Mucus glycoproteins in bovine trachea: identification of the major mucin populations in respiratory secretions and investigation of their tissue origins

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Bovine respiratory secretions were separated into gel and sol phases to allow the identification of the gel-forming mucins. Mucins were subsequently isolated from the surface epithelium and submucosal tissue to investigate the tissue origins of the species in the secretions. Density-gradient centrifugation revealed 'high-density' and 'low-density' mucins in the gel phase of the secretions. The 'high-density' mucins were large, composed of subunits joined by disulphide bonds and contained two highly glycosylated domains of apparently different lengths, whereas the 'low-density' mucins were smaller and monomeric. The sol also contained both 'high-density' and 'low-density' species. A 'high-density' mucin similar to that in the gel was isolated from

the surface epithelium, suggesting that the goblet cells produce large, gel-forming mucins. A second 'high-density' species was released from the submucosal tissue after reduction/alkylation, indicating that large mucins from the submucosal glands may also be a component of the mucus gel. In addition, two small, 'low-density' mucins were obtained from the submucosal tissue. One species was associated with the gel phase but was also present in the sol, whereas the other was present only in the sol. Bovine respiratory-tract secretions thus comprise a complex mixture of large gel-forming mucins originating from the goblet cells and submucosal glands, and smaller 'soluble' species from the submucosal glands which may interact with the gel.

INTRODUCTION

The epithelial surface of the respiratory tract is protected by a layer of mucus, the major macromolecular components of which are mucus glycoproteins or mucins. The mucin superfamily is considered to comprise two groups of glycoproteins, the very large, oligomeric secreted species, which endow the mucus gel with the mechanical properties essential for, for example, mucociliary transport, and monomeric, cell surface-associated mucins. The gel-forming mucins have M_r values of between 10×10^6 and 25×10^6 Da, and electron microscopy, as well as biochemical investigations, have shown them to be linear thread-like structures composed of subunits linked end-to-end by disulphide bonds [1–4]. Mucin apoproteins contain regions which are rich in serine, threonine and proline residues (STP-rich domains) and are separated by domains with a more conventional amino acid composition. The STP-rich domains are enriched in potential O-glycosylation sites and correspond to the highly glycosylated regions within the mature mucin molecule. This basic structure has been shown for mucins from a wide range of sources, including the respiratory tract, stomach and cervix (for reviews see [5–7]).

Recently, cDNA cloning studies have confirmed and extended our understanding of mucin structure. Eight human mucin genes (MUC1–MUC7) have so far been identified (for a review see [8]). Most mucin genes contain tandemly repeated sequences giving rise to repetitive peptide sequences in the STP-rich domains of the apoprotein; however, at least one gene containing degenerate tandem repeats has been identified [9]. Several mucin genes are expressed in the airways [10,11]: MUC2 and MUC5AC in goblet cells, MUC3 and MUC4 over the surface epithelium and MUC5B in the mucous gland acini. In addition, MUC4, MUC5B and MUC5AC are expressed along the ducts of the submucosal glands. These data suggest that airway secretions could contain mucins with several different apoproteins.

Organ cultures of both human [12–14] and animal tissue [15–17] have been used as models to investigate the mechanisms controlling airway mucin synthesis and secretion. In many studies, radiolabelled mucin precursors have been introduced for relatively short periods, and mucin secretion has been expressed in terms of released radiolabelled macromolecules. Since several different mucins are expressed in airway tissue, radiolabels will be selectively incorporated into those with a high turnover rate, and measurements of the amount of radiolabel released are likely to reflect this bias. Furthermore, it has been shown that large oligomeric mucins may be stored within secretory granules for days, suggesting that they would not become radiolabelled during the short labelling periods used in such experiments [18]. Thus, if metabolic studies on the mucus-forming mucins are envisaged, it is important to ascertain that these macromolecules are actually radiolabelled. Here we have characterized the major mucin populations present in bovine tracheal secretions and investigated their tissue origins. This study will provide a reference point for studies on the biosynthesis and secretion of large oligomeric mucins from the same tissue in organ and cell culture.

MATERIALS AND METHODS

Materials

Di-isopropyl phosphorofluoridate (DFP), CHAPS and guanidinium chloride (practical grade) were purchased from Fluka. Stock solutions (approx. 8 M) of guanidinium chloride were treated with charcoal and filtered through a PM 10 filter (Amicon) before use. Urea was bought from Acros Organics, and guanidinium chloride (ultrapure) and *N*-ethylmaleimide (NEM) were from BDH Chemicals. Dithiothreitol (DTT) was obtained from Merck, and trypsin (EC 3.4.21.4, type XIII, tosylphenylalanyl chloromethyl ketone-treated), deoxyribonuclease I (EC 3.1.21.1, type IV) and iodoacetamide (IAA) were from Sigma Chemical Company. Sepharose CL-2B and Sephacryl S-500 HR, as well as

Abbreviations used: DFP, di-isopropyl phosphorofluoridate; DTT, dithiothreitol; IAA, iodoacetamide; NEM, *N*-ethylmaleimide; PAS, periodic acid/Schiff; PBS, 0.15 M sodium chloride/10 mM sodium phosphate buffer, pH 7.4; STP-rich domains, domains rich in serine, threonine and proline residues.

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a Mono Q HR 5/5 column, were obtained from Pharmacia. All other reagents were of analytical reagent or equivalent quality.

Collection of mucus by lavage

Bovine tracheas were obtained from the local abattoir within 1 h post mortem and rinsed with 0.15 M sodium chloride/10 mM sodium phosphate buffer, pH 7.4 (PBS). The lumen was filled with 20 ml of PBS containing 1 mg/ml glucose and both ends of the trachea were sealed. The trachea was then laid in a moist chamber on an orbital shaker at room temperature for 1 h and rotated 90° four times during the hour so that all surfaces were bathed in the PBS for at least 15 min. After this time, the PBS was collected and proteinase inhibitors DFP (0.1 mM), NEM (5 mM) and Na₂EDTA (5 mM) were added before centrifugation in a Beckman J2-MC 20 centrifuge (JA 20 rotor, 18000 rev./min, 4 °C, 45 min). Four samples of pooled material (2–100 animals) were investigated and the data shown were obtained from a pool of 27 tracheas. After decantation, solid guanidinium chloride (ultrapure) was added to the sol phase to give a 6 M solution. This material was concentrated in an Amicon ultrafiltration cell using a PM 10 filter at 4 °C. The gel phase was stirred gently overnight in 6 M guanidinium chloride/5 mM EDTA/10 mM sodium phosphate buffer, pH 6.5 (extraction buffer), containing 5 mM NEM. After centrifugation in a Beckman J2-MC centrifuge (JA 20 rotor, 18000 rev./min, 4 °C, 45 min), the supernatant from the gel (S1) was poured off and new extraction buffer (5 ml) was added. The gel phase was then extracted as above to give a total of four supernatants (S1–S4). Finally, 6 M guanidinium chloride/5 mM Na₂EDTA/10 mM Tris/HCl buffer, pH 8.0, containing 10 mM DTT was added to the extraction residue for 5 h at 37 °C, followed by 25 mM IAA for 12 h in the dark at room temperature to solubilize the material. After centrifugation (under the conditions outlined above) the supernatant (P) was retained while the small residual pellet was discarded.

Extraction of mucins from the surface epithelium and submucosal tissue

Bovine tracheas obtained as above were rinsed with PBS, freed from surrounding connective tissue and opened along the dorsal aspect. The luminal surface was removed by scraping the mucosa with a microscope slide. The submucosal tissue was then dissected from the underlying cartilage. Samples, referred to below as 'surface epithelium' and 'submucosal tissue' respectively, were frozen immediately in liquid nitrogen and the submucosal tissue was pulverized in liquid nitrogen, using a tissue pulverizer (Retsch), before extraction. Both samples were thawed slowly in the presence of DFP to give a final concentration of 1 mM before the addition of extraction buffer containing protease inhibitors (see above). The surface epithelium was suspended with a Dounce homogenizer (loose pestle, three strokes), whereas the submucosal tissue was dispersed by passing the material through a syringe, and both were then stirred gently overnight at 4 °C. Samples were spun in a Beckman J2-MC 20 centrifuge (JA 20 rotor, 17000 rev./min, 4 °C, 45 min) and the resulting pellets re-extracted twice in extraction buffer. Supernatants from successive extractions were pooled. The residues remaining after the extractions were solubilized by reduction with 10 mM DTT in 6 M guanidinium chloride/5 mM Na₂EDTA/10 mM Tris/HCl buffer, pH 8.0, for 5 h at 37 °C, and alkylated with 25 mM IAA for 12 h in the dark at room temperature. Six samples of pooled material (1–15 animals) were investigated and data from a pool of three tracheas are shown.

Purification of mucins

Extracts from the secretions and tissue were subjected to isopycnic density-gradient centrifugation in a Beckman L8-60M centrifuge (50.2 Ti rotor, 36000 rev./min, 15 °C, 80 h) in CsCl/4 M guanidinium chloride (initial density 1.39 g/ml). Mucins were pooled as shown in Figures 1(a)–1(f). Pooled material from the CsCl/4 M guanidinium chloride gradients was subjected to a second isopycnic density-gradient step in CsCl/0.5 M guanidinium chloride containing 0.01 % (w/v) CHAPS (initial density 1.50 g/ml) in a Beckman L8-60M centrifuge (50.2 Ti rotor, 36000 rev./min, 15 °C, 80 h). After this second density-gradient step, fractions were pooled as shown in Figures 2(a)–2(h) and concentrated where necessary in an Amicon ultrafiltration cell using a PM 10 filter before dialysis against extraction buffer.

Preparation of reduced subunits and high-*M_r* mucin glycopeptides

Samples were fragmented to subunits by reduction of disulphide bonds in 6 M guanidinium chloride/5 mM Na₂EDTA/10 mM Tris/HCl buffer, pH 8.0, containing 10 mM DTT for 5 h at 37 °C, and subsequent alkylation was performed with IAA (2.5 molar excess over DTT) for 12 h at room temperature in the dark. For the preparation of high-*M_r* glycopeptides, samples were then dialysed against 0.1 M Tris/HCl buffer, pH 8.0, and digested with trypsin (approx. 50 µg of trypsin/mg of subunits) for 5 h at 37 °C before being dialysed exhaustively against water and lyophilized.

Gel chromatography and ion-exchange HPLC

Gel chromatography on Sepharose CL-2B was performed on a column (90 cm × 1.6 cm) eluted at a rate of 5 ml/h with 4 M guanidinium chloride, pH 7, and fractions (2 ml) were collected. Chromatography on the Sephacryl S-500 HR column was performed with a system consisting of an LKB model 2152 control unit, a Pharmacia V-7 injector and an LKB model 2150 titanium-head pump using Teflon tubing for the solvent stream. The column was eluted with 4 M guanidinium chloride, pH 7, at a rate of 0.15 ml/min, effluents were monitored with an LKB model 2151 variable-wavelength monitor at 280 nm and fractions (1.0 ml) were collected. Ion-exchange HPLC was performed on a Mono Q HR 5/5 column connected to the system mentioned above with a 2040-203 LKB mixing valve and gradients were formed on the low-pressure side. The column was eluted at a rate of 0.5 ml/min with 6 M urea/0.1 % (w/v) CHAPS/10 mM piperazine/perchlorate buffer, pH 5 (starting buffer), for 10 min followed by a linear gradient (60 min) to a final concentration of 0.4 M LiClO₄ in starting buffer, and fractions (0.5 ml) were collected.

Rate-zonal centrifugation

Centrifuge tubes (14 ml) were loaded from the bottom with linear gradients of 6–8 M guanidinium chloride, and mucin solutions (100–200 µl) were layered onto the top of the gradients [19]. Centrifugation was performed in a Beckman Optima L-70 ultracentrifuge (SW40.1 Ti rotor, 40000 rev./min, 2 h 45 min, 20 °C) and the tubes were emptied from the top into 500 µl fractions.

Analytical methods

Sialic acid in fractions from the density-gradients and gel-chromatography runs was measured with an automated version [20] of the method described by Jourdain et al. [21]. Aliquots from the density-gradient, gel chromatography and rate-zonal

gradient fractions were slot-blotted onto nitrocellulose membranes using a Schleicher & Schuell slot-blot apparatus and stained with periodic acid/Schiff (PAS) [22]. Membranes were scanned using a scanning densitometer (Hoefer) in the reflectance mode. All measurements were within the linear range as assessed by comparison with a respiratory mucin standard. The densities of fractions from the density gradients were measured using a Carlsberg pipette as a pycnometer.

RESULTS AND DISCUSSION

Isolation of mucins from tracheal secretions and tissue

Airway secretions were separated into the gel and sol phases using high-speed centrifugation, and mucins were isolated from them by repeated extraction in 6 M guanidinium chloride followed by density-gradient centrifugation in CsCl/4 M guanidinium chloride. In the first extract of the gel phase (S1), two major mucin populations were present (Figure 1a). The predominant sialic acid-containing, 'high-density' population (buoyant density 1.48 g/ml) coincided with DNA absorbing strongly at 280 nm. This material was resolved from a second, sialic acid-containing, 'low-density' mucin population at 1.38 g/ml. In addition, material reacting in the sialic acid assay was found at the top of the gradient at a density expected for proteins and glycolipids. PAS staining followed the pattern shown for sialic acid. In the subsequent three extractions (S2–S4) of the gel, the 'high-density' population was seen in decreasing amounts, while the 'low-density' mucins were absent (results not shown). Reduced mucins obtained by reduction/alkylation of the residue remaining after repeated extraction (P) appeared at a buoyant density of 1.48 g/ml, and PAS-reactivity again followed the assay for sialic acid (Figure 1b). Density-gradient centrifugation of the sol phase from the secretions contained two populations of mucins with buoyant densities similar to those of the 'high-density' and 'low-density' mucins from the gel phase (Figure 1c). These results are representative of those obtained for the three other samples studied (results not shown).

To investigate the tissue origins of the mucin populations in the secretions, mucins were isolated from the surface epithelium and submucosal tissue. In the extract from the surface epithelium, the major sialic acid- and PAS-reactive material coincided with DNA at a density of 1.48 g/ml (Figure 1d). A second sialic acid- and PAS-reactive population at a density of 1.46 g/ml was partially resolved from that at 1.48 g/ml. In addition, some PAS-reactive material was found at a density greater than 1.60 g/ml, similar to that expected for glycogen. After repeated extractions of the surface epithelial tissue, the residue contained only small amounts of material, and this was not studied further. The extract from the submucosal tissue contained mucins with a broad (1.34–1.47 g/ml) distribution of buoyant densities, which were almost completely resolved from DNA (Figure 1e). Again, some PAS-reactive material was found at a density greater than 1.60 g/ml. The major part of the material solubilized by reduction/alkylation from the submucosal tissue residue occurred together with DNA at a density of 1.47 g/ml (Figure 1f). Similar results to those presented above were obtained for all of the pooled samples studied (results not shown).

The 'high-density' mucins from the first three extractions of the gel phase were pooled and subjected to a second density-gradient centrifugation step in 0.5 M guanidinium chloride/CsCl. As expected, the mucins had a higher buoyant density in this solvent than in 4 M guanidinium chloride/CsCl [23], and appeared as a broad distribution between 1.48 and 1.60 g/ml, well separated from DNA (Figure 2a). The 'low-density' population from the gel appeared as a narrower band in 0.5 M guanidinium

Figure 1 Isopycnic density-gradient centrifugation in CsCl/4 M guanidinium chloride of lavage samples (a–c) and tissue extracts (d–f)

Secretions harvested by tracheal lavage were separated into gel and sol phase. (a) The first extract (S1) of the gel phase; (b) the extraction residue from the gel phase (P); (c) the sol phase; (d) extract of the surface epithelium; (e) submucosal tissue extract; and (f) residue from the submucosal extract. Following extraction with 6 M guanidinium chloride/10 mM phosphate buffer, pH 6.5, the pellets remaining from the gel phase, and the submucosal tissue were solubilized by reduction/alkylation. After centrifugation in 4 M guanidinium chloride/CsCl in a Beckman L8-60M centrifuge (50.2 Ti rotor, 36 000 rev./min, approx. 80 h, 15 °C; starting density 1.39 g/ml), fractions were collected from the bottom of the tubes and analysed for sialic acid (●), PAS reactivity (Δ) and absorbance at 280 nm (—). Fractions were plotted against density and pooled as indicated by the horizontal bars.

chloride/CsCl at a lower buoyant density (1.39–1.48 g/ml) than the 'high-density' population (Figure 2b). Reduced mucins, isolated by reduction/alkylation from the extraction residue, also appeared as a unimodal peak with a buoyant density of 1.55 g/ml (Figure 2c).

The mucins from the sol phase were pooled as separate 'high-' and 'low-density' populations after centrifugation in 4 M guanidinium chloride/CsCl. Centrifugation in 0.5 M guanidinium chloride/CsCl revealed the presence of mucins with a broad range of buoyant densities between 1.46 and 1.56 g/ml in the 'high-density' pool (Figure 2d). The distribution thus had a similar appearance to that of 'high-density' mucins from the gel phase, although the molecules occurred at a slightly lower density. The 'low-density' mucins from the sol (Figure 2e) banded at similar density (1.39–1.48 g/ml) to the 'low-density' population from the gel phase. The sol phase thus contained at least two different mucin populations as shown by their differing buoyant densities, although density-gradient centrifugation did not allow us to determine whether these were the same populations as those in the gel.

Figure 2 Isopycnic density-gradient centrifugation in CsCl/0.5 M guanidinium chloride

Fractions from the 4 M guanidinium chloride/CsCl gradients were pooled as shown in Figure 1 and dialysed against 0.5 M guanidinium chloride. Centrifugation was carried out after the addition of CsCl and 0.01% (w/v) CHAPS in a Beckman L8-60M centrifuge (50.2 Ti rotor, 36 000 rev./min, approx. 80 h, 15 °C; starting density 1.50 g/ml) for: (a) the 'high-density' mucins from the gel phase; (b) the 'low-density' mucins from the gel phase; (c) reduced mucins from the gel phase extraction residue; (d) the 'high-density' mucin from the sol phase; (e) the 'low-density' mucin from the sol phase; (f) mucins extracted from the surface epithelium; (g) mucins extracted from the submucosal tissue; and (h) reduced mucins from the submucosal tissue extraction residue. Fractions were collected from the bottom of the tubes and analysed for sialic acid (●), PAS reactivity (Δ) and absorbance at 280 nm (—). Fractions were plotted against density and pooled as indicated by the horizontal bars.

Mucins from the surface epithelium had a range of densities between 1.48 and 1.56 g/ml and were completely resolved from DNA (Figure 2f). To ensure that the peak with a high absorbance at 280 nm contained only DNA, fractions between 1.58 and 1.68 g/ml were pooled as shown in Figure 2(f) and treated with deoxyribonuclease. Rate-zonal centrifugation showed that this treatment caused complete fragmentation, whereas reduction did not affect the size, suggesting that the material is DNA and does not contain mucins (results not shown). Thus the surface epithelial extract appears to contain one predominant mucin population with a buoyant density similar to that of the 'high-density' mucin from the gel phase of the secretions.

In the guanidinium chloride extract from the submucosal tissue, the material showed a tendency to separate into two peaks (Figure 2g) which occurred in positions similar to the 'low-density' mucins present in the gel and sol phases of the secretions.

Figure 3 Rate-zonal centrifugation

'High-density' mucins from the gel phase (a) and the surface epithelial extract (b) were subjected to rate-zonal centrifugation as described in the text, before (●) and after (○) reduction/alkylation. Fractions were analysed for PAS reactivity.

To ensure that the material absorbing strongly at 280 nm in the CsCl/4 M guanidinium chloride gradient was only DNA, fractions between 1.47 and 1.52 g/ml were pooled, as shown in Figure 1(e). After a second density-gradient centrifugation step in 0.5 M guanidinium chloride/CsCl, treatment with deoxyribonuclease caused complete fragmentation, as shown by rate-zonal centrifugation, whereas reduction with DTT did not affect the size, suggesting that the material is DNA and does not contain mucins (results not shown). Thus two populations of 'low-density' mucins appear to originate from the submucosal tissue.

Reduced mucins obtained by reduction/alkylation of the extraction residue from the submucosal tissue occurred between 1.50 and 1.60 g/ml and were separated from a peak with high absorbance at 280 nm and a density expected for DNA (Figure 2h). These macromolecules thus had a higher buoyant density than the 'soluble' mucins from both the surface epithelium (Figure 2f) and the submucosal tissue (Figure 2g), although they were similar to those from the extraction residue from the secreted gel (Figure 2c). The difference in buoyant density between mucins from the submucosal tissue extraction residue and the soluble species from the surface epithelium did not appear to be due to reduction/alkylation, since reduced subunits from soluble mucins in the surface epithelium appeared at the same buoyant density (1.50 g/ml) as the cognate whole mucins (results not shown). The extraction residue from the submucosal tissue thus contains a population of mucins which differ from those which are soluble, and it appears likely that the submucosal tissue contains at least three mucin populations, two of which have relatively low buoyant densities and can be extracted with 6 M guanidinium chloride, while the third has a higher buoyant density and is only released from the tissue by reduction.

Figure 4 Gel chromatography on Sepharose CL-2B

Mucins pooled from the density-gradients were subjected to gel chromatography on a Sepharose CL-2B column (90 × 1.6 cm) eluted with 4 M guanidinium chloride, pH 7, at a flow rate of 5 ml/h: (a) 'high-density' mucins and (b) 'low-density' mucins from the gel phase; (c) 'high-density' mucins and (d) 'low-density' mucins from the sol phase; (e) 'high-density' mucins and (f) 'low-density' mucins from the submucosal tissue. Fractions (2 ml) were analysed for sialic acid (●). V_0 and V_t are the void volume and the total volume respectively.

Mucin extractability

The amount of 'high-density' (1.54–1.43 g/ml) and 'low-density' (1.42–1.35 g/ml) mucin in each of the four 6 M guanidinium chloride extractions (S1–S4) of the gel phase and the extraction residue (P) was estimated as the amount of sialic acid-containing material within the appropriate density band after density-gradient centrifugation in 4 M guanidinium chloride/CsCl. This showed that most (66%) of the 'high-density' mucin from the gel phase appeared in the first extract with smaller amounts being solubilized by subsequent steps. A significant amount (approx. 20%) of the 'high-density' mucins remained in the 'insoluble' residue. In contrast, all the 'low-density' mucin was solubilized in the first extract. The relative amounts of mucins in each of the four extracts of the surface epithelium and submucosal tissue, as well as the respective extraction residues, were also investigated. In the surface epithelium, approx. 80% of the mucins were solubilized in the first two extractions, with decreasing amounts solubilized by further extractions. Less than 5% remained in the extraction residue (results not shown). This suggests that the 'high-density' mucin from the surface epithelium is soluble in 6 M guanidinium chloride. In contrast, in the submucosal tissue most of the 'low-density' monomeric species (approx. 80%) were obtained in the first two extracts, with smaller amounts

Figure 5 Ion-exchange HPLC on a Mono Q column

Reduced mucins prepared by reduction/alkylation were subjected to ion-exchange HPLC on a Mono Q 5/5 HR column as described in the text: (a) subunits from the 'high-density' mucin from the gel phase; (b) subunits from the 'high-density' mucin from the surface epithelium; and (c) reduced mucins from the extraction residue from the submucosal tissue. Fractions (0.5 ml) were analysed for PAS reactivity (Δ). The broken line shows the nominal LiClO_4 concentration gradient.

being solubilized by subsequent steps, whereas virtually all (80–100%) of the 'high-density' mucins were present in the extraction residue and could only be released by reduction (results not shown). Similar results were obtained when PAS reactivity was used as a marker for mucins.

Size and oligomeric structure of whole mucins

The size and oligomeric nature of the 'high-density' mucins from the gel phase, as well as those isolated from the surface epithelium, were investigated using rate-zonal centrifugation. The 'high-density' mucins from the gel had a range of molecular sizes as shown by their broad distribution over the gradient (Figure 3a). Reduction gave rise to a much narrower distribution of smaller fragments, indicating that the mucins are composed of subunits linked by disulphide bonds. A similar pattern was seen for the mucins extracted from the surface epithelium (Figure 3b).

'High-density' mucins from the gel phase eluted, as expected, in the void volume of a Sepharose CL-2B column (Figure 4a). The cognate 'low-density' mucins were much smaller (Figure 4b) and the elution position did not change after reduction and alkylation (results not shown), suggesting that they are not composed of subunits. The 'high-density' material from the sol phase contained mucins which eluted in the void volume, as well

Figure 6 Gel chromatography on Sephacryl S-500 HR of high- M_r glycopeptides

High- M_r glycopeptides, prepared after reduction/alkylation followed by trypsin digestion, as described in the text, from: (a) the 'high-density' mucin from the gel phase, (b) 'high-density' mucins from the surface epithelium, and (c) reduced mucins from the extraction residue from the submucosal tissue, were chromatographed on a Sephacryl S-500 HR column eluted with 4 M guanidinium chloride, pH 7.0, at a rate of 0.15 ml/min. Fractions (1.0 ml) were analysed for sialic acid (●). V_0 , void volume.

as much smaller glycoproteins (Figure 4c). The mucins eluting with the void volume may thus be similar to those found in the gel phase of the secretions. The 'low-density' material from the sol was partially separated into two populations which were both fully included on the column (Figure 4d). The predominant population eluted at a position similar to that of the 'low-density' mucin from the gel phase (Figure 4b). A lesser amount of a smaller mucin, which was not present in the gel phase, was also found. Thus this population of small glycoproteins appeared to be present only in the sol. The two soluble mucins from the submucosal tissue, which were partially separated in the 0.5 M guanidinium chloride/CsCl density gradient, could be further separated by gel chromatography (Figures 4e and 4f). The population with a higher buoyant density eluted in a position similar to the mucins found only in the sol phase of the secretions, whereas the species with lower buoyant density was similar in size to the 'low-density' population found in both the gel and sol phases. The elution positions of the 'low-density' mucin populations from the submucosal tissue showed no change after reduction, suggesting that they were not composed of subunits (results not shown). Thus, in addition to a mucin which can only be extracted by reduction/alkylation, the submucosal tissue gives rise to at least two populations of lower M_r , monomeric glycoproteins with different buoyant densities. The denser population is smaller and is found in the sol phase of the secretions,

while the lower-density population is larger and occurs in both the gel and sol phases.

Charge density of reduced mucins

In an attempt to determine whether the 'high-density' population in the gel was composed of one or more populations, the charge density of the cognate subunits was compared with that of subunits from the 'high-density' mucins from the surface epithelium and reduced mucins from the submucosal tissue residue. After ion-exchange HPLC, reduced/alkylated material from each source eluted in a similar position at approx. 0.2 M LiClO₄ (Figures 5a–5c), suggesting that mucins from the surface epithelium and submucosa do not differ significantly in their charge density.

Size of the highly glycosylated regions

To investigate the size of the highly glycosylated regions of the 'high-density' mucins from the secretions, the surface epithelium and the submucosal tissue, high- M_r glycopeptides were subjected to gel filtration on a Sephacryl S-500 HR column. Each mucin gave rise to two populations of high- M_r glycopeptides which were both included on the gel (Figures 6a–6c). The relative proportions of the two populations were similar in all cases. 'High-density' mucins from the secretions and those from both the surface epithelium and submucosal tissue residue thus contain highly glycosylated regions of two different lengths, suggesting the presence of two different STP-rich domains within them. Thus the large 'high-density' mucins from the surface epithelium and submucosal glands appear to have a similar macromolecular architecture.

CONCLUSION

Tracheal lavage was used to sample the mucins present in airway secretions. Separation into a gel and a sol phase allowed the identification of the mucin species which were gel-forming, as distinct from the 'sol' phase which probably contained a mixture of non-gel-forming mucins, gel-forming mucins in equilibrium with the gel phase, and degraded species. Isolation of mucins from the surface epithelium and submucosal tissue was used to enrich the samples in the products of the goblet cells and submucosal glands respectively, and thereby to investigate the tissue origins of the species present in the secretions. These studies revealed the presence of several mucin populations, differing in buoyant density, size, molecular architecture and tissue origin.

The major population in the gel phase of the secretions comprised a large polydisperse mucin composed of subunits linked by disulphide bonds. It is thus like mucins from other sources including human respiratory tract [24,25], human cervix [1] and pig stomach [26], which have similar molecular organization although are likely to have different apoproteins. The apoproteins of respiratory mucus glycoproteins typically contain alternating highly and sparsely glycosylated regions, which after proteolysis give rise to high- M_r glycopeptides [24,25]. Proteolytic fragmentation of the mucins studied here gave rise to two high- M_r glycopeptide populations. Separation by gel chromatography is dependent upon the hydrodynamic volume of the glycopeptides, which is most affected by substitution with the initial *N*-acetylgalactosamine residue [27]. Extension of the oligosaccharide side chains does not have a significant effect upon the apparent size of the fragments, and thus differences in elution position should reflect differences in the length of the glycopeptides. The data therefore point to the presence of two STP-rich domains of different lengths within these mucins.

A population with a similar structure and buoyant density to the 'high-density' mucin from the secretions was isolated from the surface epithelial tissue. Since the major secretory cells in the surface epithelium are goblet cells, it is highly likely that this mucin originates from them. However, the range of buoyant densities and the molecular size distribution of the 'high-density' mucins from the gel phase were greater than those for the mucins from the surface epithelium, and it is therefore likely that the gel contains more than one 'high-density' mucin species. A possible candidate for the other component is the 'high-density' mucin from the submucosal tissue. This mucin was not extractable by treatment of the tissue with 6 M guanidinium chloride but was isolated after reduction/alkylation of the residue. It is currently not known whether the 'insolubility' in 6 M guanidinium chloride reflects an intrinsic property of the submucosal 'high-density' mucins or whether the intact molecules are simply too large to escape from the connective-tissue network during the isolation procedure. However, the presence of mucins with a similar buoyant density to that of the 'high-density' mucins from the submucosa in the extraction residue from the gel phase, which could not be solubilized even after four extractions, suggests that at least one mucin population is, to some degree, 'insoluble' in 6 M guanidinium chloride. MUC2, the major mucin from human intestine has also been shown to form a complex which cannot be solubilized in 6 M guanidinium chloride [28]. Since MUC2 has been shown to be expressed in the airways, it is possible that this material may be a bovine equivalent of the MUC2 gene product, although this glycoprotein was not found to be a major component of human respiratory secretions [29]. Since it was isolated as monomers, it was not possible to determine whether the mucin is present as an oligomeric structure within the tissue, however, the charge density and length of the glycosylated regions are similar to those of the surface epithelial mucin. In respiratory secretions from chronic bronchitic and normal individuals, the mucins give rise to two populations of high- M_r glycopeptides [25]. Furthermore, the two different populations of mucins with different apoproteins that have been identified in both chronic bronchitic and normal secretions [29,30] have both been shown to give rise to two populations of differently sized high- M_r glycopeptides (J. R. Davies, unpublished work). The human respiratory tract thus secretes two mucins whose apoproteins share common structural features although they are the products of different genes.

In addition to the large mucins, the gel phase contained a population of 'low-density', low- M_r mucins which were not composed of subunits and which originated from the submucosal tissue. These mucins were also present in the sol phase of the secretions, so the degree to which they are truly 'associated' with the gel phase remains unclear. The fact that all of this mucin was released in the first extraction suggests that it is not an integral component of the gel, but rather may interact with the network formed by the large 'high-density' mucin.

The sol phase contained a mixture of macromolecules. In addition to mucins similar in size and buoyant densities to the 'high-' and 'low-density' mucins from the gel phase, one low buoyant density, low- M_r species was enriched in the sol phase. This mucin was shown to originate from the submucosal tissue; however, since there are at least two populations of secretory cells in the submucosa, the mucous cell and the serous cells, the cellular origin of the submucosal mucins could not be identified.

This study highlights the complexity of airway secretions. It was conducted to identify the major mucin species present in bovine airways to provide a reference point for secretion studies

of the same tissue in cell and organ culture. We have shown that several different mucins are present in the secretions. The gel phase is composed of large subunit-based mucins which probably originate from both the goblet cells and the submucosal glands. In addition, two populations of smaller mucins originating from the submucosal tissue were present. One appeared mainly in the sol phase whilst the other was distributed between the gel and sol phases. Respiratory tract secretions are thus a mixture of different components produced by different cells whose secretion is under differential physiological regulation. The mechanisms controlling the release of these components under different conditions remain the focus for future investigations.

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