Mucin biosynthesis and secretion in tracheal epithelial cells in primary culture

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INTRODUCTION

Mucous secretion on the surface of the respiratory tract interacts with cilia to promote the removal of inhaled irritants, particles and pathogens. In addition to the degree of hydration, the physical properties of the secretion are determined largely by the content of gel-forming mucus glycoproteins (mucins). Mucins belong to a family currently encompassing 13 known gene products (MUC1–MUC12), which include both large, secreted macromolecules (encoded by the MUC2, MUC5AC and MUC5B genes and probably also by MUC6 [1–3]) and those with transmembrane domains (encoded by the MUC1, MUC3, MUC4 and MUC12 genes [4–7]). All mucins contain one or more regions within the apoprotein with sequences rich in serine or threonine residues that are heavily substituted with O-glycans (reviewed in [8]). In the gel-forming, secreted species both the N- and C-terminal regions of the apoprotein contain cysteine-rich domains that facilitate disulphide-bond-mediated oligomerization of mucin ‘monomers’ into large oligomeric molecules [9–12].

In the airway mucosa, histochemical staining shows mucins to be produced by both goblet cells in the surface epithelium and mucous cells in the submucosal glands. Immunohistochemical staining and hybridization in situ have both indicated that the major mucin from the goblet cells is MUC5AC, whereas MUC5B is produced by the submucosal glands [3,13–15]. However, at least four additional mucin genes are expressed in airway tissue [16]: MUC1 and MUC4 in surface epithelial cells, MUC2 in the goblet cells [13,17] and MUC7 in the serous cells of the submucosal glands [15]. The MUC4 mucin, like MUC1, contains a transmembrane domain [6] and is likely to be mainly cell-associated. However, sequences predicting a range of splice variants of MUC4, including those with the potential to be shed from the epithelium by proteolysis and those lacking the transmembrane sequences, have been identified, suggesting that the mucin might be present in several forms in the airways [18]. The role of such molecules in mucosal defence is not currently well understood.

To develop a model for studies of the synthesis and secretion of mucins, we have previously used bovine tracheal tissue in organ culture, in which the large oligomeric mucins were shown to be poorly radiolabelled but high-molecular-mass ‘monomeric’ mucin-like molecules became heavily labelled with [35S]sulphate [19]. Mucin-like components have also been identified from cat, canine and hamster trachea [20–22]. Because these molecules are highly labelled but seem to be present in small amounts, they are likely to be over-represented when radiolabelling is used to study the release of mucins from airway tissues. Many studies of mucin secretion have been undertaken with cells in culture (see, for instance, [23,24]); to investigate whether mucin-like components might be present in such models, we have studied the release of both radiolabelled and non-radiolabelled mucins from primary cultures of bovine tracheal epithelial cells. A sialic-acid-rich oligomeric mucin and a lower-buoyant-density periodate–Schiff (PAS)-reactive component were detected with chemical analyses but neither became radiolabelled with [35S]sulphate. However, as in bovine tracheal tissue in culture, mucin-like molecules that were highly labelled with [35S]sulphate were identified. Neither the secreted form nor the intracellular form of the radiolabelled component seemed to contain hydrophobic domains, suggesting that the mucin might not be membrane-associated. Thus, whereas bovine cells in primary culture produce...
large oligomeric mucins, the major component to become radiolabelled with [35S]sulphate is a ‘monomeric’ mucin-like molecule.

**EXPERIMENTAL**

**Materials**

F-12 medium (Ham) with l-glutamine, minimal essential medium (MEM) (Joklik’s modification), amphotericin B (Fungizone), gentamicin sulphate, streptomycin, penicillin and foetal calf serum (FCS) were bought from Life Technologies. Transferrin, retinoic acid, cortisol (hydrocortisone; water-soluble), sodium selenite, epidermal growth factor, insulin (bovine), trypsin [EC 3.4.21.4, type XIII, toslyphenylalanlyl-chloromethane (‘TPCK’)-treated], protease (type XIV), deoxyribonuclease I (type IV), collagen (type 1) and Triton X-114 were from Sigma Chemical Co. Vitrogen 100 was a product of Collagen Corp. (Pato Alto, CA, U.S.A.) and Matrigel EHS were from Amersham Pharmacia Biotech. All l-methyl sodium metaperiodate and the oxidation reaction was described for the ECL glycoprotein detection system. The antibodies were used to ensure that readings from the PAS staining were specific for mucin. PBS [0.15 M NaCl, 25 mM sodium metaperiodate in 0.1 M sodium acetate buffer, pH 5.5 (100 ml), 5 mM sodium phosphate buffer (pH 7.4)] were purchased from Fluka, and stock solutions of GdmCl were treated with charcoal and filtered through a PM10 filter (Amicon) before use. Na$_2$SO$_4$ and l-$^3$H$^3$-proline carrier-free, Sepahcyr S-500 HR and octyl-Sepharose CL-4B were from Amersham Pharmacia Biotech. All other reagents were of A.R. or equivalent quality.

**Analytical methods**

Fractions from the density gradients were analysed for density by weighing aliquots and by measuring $A_{280}$. Carbohydrate was detected by slot-blotting and staining with PAS [25] or as periodate-oxidizable structures by the method of Devine [26]. Aliquots (100 ml) were coated on multi-well assay plates (Falcon) and left overnight at room temperature; after being washed with PBS [0.15 M NaCl, 5 mM sodium phosphate buffer (pH 7.4)] containing 0.05% (v/v) Tween 20, the plates were treated with 25 mM sodium metaperiodate in 0.1 M sodium acetate buffer, pH 5.5 (100 ml at room temperature for 20 min). After washing, the wells were incubated with biotin hydrazide (2.5 ml in 1 M sodium acetate buffer, pH 5.5) for 1 h. Alkaline-phosphatase-conjugated streptavidin (dilution 1:20000) in PBS containing 0.05% (v/v) Tween 20 and 0.5% (w/v) BSA was added for 1 h. Reactivity was expressed as $A_{280}$ after 1 h of incubation with nitrophenyl phosphate (2 mg/ml in 1 M diethanolamine/HCl buffer, pH 9.8, containing 0.5 mM MgCl$_2$). Standards of respiratory mucin were used to ensure that readings from the PAS assay were within the linear range. Sialic acid was detected as described by Davies et al. [27] or with a sialic-acid-selective version of the glycan detection method outlined above, as described for the ECL* glycoprotein detection system (Amersham Life Science). In brief, samples were treated with 1 mM sodium metaperiodate and the oxidation reaction was performed on ice in the dark. All other conditions were as above. Aliquots (50 ml) from the radiolabelled fractions were mixed with 5 ml of scintillation fluid (Ready Safe; Beckman Instruments) and counted in an LKB 1214 /-scintillation counter.

**Isolation and culture of cells**

Bovine trachea were obtained from a local abattoir within 1 h of the death of the animals and freed from surrounding connective tissue. The mucosal surfaces were rinsed with ice-cold PBS and covered with MEM supplemented with penicillin (1 i.u./ml), streptomycin (1 $\mu$g/ml), gentamicin sulphate (50 $\mu$g/ml) and amphotericin B (2.5 $\mu$g/ml), approx. 10 ml per 10 cm trachea, for 1 h at 4 °C. Trachea were then incubated overnight with a 0.1% protease solution in MEM at 4 °C. After termination of the digestion with FCS [final concentration 10% (v/v)], cell suspensions harvested from several tracheas were combined and treated for 10 min with 0.01% deoxyribonuclease I to prevent cell clumping. Cells were centrifuged at 150 g for 10 min at 20 °C. The pellet was resuspended in ice-cold MEM containing 10% (v/v) FCS and the process was repeated twice. In experiments to isolate mucins from the cells before culture, cells harvested from six tracheas (pieces 15–20 cm long) were mixed with 6 M GdmCl/10 mM sodium phosphate buffer, pH 6.5, containing 5 mM sodium EDTA, 5 mM N-ethylmaleimide, 0.1 mM di-isospropyl phosphofluoridate (DFP) and 0.01% CHAPS for approx. 30 min. Samples were then dialysed exhaustively against 6 M GdmCl in 10 mM sodium phosphate buffer, pH 6.5, containing 5 mM sodium EDTA and 0.01% CHAPS, then subjected to isopycnic density-gradient centrifugation in CsCl/4 M GdmCl/sodium phosphate buffer (pH 6.5) containing 5 mM sodium EDTA and 0.01% CHAPS (36000 rev./min, 15 °C, 80 h, 50.2 Ti rotor, Beckman Optima L-70 centrifuge, initial density 1.39 g/ml). For culture, the pellets of harvested cells were suspended in F-12 medium (37 °C) containing transferrin (10 $\mu$g/ml), cortisol (0.5%), sodium selenite (30 nM), retinoic acid (10 ng/ml), insulin (5 $\mu$g/ml), penicillin (1 i.u./ml), streptomycin (1 $\mu$g/ml) and amphotericin B (2.5 $\mu$g/ml) (supplemented medium), with gentamicin (50 $\mu$g/ml) and 1% (v/v) FCS. Dissociated cells, at a density of 1.5 × 10$^5$ cells/ml, were plated on 60 mm tissue-culture-treated plastic dishes (Costar Corp.).

For experiments with different substrates, tissue-culture-treated plastic dishes were either left uncoated or coated with collagen (type 1 from calf skin), Matrigel or Vitrogen 100. Collagen was obtained as a 0.1% solution in 0.1 M acetic acid and, for the thin layers (7 $\mu$g/cm$^2$), was diluted 1:20 in distilled water. Matrigel was also diluted 1:20 in culture medium, whereas Vitrogen 100 (8 parts) was mixed with sterile 1.5 M NaCl/0.1 M sodium phosphate buffer, pH 7.4 (1 part) and 0.1 M NaOH (1 part) to give a final collagen solution of 2.5 mg/ml. For the cultures grown on thicker layers (28 $\mu$g/cm$^2$), the collagen was diluted 1:5 in distilled water. Dishes were coated with 4 ml of the solutions and incubated overnight at 37 °C. After the removal of excess solution, dishes were equilibrated with F-12 medium for 1 h and cells were left for 3 h at 37 °C to attach to the substrate. The medium was then replaced with supplemented medium containing isopycnic density gradient centrifugation (5 mg/ml) and the cells were grown at 37 °C in a humidified air/CO$_2$ (19:1) atmosphere. Medium was changed after 24 h and every other day thereafter.

**Radiolabelling of cell cultures**

Cells were plated on collagen-coated tissue culture dishes (7 $\mu$g/cm$^2$) and left to attach as described above. The medium was then replaced with F-12 medium (supplemented as above) containing Na$_2$SO$_4$ (50 $\mu$Ci/ml) and [3$^3$H] proline (5 $\mu$Ci/ml). Cells were grown at 37 °C in a humidified air/CO$_2$ (19:1) atmosphere. Medium was harvested after 1, 3, 5 and 7 days and replaced each time (except on the seventh day) with medium containing the radiolabels.

**Isolation and purification of mucins from the culture medium and cell layers**

After centrifugation at 100 g for 5 min to remove cell debris, solid GdmCl and 1% (w/v) CHAPS were added to the medium.
were analysed for radioactivity. Medium and cell layer samples were then dialysed exhaustively against 6 M GdmCl in 10 mM sodium phosphate buffer, pH 6.5, containing 5 mM sodium EDTA. After dialysis, 5 mM N-ethylmaleimide, 0.1 mM DFP and 0.01 % CHAPS were added. The samples were then subjected to isopycnic density-gradient centrifugation in CsCl/4 M GdmCl/10 mM sodium phosphate buffer (pH 6.5) containing 5 mM sodium EDTA and 0.01 % CHAPS. The samples were subjected to isopycnic density-gradient centrifugation in CsCl/4 M GdmCl/10 mM sodium phosphate buffer (pH 6.5) containing 5 mM sodium EDTA and 0.01 % CHAPS. After dialysis against 0.5 M GdmCl/10 mM sodium phosphate buffer/5 mM sodium EDTA, the samples were subjected to a second centrifugation run in CsCl/0.5 M GdmCl/0.01 % CHAPS as above (initial density 1.50 g/ml).

Rate-zonal centrifugation
Centrifuge tubes (12 ml) were loaded from the bottom with a linear gradient (6–8 M) of GdmCl [28]. Native and reduced mucin samples in 5 M GdmCl (400 μl) were layered onto the gradients and centrifuged was performed in a Beckman SW 40.1 Ti rotor [40000 rev./min (197000 gav), 20 °C, 2 h 45 min]. Tubes were emptied from the top and fractions (400 or 500 μl) were analysed for carbohydrate and radioactivity.

Degradative methods and gel chromatography
Samples were reduced for 5 h at 37 °C with 10 mM dithiothreitol in 6 M GdmCl/0.1 M Tris/HCl buffer (pH 8.0) and alkylated for 15 h at room temperature with 25 mM iodoacetamide in the dark in the same buffer. High-molecular-mass glycopeptides were then obtained by dialysis against 0.1 M NaH₂HCO₃, pH 7.0, and treatment overnight with 100 μg of trypsin at 37 °C. After treatment with 1 mM DFP to inactivate the trypsin, glycopeptides from the radiolabelled material were freeze-dried and digested with chondroitin ABC lyase (10 m-units per sample; overnight; 37 °C) or with heparan sulphate lyase (10 m-units per sample; overnight; 37 °C) in 0.1 M Tris/acetate buffer, pH 7.3, containing 10 mM sodium EDTA or with heparan sulphate lyase (10 m-units per sample; overnight; 37 °C) in 0.1 M Tris/acetate buffer, pH 7.3, containing 10 mM sodium EDTA or with heparan sulphate lyase (10 m-units per sample; overnight; 37 °C) in 0.1 M Tris/acetate buffer, pH 7.3, containing 10 mM sodium EDTA. Alkaline borohydride treatment was performed after the addition of equal volumes of water (adjusted to pH 9.0 with NaOH) and 2 M NaBH₄ to 0.1 M NaOH for 45 h at 45 °C. Samples were then neutralized with acetic acid, after which they were subjected to gel chromatography on a Sephacryl S-500HR column eluted with 4 M GdmCl, pH 7.0, at a rate of 0.15 ml/min. Fractions were analysed for radioactivity.

Hydrophobic interaction chromatography and phase-separation analysis of radiolabelled molecules
Samples of radiolabelled mucins from the medium harvested after 3 days in culture, and cell extract after radiolabelling for 7 days, were subjected to hydrophobic interaction chromatography on an octyl-Sepharose CL-4B column. Samples dialysed into 4 M GdmCl/10 mM sodium phosphate buffer (pH 6.5) were loaded onto the column, which was eluted with 4 M GdmCl/10 mM sodium phosphate buffer (pH 6.5) for 10 min followed by a linear gradient of this buffer to 10 mM sodium phosphate, pH 6.5 over the following 20 min. Fractions (0.5 ml) were collected and analysed for radioactivity. The same material was also subjected to phase separation by the method of Bordier [29]. After dialysis against 10 mM Tris/HCl buffer, pH 7.5, containing 0.5 M NaCl, samples were mixed with Triton X-114 at room temperature to give a final concentration of 1.5 % (v/v) Triton. The mixture was equilibrated on ice for 30 min and then incubated at 37 °C for 10 min to precipitate the detergent. The two phases were separated by centrifugation (2000 g, 10 min, 37 °C) and the precipitate was re-extracted twice with 100 μl of 0.006 % Triton X-114. Aliquots from the aqueous and detergent phases were then analysed for radioactivity.

Figure 1 Isopycnic density-gradient centrifugation in CsCl/4 M GdmCl (a) and CsCl/0.5 M GdmCl (b), re-centrifugation in CsCl/4 M GdmCl (c) and rate-zonal centrifugation (d) of extracts from bovine epithelial cells before culture
(a–c) Cell extracts were subjected to density-gradient centrifugation in a Beckman Optima centrifuge (36000 rev./min, 15 °C, approx. 80 h, Beckman 50.2 Ti rotor) in CsCl/4 M GdmCl (starting density 1.39 g/ml), CsCl/0.5 M GdmCl (starting density 1.5 g/ml) containing 0.01 % CHAPS and then again in CsCl/4 M GdmCl. Fractions were analysed for density (•), sialic acid (●), carbohydrate [PAS assay (▲) and glycan detection method (○)] and radioactivity (A). Fractions pooled as shown in (b) were subjected to rate-zonal centrifugation before (▲) and after (○) reduction as described in the text.
RESULTS

Mucins present in isolated bovine epithelial cells before culturing

Microscopic examination showed the cell isolates to contain a mixture of epithelial cells (ciliated, goblet and basal cells), whereas the submucosa was intact (results not shown). After extraction of the cells in GdmCl, density-gradient centrifugation in CsCl/4 M GdmCl revealed two partly resolved peaks reacting with the PAS and sialic acid assays (Figure 1a). The ‘high-density’ population at 1.48 g/ml showed a stronger reactivity with the sialic acid assay relative to PAS and coincided with a high A$_{280}$, most probably because of the presence of DNA, which is known to appear at this density [30]. A second, ‘low-density’, population at 1.42 g/ml showed a greater relative reactivity with the PAS assay. Mucin-containing fractions were pooled and re-centrifuged in CsCl/0.5 M GdmCl, in which the mucins were separated from DNA but there was no separation between the populations as demonstrated with the PAS and sialic acid assays (Figure 1b). Because the separation between PAS-reactive and sialic-acid-rich components was apparently better in the first gradient, fractions from the CsCl/0.5 M GdmCl gradient were pooled and re-centrifuged in CsCl/4 M GdmCl. This clearly separated the ‘high-density’ sialic-acid-rich mucins (1.46 g/ml) from the ‘low-density’ population at 1.40 g/ml, which showed a greater relative reactivity with the PAS and glycan detection methods (Figure 1c). Rate-zonal centrifugation of material from the CsCl/0.5 M GdmCl gradient revealed a very broad distribution of PAS reactivity, indicating that the intracellular mucins had a broad range of molecular sizes (Figure 1d). After reduction, one peak was present closer to the top of the gradient, suggesting that the material comprised large oligomeric mucins composed of subunits linked by disulphide bonds. However, the ‘shoulder’ present in the distribution suggests that there might be a partial separation into two species possibly originating from the two populations identified by density-gradient centrifugation (Figure 1c). Thus, before culture, two intracellular populations were identified: a ‘high-density’ one that contained relatively more sialic acid and a lower-density one that reacted more strongly with the PAS assay.

Investigation of mucin production on different substrates

Cells were seeded directly on plastic dishes or dishes coated with collagen (7 μg/cm²), Matrigel or Vitrogen and grown to confluence (3 days) in serum-free medium. The layers had the typical ‘cobblestone’ morphology of epithelial cells and PAS-positive cells with a granular appearance were seen, suggesting the presence of mucin-like material within them (results not shown). Medium was collected over the 2-day period after confluence to ensure that the cell number was approximately the same in each dish; the cell layers were harvested at the end of the 5 days in culture. The medium and cell-layer extracts were subjected to density-gradient centrifugation in CsCl/4 M GdmCl. In the medium from cells grown on all substrates, a peak of sialic-acid-reactive and PAS-reactive material similar to the ‘high-density’ population present in the cells before culture was seen at 1.46 g/ml, whereas the ‘low-density’ population seen before culture was absent. Results for cells grown on plastic are shown...
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Figure 3 Isopycnic density-gradient centrifugation in CsCl/0.5 M GdmCl of medium (a–d) and cell extracts (e–h) from cells grown on collagen type 1 for 1 day (a, e), 3 days (b, f), 5 days (c, g) and 10 days (d, h)

The medium and cell layers were harvested at different time points and subjected to density-gradient centrifugation first in CsCl/4 M GdmCl (results not shown) and then in CsCl/0.5 M GdmCl containing 0.01% CHAPS in a Beckman Optima centrifuge (36000 rev./min, 15 °C, approx. 80 h, Beckman 50.2 Ti rotor). Fractions were analysed for density ( ), sialic acid ( ), hexose ( ) and A₂₈₀ (line without points). Values are corrected to account for the different lengths of the collection periods.

in Figure 2(a) (left panel). The amount of DNA, as revealed by \textit{A}_{	ext{gdn}}, was low; material probably representing low-buoyant-density proteins and glycolipids was found at the top of the gradients. Because the major mucin produced after 5 days in culture was the sialic-acid-rich one, to compare the relative amounts produced by cells on the different substrates we integrated the area under the sialic acid distribution (1.40–1.48 g/ml) and related it to that for cells grown on plastic. This showed that the amount of sialic-acid-rich mucin material released was greater when a collagen, Matrigel or Vitrogen substrate was used (Figure 2a, right panel). In the extracts from the cells, a peak of PAS reactivity and sialic acid reactivity as well as DNA was separated from large amounts of low-buoyant-density material in the CsCl/4 M GdmCl gradient (results not shown). Fractions between 1.35 and 1.50 g/ml were re-centrifuged in CsCl/0.5 M GdmCl, in which the mucins from cells grown on plastic showed a broad distribution in the gradient (1.43–1.58 g/ml approx.) and were separated from DNA at a density of 1.62 g/ml (Figure 2b, left panel). Results from cells grown on collagen, Matrigel or Vitrogen were similar to those from cells grown on plastic (results not shown). The level of reactivity with the sialic acid assay was estimated as the area under the curves between 1.43 and 1.58 g/ml, related to the level for cells grown on plastic and plotted for each substrate. The relative amount of intracellular material varied between cells grown on the collagen, Matrigel and Vitrogen substrates but showed no major differences from that for cells grown on plastic (Figure 2b, right panel). Cells grown on a thicker collagen substrate (28 μg/cm²) did not differ from those on the thin collagen layer (7 μg/cm²), Matrigel or Vitrogen (results not shown). The presence of a substrate was therefore a significant factor for the production and secretion of the sialic-acid-rich mucin by these cells but the nature of the substrate seemed to be less important. With collagen, contrary to a previous report [31], the thickness of the layer did not affect the result; thin collagen layers (7 μg/cm²) were therefore used in subsequent experiments.

Investigation of mucin release over time

Medium and cells were harvested from cultures 1 day (pre-confluence), 3 days (confluence), 5 days (2 days after confluence) and 10 days (7 days after confluence) after plating. In the medium after 1 day, in addition to the ‘high-density’ population, small amounts of the ‘low-density’ population seen before culture were present in the CsCl/4 M GdmCl gradient (results not shown). However, at later time points (5 and 10 days) only the ‘high-density’ population was present. Fractions between 1.35 and 1.50 g/ml were pooled (results not shown) and subjected to a second density-gradient step in CsCl/0.5 M GdmCl. Here, after 1 day, sialic acid and PAS reactivities showed a broad peak of mucins between 1.47 and 1.53 g/ml (Figure 3a). However, after 3 days, the sialic acid distribution was sharper and the level had increased. This trend continued at 5 and 10 days, which was consistent with an increase in secretion of the sialic-acid-rich mucin and a decrease in release of the PAS-reactive component (Figures 3b–3d). Both the ‘high-density’ and ‘low-density’ populations were present in the CsCl/4 M GdmCl gradient of material from the cell layer after 1 day in culture but the relative
amount of the ‘low-density’ population had decreased after 5 days (results not shown). In CsCl/0.5 M GdmCl, the cell layer contained a broad peak of reactivity with the PAS and sialic acid assays between 1.42 and 1.50 g/ml, well separated from DNA at 1.60 g/ml (Figure 3e). Over time, there was a relative increase in reactivity with the sialic acid assay (Figures 3f–3h). These results suggest that the ‘low-density’ PAS-reactive population is present in the cells before culture and that small amounts of this material are released into the medium in the early stages of culture. However, production of the sialic-acid-rich component seems to be initiated after confluence and increases for up to 10 days of culture.

Characterization of the secreted mucins

Mucins secreted into the medium after 3 days were subjected to rate-zonal centrifugation to investigate the size distribution. The native molecules were separated into two distributions. The sialic-acid-selective glycan detection assay revealed molecules spread over the gradient, suggesting the presence of sialic-acid-containing mucins with a broad range of molecular sizes (Figure 4a). These molecules thus represent the largest species found in the cells before culture, which became smaller on reduction. It therefore seems that the sialic-acid-rich mucins are large oligomeric molecules made up of subunits linked by disulphide bonds. However, the major part of the PAS-reactive material was found as a peak towards the top of the gradient, indicating that these molecules were smaller than the sialic-acid-rich ones. After reduction, the position of the PAS-reactive molecules did not change (Figure 4a); they were therefore unlikely to be composed of disulphide-bond-linked subunits. The same material was also subjected to gel chromatography on a Sephacryl S-500HR column. After reduction, the molecules were eluted mainly in the void volume, suggesting that they were relatively large (Figure 4b). After digestion with trypsin the material was partly included, indicating the presence of relatively large, trypsin-resistant, glycosylated domains within the molecules (Figure 4c). Thus the sialic-acid-rich mucins seem to be large and oligomeric, whereas the PAS-reactive species, although ‘monomeric’, contain large glycosylated domains.

Radiolabelling of mucins

To investigate whether mucins identified with the sialic acid and PAS assays could be radiolabelled, cells were grown in the presence of [H]proline and [35S]sulphate over a 7-day period and the medium was harvested after 1, 3, 5 or 7 days. Cell extracts were made after the 7 days in culture. Little material was secreted over the first 24 h but there was a clear separation into ‘high-density’ and ‘low-density’ species similar to those from the cells before culture. This material was not studied further. After 3 days, mainly the major sialic-acid-rich population similar to that identified previously was present at 1.46 g/ml (Figure 5a). In addition, a large peak of [35S]sulphate-labelled material at 1.50 g/ml was partly separated from the molecules identified by the chemical analyses. Some [H]proline was associated with the main mucin band at 1.46 g/ml, although most of the radiolabel was found in low-buoyant-density material at the top of the gradient. After 5 and 7 days the pattern was similar to that seen at 3 days, although the relative amount of [35S]sulphate label present in the density gradients declined over time (Figures 5b and 5c). Mucin-containing fractions were pooled as shown in Figures 5a–5c and re-centrifuged in CsCl/0.5 M GdmCl. The major population identified by the chemical assays was, as expected, found between 1.45 and 1.55 g/ml (Figures 5d–5f). [H]Proline was present as a peak associated with the mucins detected with the PAS and sialic acid assays, whereas there was a partial separation between these and the [35S]sulphate distribution. In the samples from 3 and 5 days, the [35S]sulphate-labelled material showed a partial separation into a ‘high-density’ peak at the bottom of the gradient and material shifted out from the mucin peak at 1.53 g/ml (Figures 5d and 5e). After 7 days the intracellular fraction contained predominantly the ‘high-density’ sialic-acid-rich population at 1.46 g/ml, whereas the lower-density PAS-reactive population was absent (Figure 5g). The mucins were partly separated from a [35S]sulphate peak on the high-density side of the distribution, similarly to that seen in the medium, as well as some material that trailed into the lower-buoyant-density side of the gradient. Almost all of the [H]proline was found with low-buoyant-density material at the top of the gradient. Fractions were pooled as shown in Figure 5g and subjected to density-gradient centrifugation in CsCl/0.5 M GdmCl. This revealed a similar pattern to that
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Figure 5 Isopycnic density-gradient centrifugation of medium (a–c) and cell extract (g) in CsCl/4 M GdmCl and of medium (d–f) and cell extract (h) in CsCl/0.5 M GdmCl from cells radiolabelled for 7 days with [3H]proline and [35S]sulphate

Medium was harvested after 3 days (a, d), 5 days (b, e) and 7 days (c, f); extracts from the cells were prepared after 7 days in culture (g, h). Samples were subjected to density-gradient centrifugation in CsCl/4 M GdmCl containing 0.01% CHAPS in a Beckman Optima centrifuge (36000 rev./min, 15 °C, approx. 80 h, initial density 1.40 g/ml, Beckman 50.2 Ti rotor). Fractions were pooled as shown (a–c and g) and re-centrifuged in CsCl/0.5 M GdmCl as described above (initial density 1.50 g/ml). Fractions were analysed for density (+), sialic acid (E), hexose (-), A280 (line without points), [3H]proline (D) and [35S]sulphate (U).

found in the medium with a peak of [35S]sulphate-labelled material partly separated from the mucins. However, the relative amount of the [3H]proline associated with the mucin band was much greater than in the secreted material in the medium (Figure 5h). Thus the major [35S]sulphate-labelled molecules had a higher buoyant density than either of the components identified previously with the chemical assays.

Investigation of the size and molecular nature of the radiolabelled molecules

Fractions from the medium collected after 3 days were pooled (Figure 5d) and subjected to rate-zonal centrifugation before and after reduction. In the ‘native’ sample, [35S]sulphate-labelled molecules sedimented as a distinct population towards (but not at) the top of the gradient, suggesting that they were larger than the PAS-reactive molecules (Figure 6a). After reduction, the position of the radiolabelled molecules was unchanged, indicating that they were not oligomeric species composed of subunits linked by disulphide bonds (Figure 6b). The same fractions were also subjected to gel chromatography on a Sephacryl S-500 HR column. This showed that most of the radiolabelled material, as expected from the rate-zonal gradient, was eluted with the void volume (Figure 7a). Digestion with trypsin of reduced material from the void volume gave rise to two partly separated populations of high-molecular-mass glycopeptides that were included on the gel (Figure 7b). After digestion of high-molecular-mass glycopeptides with heparan sulphate lyase or chondroitin ABC lyase, the elution position was unchanged, suggesting that the material did not consist of proteoglycans containing heparan sulphate or chondroitin/dermatan sulphate side chains (results not shown). Treatment of the glycopeptides with alkaline borohydride caused complete fragmentation, as shown by the elution of all the material close to the total volume (Figure 7c). The peak of [35S]sulphate-labelled material was eluted a little earlier than that radiolabelled with [3H]proline, suggesting a separation between glycans and the protein core. The [35S]sulphate-labelled population therefore seems to comprise mucin-like molecules with large glycosylated domains containing O-linked glycans.

DISCUSSION

To provide a reference point for characterization of the glycoproteins produced by bovine tracheal cells during culture the mucins present in the cells were investigated in extracts made before culture. Two populations of molecules were identified: one that was relatively sialic-acid-rich and a second, at a lower
density, that reacted more strongly with the PAS assay. The ‘high-density’ sialic-acid-rich molecules were composed of disulphide-bond-linked subunits and had a similar buoyant density to the major population identified in the gel phase of secretions and lavage fluid from bovine trachea, as well as extracts from the surface epithelium of the tissue [19,32]. It is therefore likely that the sialic-acid-rich molecules correspond to the major oligomeric mucin population identified previously from the surface epithelium of bovine trachea. The ‘low-density’ population had a buoyant density consistent with that of a mucin, was glycosylated as shown by its reactivity with the PAS assay, and contained relatively large trypsin-resistant fragments, suggesting the presence of large glycosylated domains. However, the molecules were small and ‘monomeric’. This population is also similar in nature to one present in secretions and extracts of the surface epithelium from bovine trachea [19]. In human airway epithelium, several mucin genes are expressed, including MUC1, MUC4, MUC5AC, MUC8 and MUC11 [7,13,33], suggesting that several glycoproteins are produced in the tissue. MUC5AC has been identified as a gel-forming oligomeric glycoprotein from the goblet cells [2] and it is therefore possible that the sialic-acid-rich population is a bovine equivalent of MUC5AC. MUC1, MUC4 and MUC11 are thought to be ‘monomeric’ species and are therefore candidates for the human equivalents of the PAS-reactive component. However, with the exception of MUC1, the biochemical nature of the mucins encoded by these genes is currently unclear. Another possibility is that the PAS-reactive component represents a ‘small’ form of an oligomeric mucin because MUC5AC has recently been shown to be secreted in a less oligomeric form from cells early in culture than that released at later time points [34].

Although small amounts of the PAS-reactive component were released in the early stages of culture (before confluence), the predominant glycoprotein secreted into the medium was the ‘high-density’ sialic-acid-rich one. Release of these molecules increased after confluence, in agreement with reports that the secretory activity of cultivated cells increases after the cells become confluent [35]. These results thus suggest that bovine cells in primary culture are a good model for studies of the synthesis and secretion of at least one, large gel-forming mucin produced in the airway epithelium.

To investigate whether the mucins identified were those that became radiolabelled, cells were grown in the presence of [3H]proline and [35S]sulphate. Most of the [3H]proline was present in low-buoyant-density proteins rather than the mucins, in keeping with autoradiographic investigations, in which [3H]proline was taken up mainly by non-goblet epithelial cells in bovine tracheal tissue in culture or cat trachea [19,20]. However, [35S]sulphate was incorporated into ‘high-density’ molecules that were present intracellularly and secreted. The amount of [35S]sulphate-labelled material released declined over time, but, because the molecules did not react with the chemical assays it was not possible to ascertain whether this reflects a decrease in the total amount of radiolabelled material or a decrease in the specific radioactivity.
of the molecules. Neither radiolabel was taken up by the major components identified with the chemical assays. Although this would be expected for the ‘low-density’ mucins, which did not seem to be synthesized in culture, the reasons for the lack of uptake into the sialic-acid-rich mucin are unclear. It is possible that the sialic-acid-rich mucin contains little sulphate, or that all the intracellular [35S]sulphate is rapidly incorporated into other molecules. The radiolabelled molecules had a higher buoyant density than the sialic-acid-rich mucins and were not oligomeric molecules composed of subunits linked by disulphide bonds. They were not proteoglycans with heparan sulphate or chondroitin/dermatan sulphate side chains and seemed to be mucin-like in that they contained O-linked glycans. The uptake of large amounts of radiolabel by the molecules that could not be detected with the chemical assays suggests that they have a high specific activity. Highly radiolabelled, ‘monomeric’ mucin-like molecules of this nature have also been described in secretions from cat, dog and hamster trachea [20–22] as well as in a cell line from human endometrial adenoma [36]. It is not known whether molecules of this nature are present in human airway but both MUC1 and MUC4, which are expressed in epithelial cells [13,17], seem to be potential candidates for such ‘monomeric’ mucins. However, because neither the mucin-like molecules from tissue nor those secreted into the medium seemed to contain hydrophobic domains, the molecules might not be membrane-associated and in this respect they might differ from at least the most common splice variants of MUC1 and MUC4 [4,6,18].

Metabolic labelling with radiolabelled precursors has been favoured as a method for the detection of glycoproteins in studies of mucin synthesis and/or secretion in various cell-based and tissue-based models. Results obtained in such investigations have sometimes been extrapolated to include the large oligomeric and tissue-based models. Results obtained in such investigations of mucin synthesis and expression of human tumor-associated polymorphic epithelial mucin. J. Biol. Chem. 265, 15286–15283


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