecipitation was run on 6% acrylamide SDS–PAGE gels under reducing conditions, transferred to PVDF membranes, and probed with GNA lectin. Lane 1, N+2TR+C protein purified from S9 cells (transient transfection); lane 2, N+2TR+C protein purified from stably transfected IB3-1 cells; lane 3, N+C protein purified from stably transfected S9 cells; and lane 4, N+C protein purified from stably transfected IB3-1 cells. The adjacent membrane contains FLAG immunoprecipitated material from lysates of S9 and IB3-1 cells transfected with the empty vector, run under identical conditions, and probed with GNA lectin. Recombinant products detected in the transfected cells are indicated with arrowheads and correspond to the cleavage product of the C-terminus protein (molecular mass of 100–150 kDa), the N-terminus fragment (molecular mass of 150–250 kDa), monomeric protein (molecular mass of >250 kDa), and reduced high molecular mass protein (molecular mass of >250 kDa). (B–D) PNGase F treatment of recombinant MUC5AC N+C and MUC5AC N+2TR+C proteins. Immunoprecipitated proteins separated on 6% acrylamide SDS–PAGE gels under reducing conditions were transferred to PVDF membranes and probed with anti-His antibody. (B and C) Immunoreactivity of recombinant proteins from IB3-1 and S9 cells, respectively. Lane 1, recombinant N+C protein; lane 2, PNGase F-treated recombinant N+C protein; lane 3, recombinant N+2TR+C protein; and lane 4, PNGase F-treated recombinant N+2TR+C protein. Recombinant products detected in transfected cells are indicated by arrowheads and correspond to the cleavage product of the C-terminus protein (molecular mass of 100–150 kDa), monomeric protein (molecular mass of >250 kDa), and reduced high molecular mass protein (two bands with molecular masses of >250 kDa). (D) Immunoreactivity of immunoprecipitated lysates from IB3-1 and S9 cells transfected with empty vectors.

treatment of the proteins removed all reactivity with GNA lectin (results not shown).

Secretion of Recombinant MUC5AC Protein. A major issue when working with recombinant mucins has been difficulty in getting the recombinant protein secreted from the cells.[31] We assessed the effect of growth conditions on protein secretion from cells stably expressing the N+2TR+C construct. IB3-1 cells stably expressing the N+2TR+C construct were grown as monolayers on both plastic tissue culture plates and on transwell inserts. Culture medium was collected from cells, western blotted, and probed with anti-His and -FLAG antibodies. Recombinant mucin protein was detected only in the culture medium of cells grown on transwell filters. Immunofluorescence microscopy of non-permeabilized cells demonstrated that minute amounts of extracellular mucin could be detected on the surface of the cells after 7 days of growth on the transwell filter but increased over time with the most intense staining detected after 21 days (Figure 7A). Confocal immunofluorescence microscopy of nonpermeabilized cells suggested that small amounts of extracellular mucin could be detected on the surface of the cells grown on the transwell filters for 21 days (Figure 7B). There was no extracellular mucin detected on cells grown as monolayers on plastic (results not shown). Immunoblotting of concentrated culture supernatant from cells grown on transwell filters under reducing conditions and probed with anti-His antibody demonstrated clearly that recombinant protein was secreted from the cells and could be detected in the culture supernate. The secreted mucin detected in the culture supernate differed in molecular mass from the intracellular

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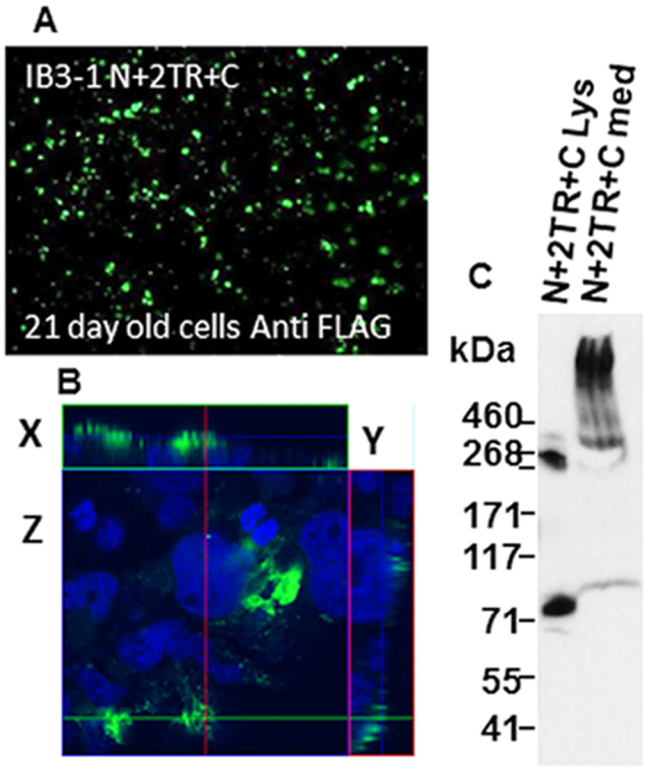

Figure 7. Secretion of recombinant MUC5AC protein. (A) A stable clone of IB3-1 cells expressing recombinant N+2TR+C protein was cultured on transwell filter inserts for 21 days, stained without permeabilization for detection of extracellular recombinant MUC5AC with anti-FLAG antibody, and revealed using an anti-mouse secondary antibody conjugated to Alexa Fluor 488. Original magnification is 200×. (B) Confocal immunofluorescent micrograph of the IB3-1 stable clone cells expressing recombinant N+2TR+C protein cultured on a transwell filter insert for 21 days, stained without permeabilization for detection of extracellular recombinant MUC5AC with anti-His antibody, and revealed using an anti-mouse secondary antibody conjugated to Alexa Fluor 488 (green). Cell nuclei were stained with DAPI (blue). (C) Western immunoblot of cell lysates (Lys) and of concentrated spent culture supernate (med) from IB3-1 cells expressing N+2TR+C protein. Samples were run under reducing conditions on 3−8% Tris acetate gels and blotted onto a PVDF membrane, which was probed with the anti-His antibody.

form (Figure 7C), suggesting that the extracellular product undergoes processing prior to or during secretion from cells. An attempt was made to detect how the mucin was processed and secreted out of the cells. Confocal microscopy of cells grown on transwell filters stained for mucin and calreticulin, a marker protein for the endoplasmic reticulum (ER), showed mucin present in granules in cells surrounded by the ER (Figure 8A). However, staining did not demonstrate mucin in the Golgi apparatus of the cells (Figure 8B). It is possible that the transit time for mucin in the Golgi apparatus is short, especially if O-glycosylation is limited, making it difficult to capture an image with mucin in the Golgi apparatus.

DISCUSSION

Cloning of versions of human MUC5AC containing both the N- and C-termini and TR regions has not previously been reported, possibly due to difficulties in cloning large mucin genes and the repetitive nature of the sequences that encode the O-glycosylation domains. This study demonstrates the

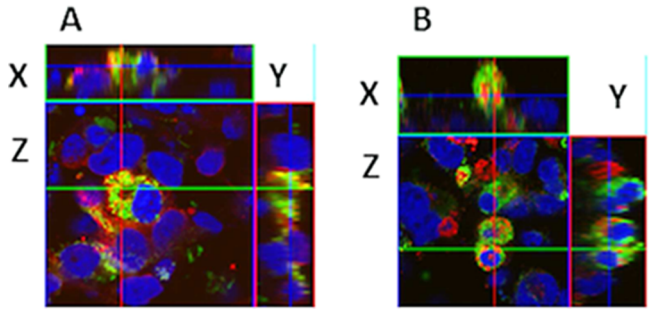

Figure 8. Confocal immunofluorescent images of IB3-1 cells stably expressing the N+2TR+C construct stained for recombinant protein with anti-His (green) and (A) anti-calreticulin to visualize the ER (red) and (B) anti-Golgi apparatus (red) in IB3-1 cells grown on transwell filter inserts.

feasibility of producing recombinant secreted mucins composed of a native signal sequence and N- and C-termini and TR sequences in mammalian cell lines. In addition, the study sheds new light on the role of the N-terminus in MUC5AC processing. It has previously been shown that the N-terminus of intestinal gel-forming mucin MUC2 forms trimers and that the CysD domain plays an essential role in this process.[8,16,20] Trimerization of the MUC2 N-terminus suggests that it aids in maintenance of the mucin network.[8] In contrast, it has been demonstrated recently that the N-terminus of another secreted mucin, MUC5B, dimerizes and that mature MUC5B forms linear polymeric structures.[13] It has previously been suggested that MUC5AC mucin is also dimeric and forms linear polymeric mucins.[32] However, our results would suggest that MUC5AC may behave more like MUC2 than MUC5B. Under nonreducing conditions and in addition to N-terminal monomers, oligomeric forms, which could be dimers and possibly even trimers, were also evident, suggesting that extracellular MUC5AC may assemble as a branched network rather than as a linear polymer in a manner analogous to that predicted for MUC2. Further studies are required to more accurately determine the molecular masses of the different forms of the N-terminus protein detected under nonreducing conditions. Unfortunately, we have not established a stable clonal population of cells expressing the N-terminus construct to date, but the generation of such a clone would allow for detailed investigation of the importance of the N-terminus for the correct biosynthesis and secretion of MUC5AC.

The sequences of MUC5AC and vWF are homologous, and MUC5AC undergoes dimerization in the ER via intermolecular disulfide bonds located in the C-terminus and forms multimers in the Golgi apparatus via disulfide bonding between cysteine residues in the N-terminus.[33] It has previously been demonstrated in colonic lines that the MUC5AC C-terminus forms dimers through its CK domain.[15] In this study, the C-terminus protein produced alone and as part of larger constructs also resulted in dimer formation (results not shown), whereas the N+C and N+2TR+C proteins formed higher order polymeric structures, suggesting that they function in a manner similar to that of native MUC5AC.[32]

The MUC5AC C-terminus has been shown previously to be posttranslationally cleaved at an Asp−Pro bond situated in a GDPH domain in the vWFD4 motif. This process occurred in the ER at neutral pH and was only modestly accelerated at a slightly lower pH.[9] In a recent proteomic analysis of MUC5AC, five different nonspecific cleavage sites on two predicted tryptic

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peptides, one from the C-terminal vWF domain and the other from the N-glycosylation-rich region, were detected, but cleavage in this GDPH sequence of the C-terminal region was not one of them.[34] However, the C-terminal, N+C, and N+2TR+C products produced in this study all underwent cleavage in this domain. This indicates that the proteins are processed in airway cell lines like previous versions of the MUC5AC C-terminus and is in accordance with the predicted model of C-terminal processing.[32] In addition, it indicates that the presence of the N-terminus and VNTR domains does not influence this process. All of the products expressed in both cell lines showed evidence of glycosylation, as demonstrated by molecular masses of recombinant proteins that were higher than those predicted for apomucin proteins alone. High molecular mass forms of recombinant N+C and N+2TR+C products detected with the native MUC5AC antibody 62M1 may be glycoforms of MUC5AC at different stages of synthesis within the ER or Golgi apparatus. N-glycosylation was confirmed by comparison of proteins after selective glycan removal using PNGase F digestion as well as detection with GNA lectin, which is suitable for identifying high mannose N-glycan chains. Extensive O-glycosylation is a major feature of mature mucins. The recombinant version of MUC5AC differs significantly from native MUC5AC, particularly in terms of the number of O-glycosylated VNTR domains. The inclusion of two TR sequences is a balance between the need for the construct to be stable and trying to generate a version of MUC5AC that can function in a manner similar to that of native MUC5AC. There is limited evidence that the N+2TR+C protein undergoes O-glycosylation. PNGase F digestion did not reduce recombinant MUC5AC proteins to their predicted peptide molecular mass. Although this may be due to incomplete PNGase F digestion caused by secondary structures preventing access to all N-glycans, in the case of the N+2TR+C protein, it could be due to the presence of O-glycans, which would add additional mass to the recombinant proteins. A comprehensive analysis of O-glycosylation was not feasible due to the limited amount of purified protein available. Future studies should focus on generating sufficient material to facilitate O-glycan structural analysis to confirm this hypothesis. It is unknown what effect the significantly reduced VNTR region has on either mucin processing or glycosylation. The MUC5AC N+C and N+2TR+C constructs appear to be processed in a similar fashion, and these steps fit well with the predicted model for native MUC5AC. However, the number of glycosylation sites being limited compared to the native protein may lead to differences in glycan structure, specificity, and quantity. Increasing the number of Cys domains interspersed with suitable VNTR sequences would increase the relevance of the construct. The constructs described here serve as a starting platform that can be modified by the addition of more relevant types and numbers of VNTR regions to explore their role in fully assembling a viscoelastic gel. It is known that the number of VNTRs in secreted mucins is heterogeneous, and a reduction and add-back approach can be used to explore the relevance of different VNTR numbers on overall mucin function and gel formation. In addition, future studies should also include detailed glycan analysis of these proteins, including assessing the effect of glycosidases, such as O-glycanase, fucosidase, and sialidase, detailed lectin-binding analysis, and ultimately mass spectrometry analysis.

The native MUC5AC signal sequence is incorporated into these proteins to facilitate correct intracellular trafficking and processing. This is similar to the approach employed to clone and express a recombinant version of MUC1 localized to the cell membrane.[35] Transfected cells grown as monolayers on plastic did not show detectable levels of secretion. However, growth of the cells on transwell filters resulted in secretion of the N+2TR+C recombinant protein, which could be detected in conditioned medium. The growth of these cells in this way may mimic cellular differentiation and normal tissue organization more closely than growth on plastic. For example, actin plays a key role in communicating signals from the extracellular matrix to the cell, and its polymerization has been shown to play a role in increasing mucin secretion.[35] However, we did not detect an increase in the transepithelial resistance of cells over time when they were cultured on transwell filters, indicating that tight junctions were not being formed. In our hands, this is similar to what occurs with A549 cells, which secrete mucin only when they are grown on transwell filters but also does not result in high transepithelial resistance in the cells. When compared to immature intracellular versions, secreted forms of the N+2TR+C protein displayed an increase in molecular mass. This may be due to altered folding as the proteins mature and are secreted but may also be due to O-glycosylation, which occurs in the Golgi late in the mucin assembly sequence.[36] These results suggest that an in-depth analysis of the growth conditions of cells and how they affect the expression and processing of recombinant mucin is warranted. Of particular interest would be the effect of growing cells using an air−liquid interface[37] and the use of three-dimensional cultures. BEAS-2B, an immortalized human bronchial epithelial cell line, was found to differentiate and produce mucin when grown in three-dimensional culture but not when grown in monolayers on plastic.[38]

Although we could detect secretion of N+2TR+C protein in the culture supernate, we could not demonstrate the formation of an adherent mucus gel on the surface of the cells. Confocal microscopy images suggested that only small amounts of mucin could be detected on the surface of the cells. IB3-1 cells do not normally make mucus, and it is likely that they are not expressing other proteins found in mucus[39] that may be required for the formation of a mucus gel. It would be interesting to look at the expression of these constructs in cells that secrete mucins, such as respiratory A549 cells[40] or some of the colonic adenocarcinoma mucus-secreting HT29 clones that have been previously characterized.[41−45]

In addition to the use of these cell lines to analyze the processing, glycosylation, and assembly of MUC5AC, other more fundamental questions related to the processing of mucin as well as the influence of VNTR number and sequence on glycosylation can potentially be addressed using the constructs described in this study. The generation of stable clones expressing each of the constructs in both IB3-1 and S9 cells would allow for investigation of the influence of CFTR on mucin processing. The purified products, as well as the cell lines, will also be of use in investigating specific mucin domains that may play a role in facilitating bacterial binding, such as the gastric pathogen *Helicobacter pylori*, which displays a specific tropism for MUC5AC expressed in the stomach.[46] MUC5AC has also been identified as a critical component of immune-regulated responses during intestinal nematode infection.[47] Aberrant expression of MUC5AC occurs in a number of different types of cancer, including that of the colon[48] and pancreas.[49] Expression of MUC5AC is upregulated in ovarian cancer[50] and downregulated in gastric cancer.[51] The potential

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to mutate specific regions of the core peptide and manipulate individual domains and residues means that these constructs are ideal for future investigations on processes such as mucin biosynthesis, MUC5AC expression in diseases such as cancer, MUC5AC pathogen interactions, and innate host defense.

■ ASSOCIATED CONTENT

Supporting Information

Detailed methods for the cloning strategy. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS

CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; CK, cysteine knot; COPD, chronic obstructive pulmonary disease; DIG, digoxigenin; ER, endoplasmic reticulum; GNA, *G. nivalis* agglutinin; His, histidine; HRP, horseradish peroxidase; NR, nonreduced; PVDF, Immobilon-P polyvinylidene difluoride; R, reduced; TR, tandem repeat; VNTRs, variable number tandem repeat regions; vWF, von Willebrand factor

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