Identification of a nonmucin glycoprotein (gp-340) from a purified respiratory mucin preparation: evidence for an association involving the MUC5B mucin

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Received on April 30, 2001; revised on June 28, 2001; accepted on July 3, 2001

Rate-zonal centrifugation of a reduced and alkylated respiratory mucin preparation identified a protein-rich fraction. This was subjected to trypsin treatment and one of the many liberated peptides was purified and its N-terminal sequence determined. The peptide was identical to a 14 amino acid sequence from the scavenger receptor cysteine-rich domain containing glycoprotein gp-340. A polyclonal antiserum, raised against the peptide, stained the serous domain containing glycoprotein gp-340. A polyclonal antiserum, raised against the peptide, stained the serous cells in the submucosal glands of human tracheal tissue. The glycoprotein was purified from respiratory mucus by density-gradient centrifugation, gel chromatography, and anion exchange chromatography. The molecule exhibited a heterogeneous distribution of buoyant density (1.28–1.46 g/ml) that overlapped with the gel-forming mucins, was included on Sepharose CL-2B and was quite highly anionic. SDS–PAGE indicated a mass greater than 208 kDa and measurements performed across the molecular size distribution indicated an average Mr of 5 × 10^5 with a range of Mr from 2 × 10^5 to 1 × 10^6. Gel chromatography of respiratory mucus extracts (“associative” and “dissociative”) indicated that this glycoprotein forms complexes that may involve the large gel-forming mucins MUC5AC and MUC5B. Rate zonal centrifugation suggested such complexes are more likely to involve MUC5B rather than MUC5AC mucins.

Key words: Gp-340/mucus/MUC5AC/MUC5B

Introduction

The respiratory mucus gel performs a number of essential functions that collectively lead to the protection of the airways. This highly hydrated gel, in conjunction with ciliated epithelial cells, forms the mucociliary “escalator,” which, along with cough, is essential for the maintenance of sterile and unobstructed airways. The polymer matrix of the mucus is provided by very large oligomeric glycoproteins known as mucus glycoproteins or mucins (for a review see Thornton et al., 1997a). At least eight mucin genes are expressed in the respiratory tract (for a review see Gendler and Rose, 1997) and the products of two of these genes, the MUC5AC and MUC5B mucins, appear to be gel-forming molecules (Hovenberg et al., 1996; Thornton et al., 1996, 1997b; Wickstrom et al., 1998).

The mucus gel provides a physical barrier to chemical and invasive biological agents and in addition contains a host of protective agents, which include lysozyme, lactoferrin, transferrin, proteinases, proteinase inhibitors and secretory IgA (for a review see Rayner and Wilson, 1997). These proteins are vital to the sterility of the airways and act to destroy bacteria or prevent their colonization of the mucosal surface and to protect against host and bacterial proteinases. These components may associate with the gel-forming mucins and thus become part of the gel network and provide an immobilized reservoir of protective factors. In support of this, previous studies have reported that proteins are released from purified respiratory mucins after treatment with reducing agents (Tabachnik et al., 1981; Ringler et al., 1988; Naziruddin et al., 1990; Gupta and Jentoft, 1992; Thornton et al., 1994), but their identity was not determined. Furthermore, recent investigations on saliva have identified a number of proteins involved in heterotypic associations with the gel-forming mucins, which in this case are the product of the MUC5B gene (Iontcheva et al., 1997; Wickstrom et al., 2000).

In a previous study of asthmatic mucins we noted that after reduction of a highly purified respiratory mucin preparation, a protein-rich fraction was generated (Thornton et al., 1994). In the present study we present data to suggest that a high-molecular weight glycoprotein (gp-340), previously identified in bronchoalveolar lavage fluid (Holmskov et al., 1997, 1999), is present in this fraction. It is likely that due to its chemical composition that this glycoprotein was present in the mucin fraction due to copurification. However, our data suggest that in respiratory mucus this glycoprotein is involved in high-molecular-weight complexes that may involve the gel-forming mucins and in particular a subpopulation of the MUC5B mucins.

Results

Fractionation of the reduced mucin preparation

Respiratory mucins were isolated by CsCl density gradient centrifugation and then radiolabeled by reductive alkylation as described previously (Thornton et al., 1994). The reduced mucin preparation was subjected to rate-zonal centrifugation and the separation was monitored with antisera recognizing the
MUC5AC and MUC5B mucins and for radioactivity (Figure 1). The MUC5AC and MUC5B mucin subunits were separated from other 14C-radiolabeled material toward the top of the gradient. Furthermore, the subunits of the two mucins were separated; the MUC5B mucin subunits are apparently more massive than those derived from the MUC5AC mucin. The 14C-radiolabeled material released by reduction had little reactivity with the MAN-5ACI antiserum, but some MAN-5BI reactivity was observed. Whether this protein-rich fraction was derived from the mucin polypeptides or comprised of other, nonmucin proteins was investigated by N-terminal sequencing of peptides produced after trypsin treatment.

Trypsin fragmentation and peptide identification

The 14C-radiolabeled material from the top of the rate-zonal gradient was pooled, dialyzed into 0.1 M ammonium hydrogen carbonate, and digested with trypsin; the digest was fractionated by reverse-phase chromatography (data not shown). A complex mixture of peptides was observed and a peptide with a mass of 1455 Da (determined by matrix-assisted laser desorption time of flight mass spectrometry [MALDI-TOF MS]) was purified by rechromatography on the reverse-phase column (data not shown). The primary sequence of the peptide was determined by automated Edman degradation (Figure 2) and found to be identical to a 14-amino-acid repeated sequence from a scavenger receptor cysteine-rich (SRCR) domain-containing protein designated gp-340 (Holmskov et al., 1997, 1999). A high degree of similarity was apparent with other members of this protein superfamily (Figure 2).

Immunolocalization

A polyclonal antiserum, raised against the peptide sequence, was used for immunolocalization studies on human tracheal tissue (Figure 3). Staining was observed mainly in the serous cells of the submucosal glands, and there was little evidence of staining within the mucous cells. However, after reduction and alkylation very weak staining was observed over the mucous cells of the submucosal glands and the goblet cells (data not shown).

Purification and characterization of gp-340

Respiratory secretions were extracted with 6 M guanidinium chloride and subjected to isopycnic density gradient centrifugation in 4 M guanidinium chloride/CsCl (Figure 4a). Reactivity with the gp-340 antiserum was seen over a broad density range (1.28–1.46 g/ml), which overlapped with the gel-forming mucins (1.33–1.55 g/ml). The MAN-gp-340-positive fractions were pooled and subjected to chromatography on Sepharose CL-2B in 4 M guanidinium chloride (Figure 4b). The small amount of gel-forming mucins still present after pooling was eluted in the void volume of the column and was
gp-340 in respiratory mucus

separated from the gp-340 antiserum reactivity that eluted in the included volume. Fractions corresponding to the gp-340 reactivity were pooled and dialyzed against 6 M urea/10 mM piperazine, pH 5.0, containing 0.02% (w/v) 3-(3-cholamidopropyl)-dimethyl-ammonio)-1-propane-sulfonate (CHAPS) prior to fractionation by anion exchange chromatography on Mono Q (Figure 4c). Two periodic acid–Schiff’s (PAS)–positive peaks were observed, and the most acidic material in the more major of the two peaks was reactive with the gp-340-antiserum. These fractions (17–22) were pooled and rerun on the column; finally fractions containing gp-340 antiserum reactivity were pooled and dialyzed either against water and lyophilized or into 6 M urea. A monosaccharide compositional analysis for the preparation is presented in Table I.

The preparation was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) followed by silver staining (Figure 5a) or western blotting with the gp-340 antiserum (Figure 5b). In both cases a broad diffuse band with an apparent \( M_r > 2.08 \times 10^5 \) that barely entered the resolving gel was observed. In contrast, western blotting after agarose gel electrophoresis identified a single diffuse band more rapidly migrating than either the MUC5AC or MUC5B mucin reduced subunits (Figure 5c).

The molecular weight and size distribution of the gp-340 preparation was analyzed by in-line light scattering measurements after gel chromatography on a Superose 6 column eluted with 0.2 M NaCl (Figure 6). The profile of gp-340 antiserum reactivity (data not shown) followed the mass profile as assessed by refractive index measurements (Figure 6). The average \( M_r \) for the distribution was approximately \( 5 \times 10^5 \) with a range from \( 2 \times 10^5 \) to \( 1 \times 10^6 \), and there was no measurable value for the radius of gyration \( (R_g) \) across the entire distribution.

Does the gp-340 form complexes with high-\( M_r \) mucins?

To ascertain if the gp-340 glycoprotein formed complexes with itself or other molecules in the secretion, we subjected a 4 M guanidinium chloride mucus extract to gel chromatography on a Sepharose CL-2B column under “dissociative” (4 M guanidinium chloride) and “associative” (phosphate buffered saline [PBS]) conditions (Figure 7a, b). As previously (Figure 4b), chromatography in 4 M guanidinium chloride yielded gp-340 reactivity only in the included volume of the column. In contrast, the extract after dialysis into PBS and chromatography in the same solvent resulted in a proportion of the gp-340 population eluting in the void volume of the column (Figure 7b). A PBS extract of mucus chromatographed under “associative” conditions (i.e., PBS) resulted in a higher proportion of the gp-340 antiserum reactivity coeluting with a PAS-rich peak in the void volume of the column (Figure 7c). Furthermore, the molecules were more spread over the included volume of the column.

**Fig. 3.** Section of human trachea stained with the MAN-gp-340 antiserum. Sections (4 \( \mu \)m) of paraffin-embedded tissue were stained with the antiserum and counterstained as described in the text.
Material from the void and included volume of the Sepharose CL-2B column (Figure 7c) was subjected to rate-zonal centrifugation performed on 10–30% sucrose gradients (Figure 8). The MAN-gp-340 reactive material from the void volume of the column had a higher sedimentation rate than the included gp-340 fraction (Figure 8a, b). There was evidence of cosedimentation of a proportion of the gp-340 population and MUC5AC and MUC5B mucins (Figure 8b). To investigate this further the void fraction was subjected to rate-zonal centrifugation under identical conditions but for a longer time (Figure 8c). The sedimentation profiles suggest that whereas a proportion of the MUC5B mucin cosediments with the MAN-gp-340 reactivity the MUC5AC mucin does not.

**Discussion**

Other studies in the literature have noted the liberation of protein components from what were believed to be substantially purified respiratory mucin preparations by treatment with reducing agents (Tabachnik et al., 1981; Ringler et al., 1988; Naziruddin et al., 1990; Gupta and Jentoft, 1992). In these studies it was not determined if these components were other proteins associated with the mucins or cleaved fragments of mucin polypeptides. More recent investigations, however, have reported heterologous associations of nonmucin proteins...
with salivary mucins (Iontcheva et al., 1997; Wickstrom et al., 2000), whereas others have demonstrated that mucin polypeptides can undergo proteolytic cleavage. For example, the 118-kDa glycopeptide isolated from rat (and human) intestinal mucins, that was once thought to be a “link” glycoprotein responsible for cross-linking mucin subunits (Fahim et al., 1987; Forstner et al., 1989; Roberton et al., 1989), has now been identified as a proteolytically derived fragment from the C-terminus of the rat Muc2 mucin (Khatri et al., 1998). Furthermore, it has been reported that the MUC5B mucin undergoes cleavage at both its N- and C-termini (Wickstrom et al., 1998; Thornton et al., 2000).

Previously we have shown that a protein-rich fraction was generated after reduction of a respiratory mucin preparation that was isolated by extraction with 6 M guanidinium chloride followed by three stages of isopycnic density-gradient centrifugation (Thornton et al., 1994). The results of the present investigation clearly show that this fraction contained a component of nonmucin origin. Its broad buoyant density range in 4 M guanidinium chloride/CsCl overlaps with that of the MUC5AC and MUC5B mucins, explaining why a proportion of it was found in the original mucin preparation. Therefore to remove this glycoprotein from mucin preparations it will be necessary to add a “dissociative” gel chromatography step on a porous media (e.g., Sepharose CL-2B) to the current CsCl density-gradient protocol. Further study of the complex pool of peptides generated by trypsin treatment of the protein-rich fraction is ongoing to ascertain the molecular identity of the parent proteins. It cannot be ruled out that this fraction may also contain or even be dominated by peptides derived from the mucins.

The isolated peptide was identical to a 14-amino-acid sequence that is repeated 12 times within the deduced primary sequence of gp-340, which is an alternatively spliced form of the DMBTI gene (Mollenhauer et al., 1997; Holmskov et al., 1999). Gp-340 was previously isolated from bronchoalveolar lavage fluid, where it was shown to bind surfactant protein-D, which is a member of the collectin family (Holmskov et al., 1997). More recently it has been shown that the polypeptide of gp-340 is likely to be identical to that of the salivary agglutinin, this latter glycoprotein has been demonstrated to bind to oral bacteria (Prakobphol et al., 2000). Immunostaining for gp-340 has been shown in the lung on alveolar macrophages and epithelial cells of the small intestine and ducts of the salivary glands (Holmskov et al., 1999). In the present study, using a polyclonal antiserum raised to the isolated 14 amino acid peptide, we have localised immunoreactivity to the serous cells of the submucosal glands.

The gp-340 preparation is polydisperse ($M_r 2.5 \times 10^5$–$1 \times 10^6$) with an average $M_r$ (in NaCl) of $5 \times 10^5$, and there is no
gradients for 1.5 h as described in chromatography (see Figure 7c) were centrifuged on 10–30% (w/v) sucrose a. Aliquots of selected fractions (Fig. 8. dashed line shows the nominal gradient. (open squares), MAN-5BI (open circles), and MAN-5ACI (closed circles). The from the top, and fractions (0.5 ml) were analyzed for reactivity with MAN-gp-340 fraction 19 was centrifuged as above but for 2.5 h. The gradients were emptied that can be detected with this technique. These findings from the deduced sequence (Holmskov et al., 1997). The average mass determined by multi-angle laser light scattering (MALLS) is higher than the mass of 258 kDa that is predicted average mass determined by multi-angle laser light scattering of interacting species under the conditions used. This is in broad agreement with previous estimates from gel chromatography under nondissociating conditions, which report a value > 1000 kDa (Holmskov et al., 1997). The broad buoyant density range observed for the glycoprotein is consistent with variable glycosylation. However, it is also possible that the molecule has a tendency to self-associate and the chromatographic profile on Superose 6 may reflect an equilibrium of interacting species under the conditions used. There was no detectable measurement of the radius of gyration (R_g) across the entire chromatographic distribution and this indicates that putative complexes would have to have R_g < 15nm, because this is the size of the smallest particles that can be detected with this technique. These findings strongly suggest that there is no tendency toward an open-ended self-association for this molecule.

A single species of gp-340 was observed after extraction of mucus with guanidinium chloride and subsequent Sepharose CL-2B gel chromatography in this “dissociative” solvent. This contrasts with the same mucus sample extracted with PBS prior to gel chromatography under “associative” conditions. In this case there is evidence for more than one population of the gp-340, one in the void volume and others included on the column. These data provide evidence that gp-340 may exist as part of a complex in mucus. The MUC5AC and MUC5B mucins also chromatograph in the void volume of Sepharose CL-2B, thus the distribution of the gp-340 may have resulted from its direct or indirect association with one or both of these gel-forming mucins. Sucrose gradient centrifugation of selected fractions from the Sepharose CL-2B gel chromatography of the “associative” mucus extracts confirms the presence of an association, which is consistent with the involvement of mucins. In particular the sedimentation data suggest that a subpopulation of the MUC5B mucins may be involved, whereas the MUC5AC mucins are not. Further studies are required to distinguish between self-association and complex formation with the MUC5B mucins. However, in support of our data it has been demonstrated that the salivary agglutinin (gp-340) (Prakobphol et al., 2000) is enriched in the gel phase of salivary mucus (Holmskov et al., 1999) where the predominant gel-forming mucin in this secretion is MUC5B (Thornton et al., 1999). It is interesting to speculate on the consequences of an interaction of gp-340 with the mucin network. Gp-340 clearly has an important antibacterial role in mucus either via its direct binding to bacteria (Prakobphol et al., 2000) or its association with the bacterial-binding collectin surfactant protein-D (Holmskov et al., 1997). Thus binding of gp-340 to the gel network increases the functionality of the mucus by facilitating clearance of sequestered bacteria from the respiratory tract via the mucociliary escalator or cough.

The peptide sequence reported here is repeated exactly 12 times in the deduced gp-340 sequence and is part of a larger motif known as the SRCR domain. The function of this motif is still not defined however; the known mammalian SRCR domain-containing proteins are found on immune cell surfaces or are secreted and suspected of being involved in host defense (Resnick et al., 1994). Other SRCR-containing proteins contain repeated sequences that are homologous to the peptide sequenced here; the three most similar, murine CRP-ductin (Cheng et al., 1996), rat ebnerin (Li and Snyder, 1995), and bovine gallbladder mucin (Nunes et al., 1995), are all expressed at epithelial surfaces. It seems likely from the data presented in this study and previously by Holmskov and colleagues (1999) that the putative gallbladder mucin is not a classical mucin but a bovine form of gp-340 or a closely related molecule.

In addition to 14 SRCR domains the deduced gp-340 polypeptide contains 2 CUB (complement subcomponents C1r/C1s, Uegf protein, and bone morphogenic protein) domains and a zona pellucida (ZP) domain (Bork and Sander, 1992; Bork and Beckmann, 1993). All of these motifs are found in molecules that are secreted or located at the cell surface (Mollenhauer et al., 1997) and are potential sites for binding proteins, carbohydrates and lipids (Moestrup et al.,

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Fig. 8. Sucrose gradient centrifugation of fractions from gel chromatography. Aliquots of selected fractions (a) 31 and (b) 19 from Sepharose CL-2B chromatography (see Figure 7c) were centrifuged on 10–30% (w/v) sucrose gradients for 1.5 h as described in Materials and methods. (c) An aliquot of fraction 19 was centrifuged as above but for 2.5 h. The gradients were emptied from the top, and fractions (0.5 ml) were analyzed for reactivity with MAN-gp-340 (open squares), MAN-5BI (open circles), and MAN-5ACI (closed circles). The dashed line shows the nominal gradient.
Recent crystal structure data on seminal fluid sperm-adhesins indicates that the CUB motif may specify sites for strong self-interaction (Varela et al., 1997; Romao et al., 1997). It is possible that any or all of these different domains (SRCR, CUB, and ZP) are sites by which the glycoprotein may interact with itself or other molecules and may provide the basis for the complexes identified by gel chromatography and rate-zonal centrifugation. By the nature of its known and potential binding abilities this glycoprotein may have numerous protective functions in the mucus. For example, the repeated SRCR motifs may provide multiple sites for interaction with toxins or pathogens. Therefore it will be important in the future to specify its functions and assay its relative level in health and disease.

Materials and methods

Agarose UltraPURE (electrophoresis grade) was from BRL (Paisley, UK). The ECL Western detection kit was from Amersham (Amersham, UK). Tween 20, CHAPS, Schiff’s reagent, α-cyano-4-hydroxy cinnamic acid, Substance P, insulin (bovine pancreas), and guanidinium chloride (practical grade) were purchased from Sigma (Poole, UK). Stock solutions of guanidinium chloride (approximately 8 M) were treated with charcoal before use. Trypsin, modified by reductive alkylation to reduce autolysis, was purchased from Promega (Southampton, UK). Strept ABCComplex/HRP kit and the biotin blocking system were from Dakopatts (Ålsjö, Sweden).

Preparation of 14C-radiolabeled reduced mucin preparation

Mucins were extracted with 4 M guanidinium chloride containing proteinase inhibitors from the mucus gel plug obtained post mortem from the lungs of an individual who died in status asthmaticus and purified by CsCl density-gradient centrifugation as described previously (Thornton et al., 1994). The mucin preparation was treated with 10 mM dithiothreitol to reduce disulfide bonds, and these were subsequently blocked with 14C-radiolabeled iodoacetamide as described previously (Thornton et al., 1994).

Rate-zonal centrifugation

The 14C-radiolabeled reduced mucin preparation was fractionated by centrifugation on preformed 6–8 M guanidinium chloride gradients using a SW40 Ti swing bucket rotor for 7.5 h at 40,000 r.p.m. as described previously (Thornton et al., 1994). Samples from gel chromatography of associative extracts of mucus were analyzed by centrifugation on preformed 10–30% (w/v) sucrose gradients using a SW40 Ti swing bucket rotor for 1.5 or 2.5 h at 40,000 r.p.m., respectively.

Isolation of tryptic peptides

The protein-rich fraction from the rate-zonal gradient was dialyzed against water and lyophilized. The material was dissolved in 0.1 M ammonium hydrogen carbonate and treated with modified trypsin for 16 h at 37°C as described previously (Thornton et al., 1997b). Tryptic peptides were chromatographed at a flow rate of 240 µl/min on a µRPC C2/C18 PC 3.2/3 column using the Pharmacia SMART system. The column elution program was 0.1% (v/v) trifluoroacetic acid (TFA) (5 min) followed by a linear gradient of 0–40% (v/v) acetonitrile in 0.1% (v/v) TFA (30 min). The column eluent was monitored for absorbance at 215 nm and fractions were analyzed by MALDI-TOF MS. Peptides were purified to homogeneity by rechromatography on the column employing shallower gradients centered on their elution point.

MALDI-TOF MS

Samples (1 µl) in 0.1% TFA containing various proportions of acetonitrile were mixed with an equal volume of 50 mM α-cyano-4-hydroxycinnamic acid and applied to a TOFspec target. Samples were analyzed by MALDI-TOF MS in positive ion mode using a VG TOFSpec-E with Substance-P (M+ 1348.7) and bovine insulin (M+ 5734.5) as internal standards. The data generated were processed using the OPUS™ peak detection program.

Gel electrophoresis

SDS–PAGE was performed with a discontinuous gel system in a 5% (w/v) resolving gel with a 3% (w/v) stacking gel. Alternatively samples were electrophoresed in 1.0% (w/v) agarose gels in 40 mM Tris-acetate/1mM EDTA, pH 8.0, containing 0.1% (w/v) SDS. Gels were analyzed by transfer of molecules to nitrocellulose by vacuum blotting prior to detection using antibodies (Thornton et al., 1995, 1996, 1997b).

Isolation of the gp-340

Respiratory secretions solubilized in 6 M guanidinium chloride were brought to 4 M guanidinium chloride and solid CsCl was added to a density of 1.4 g/ml. The sample was subjected to centrifugation in a Beckman Ti 70 rotor at 40,000 r.p.m. for 64 h at 15°C. The gp-340-containing fractions were pooled (see Figure 5a), dialyzed into 4 M guanidinium chloride, and chromatographed on Sepharose CL-2B. The column (88 cm x 2.6 cm) was eluted with 4 M guanidinium chloride at a flow rate of 22 ml/h. The gp-340-containing fractions were pooled (see Figure 5b), dialyzed into 6 M urea containing 0.02% CHAPS and subjected to anion exchange chromatography on a Mono Q HR 5/5 column. The column was eluted at a flow rate of 0.5 ml/min with a linear gradient of 0–0.4 M lithium perchlorate/10 mM piperase, pH 5.0, in 6 M urea containing 0.02% CHAPS (Thornton et al., 1996). The gp-340-containing fractions were pooled (see Figure 5c), dialyzed into 6 M urea containing 0.02% CHAPS, and rechromatographed on the Mono Q column under identical conditions. The gp-340-containing fractions were pooled, dialyzed against 6 M urea, and stored at 4°C.

Monosaccharide analysis

Samples were hydrolyzed in 2 M TFA at 100°C for 5 h, then rotary evaporated to dryness, redissolved in water, and the monosaccharides separated on a Dionex CarboPac PA1 column eluted at 1 ml/min with 21 mM sodium hydroxide. Monosaccharides were detected using a Dionex PAD II detector.

Molecular weight determination by MALLS

The gp-340 preparation was chromatographed on a Pharmacia Superose 6 HR 10/30 column eluted with 0.2 M NaCl at a flow rate of 200 µl/min. The column effluent was passed through an in-line Dawn DSP laser photometer coupled to a Wyatt/Optilab 903 interferometric refractometer to measure light scattering and sample concentration, respectively. Light
scattering measurements were taken continuously at 18 angles between 15° and 151°; the data were analyzed according to Zimm (1948).

**Analytical gel chromatography**

Respiratory secretions were chromatographed on a Sepharose CL-2B (29 cm × 1.6 cm) column eluted with either 4 M guanidinium chloride or PBS at a flow rate of 3 ml/h.

**Polyclonal antisera**

The polyclonal antisera MAN-5AC and MAN-5BI, raised against sequences within the MUC5AC and MUC5B mucins, respectively, were employed and these have been described previously (Thornton et al., 1997b; Sheehan et al., 1999). A polyclonal antiserum (MAN-gp-340) was raised to the following peptide FGQGSGP1VLDDVR conjugated to keyhole limpet hemocyanin. The antiserum was used at a dilution of 1:2000 for immunoblots and western blots and was more effective against reduced samples. Inhibition of antisemr reactivity in both immunoblots and western blots could be achieved by preincubation with the peptide antigen (2 µg/ml for 1 h at room temperature).

**Immunolocalization**

Human trachea was fixed in 10% neutral buffered formal saline and embedded in paraffin prior to the preparation of 4-µm sections. The slides were dewaxed, rehydrated, and treated for 10 min with 10 mM sodium citrate buffer, pH 6, at 100°C in a microwave oven. Endogenous peroxidase activity was quenched with 3% H2O2 for 30 min, and the sections were then blocked with normal goat serum (diluted in 0.15 M NaCl/0.05 M Tris–HCl buffer, pH 7.4) for 1 h. Endogenous biotin was blocked by treatment with the Dako biotin blocking kit used according to the manufacturer’s instructions. Sections were then incubated with the MAN-gp-340 antiserum (1:2000 in 0.15 M NaCl/0.05 M Tris–HCl buffer, pH 7.4 containing 0.05% bovine serum albumin) for 1 h in Coverplate immunostaining chambers (Shandon, Pittsburgh, PA). After the detection of antibody binding using the Dako Strept ABCComplex/HRP kit with diaminobenzidine as the substrate, sections were counterstained with Mayers hematoxylin.

**Acknowledgments**

The authors wish to thank Prof. Tim Hardingham for reading the manuscript and the Wellcome Trust and the National Asthma Campaign for financial support. J.R.D. wishes to thank the Swedish Medical Research Council (grant nos. 7902, 9711), Riksförbundet Cystisk Fibros, the Swedish Fund for Research without Animal Experiments, Greta and Johan Kocks Stiftelse, and the Medical Faculty of Lund. N.K. has a BBSC/CASE in collaboration with Beckman Instruments Ltd.

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**gp-340 in respiratory mucus**


