Biosynthesis of mucins in bovine trachea: identification of the major radiolabelled species

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Bovine trachea in organ culture secretes mucus containing a 'high-density' (1.46 g/ml) and a 'low-density' (1.37 g/ml) mucin similar to those identified previously in bovine respiratory secretions [Hovenberg, Carlstedt and Davies (1997) *Biochem. J.* **321**, 117–123]. After pulse-labelling, autoradiography showed uptake of [³⁵S]sulphate by both epithelial goblet cells and submucosal glands, while [³H]proline was mainly incorporated into the ciliated surface epithelial cells. After 24 h of radiolabelling, neither the high- nor the low-density mucin in the secreted mucus gel was heavily radiolabelled with the precursors. In contrast, a population of molecules banding at 1.50 g/ml was heavily radiolabelled with [³⁵S]sulphate. This component was smaller than the high-density mucin from the mucus gel and was insensitive to reduction or digestion with chondroitin ABC lyase

or heparan sulphate lyase. The molecules yielded two populations of high-*M_r* glycopeptides upon trypsin digestion, were sensitive to keratanase and endo- β -galactosidase digestion and contained O-linked glycans. Extracts of the surface epithelium and submucosal tissue after radiolabelling showed that the high- and low-density mucins in the tissue were also poorly radiolabelled. Thus, under these conditions, the radiolabelled precursors were not effectively incorporated into the large oligomeric mucins but into a high-*M_r* monomeric species. This study suggests that data obtained in investigations where mucins are radiolabelled and studied without further separation into distinct components may rather reflect the turnover of this 'novel' monomeric species than the large oligomeric mucins.

INTRODUCTION

Respiratory mucins belong to a family of glycoproteins which comprises both membrane-bound and large secreted species. The mucins, which are responsible for the gel-like properties of respiratory mucus, are large linear flexible molecules (*M_r* in the order of 10–30 million), consisting of a protein core which is extensively substituted with O-linked oligosaccharide side chains [1–3]. The glycan chains, which may constitute up to 80 % of the weight of the molecule, are clustered into highly glycosylated regions interspersed along the molecule with 'naked' areas almost devoid of carbohydrate (for reviews, see, for example, [4,5]).

At least eight mucin genes (*MUC1*, *MUC2*, *MUC3*, *MUC4*, *MUC5AC*, *MUC5B*, *MUC7* and *MUC8*) are expressed in the human airway (for a review, see [6]). *MUC5AC* and *MUC2* are expressed by the epithelial goblet cells, *MUC3* and *MUC4* by the surface epithelium, *MUC5B* by the submucosal glands [7] and *MUC8* by both submucosal glands and goblet cells [8]. The *MUC5AC* mucin, produced by the epithelial goblet cells [9,10] and *MUC5B* from the submucosal glands (C. Wickström, J. R. Davies, G. V. Eriksen, E. C. I. Veerman and I. Carlstedt, unpublished work) have been identified as large, secreted gel-forming mucins, while *MUC2* from human intestine is found as an 'insoluble' glycoprotein complex [11].

Studies of the regulation of mucin secretion have been carried out in various models of respiratory epithelium and many have relied upon the incorporation of radioactive glycoprotein precursors to measure mucin release (see, for example, [12–14]). In addition, metabolic radiolabelling of human airway cells in primary culture has been used to study changes in mucins associated with disease [15]. Since radiolabelled precursors will be incorporated into mucins depending upon their turnover rate, it is likely that measurement of the release of radiolabelled macromolecules will mainly reflect secretion of those species that have the highest turnover. Radiolabelling studies in a cell-line

have recently shown that synthesis and assembly of the large oligomeric mucins may take several days [16].

As a basis for investigations of biosynthesis of the large, oligomeric gel-forming mucins, we have previously characterized the major species from bovine airway secretions and investigated their tissue origins [17]. In the present study we have isolated the mucins released into the medium by bovine tracheal tissue in organ culture. Incorporation of radiolabels into the various secretory cells was studied by autoradiography, and the release of radiolabelled macromolecules into the medium, as well as those retained within the tissue, was investigated. Our data show that, while [³⁵S]sulphate was taken up into secretory cells in the epithelium and submucosal glands, labelling of the large, oligomeric mucin species identified previously in bovine trachea was poor and the predominant [³⁵S]sulphate-labelled molecules in the secreted gel are high-*M_r* monomeric species.

EXPERIMENTAL

Materials and methods

Ham's F-12 culture medium without glutamine, L-glutamine, amphotericin B (Fungizone), streptomycin and penicillin were purchased from Flow Laboratories. Transferrin, retinoic acid, cortisol (water-soluble), sodium selenite, insulin (bovine), iodoacetamide and trypsin [EC 3.4.21.4, type XIII, tosylphenylalanyl-chloromethane ('TPCK')-treated] were from Sigma Chemical Co. Chondroitin ABC lyase (EC 4.2.2.4), heparan sulphate lyase (EC 4.2.2.8) and keratanase from *Pseudomonas* sp (IFO-13309) were products of Seikagaku Kogyo Co. Endo- β -galactosidase from *Escherichia freundii* was from ICN Biomedicals. Di-isopropyl phosphofluoridate (DFP), CHAPS, sodium borohydride and guanidinium chloride (practical grade) were purchased from Fluka. Stock solutions of guanidinium chloride (approx. 8 M) were treated with charcoal and filtered through a PM10 filter

Abbreviations used: DFP, di-isopropyl phosphofluoridate; DTT, dithiothreitol; NEM, *N*-ethylmaleimide; PAS, periodic acid/Schiff's.

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(Amicon) before use. $\text{Na}_2^{35}\text{SO}_4$ and L-[5- ^3H]proline (carrier-free) were from Amersham International, *N*-ethylmaleimide (NEM) from BDH Chemicals and dithiothreitol (DTT) from Merck. Sephacryl S-500HR was obtained from Pharmacia. All other chemicals were of A. R. grade or equivalent.

Analytical methods

The density of fractions from CsCl gradients was measured using a Carlsberg pipette as a pycnometer. Sialic acid was measured with an automated version [18] of the method of Jourdain et al. [19], modified as described by Davies et al. [3]. Fractions from density and rate-zonal gradients were blotted on to nitrocellulose membranes and stained by the periodic acid/Schiff's (PAS) procedure [20]. Aliquots (50 μl) of fractions were mixed with 5 ml of scintillation fluid (Ready Safe; Beckman Instruments) and counted for radioactivity in an LKB 1214 β -scintillation counter.

Tissue culture

Bovine tracheas obtained from the local abattoir within 1 h of slaughter were rinsed with ice-cold PBS (0.15 M NaCl/10 mM sodium phosphate buffer, pH 7.4). Connective tissue was trimmed away and the tracheas opened along the dorsal aspect. Squares of tissue (7 mm $\times$ 7 mm) were dissected from the underlying cartilage and placed in Ham's F-12 medium containing L-glutamine (0.2 mM), penicillin (1 i.u./ml), streptomycin (1 $\mu\text{g}/\text{ml}$) and amphotericin B (2.5 $\mu\text{g}/\text{ml}$) for 1 h. The tissue was then rinsed twice, transferred to sterile titanium nets in sterile Petri dishes (four pieces/dish) and covered with Ham's F-12 medium supplemented with L-glutamine (0.2 mM), transferrin (10 $\mu\text{g}/\text{ml}$), cortisol (0.5 μM), sodium selenite (30 nM), retinoic acid (10 ng/ml), insulin (5 $\mu\text{g}/\text{ml}$), penicillin (1 i.u./ml), streptomycin (1 $\mu\text{g}/\text{ml}$) and amphotericin B (2.5 $\mu\text{g}/\text{ml}$). Cultures were incubated in air/CO₂ (19:1; 98% humidity, 37 °C) on a platform which rocked from side to side. In each cycle, the tissue was thus exposed to the gas phase for 60 s, followed by a brief submergence in the medium for 15 s. Ciliary beating, viewed with a dissection microscope, was used as a day-to-day criterion of tissue viability.

Isolation of radiolabelled mucins from the culture medium and tissue

$\text{Na}_2^{35}\text{SO}_4$ (50 $\mu\text{Ci}/\text{ml}$) and [^3H]proline (25 $\mu\text{Ci}/\text{ml}$) were added to the cultures in the initial medium, which was harvested 24 h later and replaced with non-radiolabelled chase medium. This was subsequently changed every 24 h until a total of 72 h of chase was reached. Medium samples were mixed with protease inhibitors (5 mM NEM, 5 mM Na₂EDTA and 0.1 mM DFP) and centrifuged to separate a gel phase and a sol (36000 rev./min; 4 °C; 45 min; Beckman 50.2Ti rotor; Beckman L-70 ultracentrifuge). Gels were solubilized in 6 M guanidinium chloride/10 mM sodium phosphate buffer, pH 6.5, with 5 mM Na₂EDTA (extraction buffer), containing 5 mM NEM and 0.1 mM DFP, and stirred gently overnight prior to dialysis against six changes of extraction buffer to remove unincorporated radioactivity. The sol phases were mixed with solid guanidinium chloride to give a 6 M solution and dialysed extensively against extraction buffer. After 72 h of chase, the surface epithelium was scraped off and the underlying submucosal tissue frozen in liquid nitrogen and pulverized using a tissue pulverizer (Retsch). Protease inhibitors (5 mM NEM, 5 mM Na₂EDTA and 0.1 mM DFP) were added and the samples solubilized in extraction buffer prior to dialysis against the same buffer. The surface

epithelium was completely solubilized by this treatment, whereas a residue remained in the submucosal tissue. After centrifugation of the submucosal extract (25 500 g; 4 °C; 45 min; Beckman JA20 rotor; Beckman J2-MC centrifuge), the supernatant was retained and dialysed against extraction buffer while the residue was solubilized by reduction with 10 mM dithiothreitol in 6 M guanidinium chloride/0.1 M Tris/HCl buffer, pH 8.0, containing 5 mM Na₂EDTA (reduction buffer) for 5 h at 37 °C, followed by alkylation with 25 mM iodoacetamide in the dark (15 h, room temperature).

Purification of mucins

Mucins were prepared from the secreted gel phase and the sol as well as the surface and submucosal tissue using isopycnic density-gradient centrifugation in CsCl/4 M guanidinium chloride as described by Carlstedt et al. [21]. Briefly, CsCl and 10 mM sodium phosphate buffer, pH 7.4, were added to give a final concentration of 4 M guanidinium chloride and a density of 1.39 g/ml. Samples were then centrifuged (36000 rev./min; 15 °C; 80 h; Beckman 50.2Ti rotor; Beckman L-70 centrifuge). Fractions from the gel were pooled as shown in Figure 2 (below) and subjected to a second centrifugation step in CsCl/0.5 M guanidinium chloride containing 0.01% CHAPS (40000 rev./min; 15 °C; 65 h; Beckman 70.1Ti rotor; initial density 1.52 g/ml).

Autoradiography

Cultures were radiolabelled with $\text{Na}_2^{35}\text{SO}_4$ (50 $\mu\text{Ci}/\text{ml}$) or [^3H]proline (25 $\mu\text{Ci}/\text{ml}$) for 24 h, after which one tissue piece was removed from each of the cultures and fixed in 10% neutral-buffered formal saline. The medium was replaced with non-radiolabelled medium and one tissue piece removed every subsequent 24 h over the following 72 h. After embedding the tissue in paraffin, sections (3 μm) were cut, stained with PAS reagent and prepared as autoradiographs using LM-1 emulsion (Amersham). The sections were then exposed in light-proof boxes for 24–48 h at 4 °C and developed with D19 developer (Kodak). Microscopy was performed with a Nikon Optifot microscope.

Rate-zonal centrifugation

High-density material from the mucus gel collected during the 24 h labelling period (Figure 5a below) was subjected to rate-zonal centrifugation before and after reduction with 10 mM DTT in reduction buffer (5 h; 37 °C), followed by alkylation with 25 mM iodoacetamide in the dark (15 h; room temperature). The material was also treated with chondroitin ABC lyase (10 munits/sample; 37 °C; overnight) after dialysis into 0.1 M Tris/acetate buffer, pH 7.3, containing 10 mM Na₂EDTA or with heparan sulphate lyase (10 munits/sample; 37 °C; overnight) after dialysis into 0.1 M Tris/acetate buffer, pH 6.8, containing 0.15 M NaCl and 5 mM CaCl₂. High-density material from the mucus gel collected during each of the 24 h chase periods was also pooled (Figures 5b–5d below) and subjected to rate-zonal centrifugation. Centrifuge tubes (12 ml) were loaded from the bottom with a linear gradient (6–8 M) of guanidinium chloride using a Hoeffer gradient maker and an LKB 2232 microperpex pump at a flow of 50 ml/h [22]. Mucin samples in 5 M guanidinium chloride (400 μl) were layered on to the gradients and centrifugation was performed in a Beckman SW 40.1Ti rotor [40000 rev./min (197000 g_{av}); 20 °C; 2 h 45 min]. Tubes were emptied from the top and fractions (500 μl) analysed for carbohydrate and radioactivity. Fractions from the rate-zonal

Figure 1 Autoradiographs of radiolabelled trachea stained with PAS reagent

Tissue labelled for 24 h with [^{35}S]sulphate (**a, c, e** and **g**) or [^3H]proline (**b, d, f** and **h**), taken after pulse-labelling (**a** and **b**) or after 24 h (**c** and **d**), 48 h (**e** and **f**) and 72 h (**g** and **h**) of chase were fixed in 10% neutral-buffered formal saline and embedded in paraffin. Sections (3 μm) were cut and stained with PAS reagent. The stained sections were then dipped in LM-1 autoradiographic emulsion and developed as described in the Experimental section. Each picture represents a montage of photographs of the surface epithelium and submucosal tissue. The bar represents 100 μm .

gradients contained increasing concentrations of guanidinium chloride (6–8 M), which led to differences between fractions in the sensitivity of the sialic acid assay. The PAS distribution in the CsCl/4 M guanidinium chloride gradients followed that of the sialic acid assay closely, and the PAS method was therefore used to detect mucins in these experiments.

Degradative methods and gel chromatography

High- M_r glycopeptides were prepared from the high-density mucins secreted during the 24 h labelling period after reduction with 10 mM DTT in reduction buffer (5 h; 37 °C) followed by alkylation with 25 mM iodoacetamide in the dark (15 h; room temperature). The reduced subunits were then dialysed against 0.1 M ammonium hydrogen carbonate, pH 7.0, and treated with 100 μ g of trypsin (5 h; 37 °C). After treatment with 1 mM DFP to inactivate the trypsin, the glycopeptides were freeze-dried and treated with keratanase (0.1 unit/sample; 12 h; 37 °C in 50 mM Tris/HCl buffer, pH 7.4) or endo- β -galactosidase (0.01 unit/sample; 12 h; 37 °C in 50 mM sodium acetate buffer, pH 5.6). Alkaline borohydride treatment was performed after the addition of equal volumes of water and 2 M NaBH₄/0.1 M NaOH for 45 h at 45 °C, and samples were then neutralized with acetic acid. The digests, as well as 'whole' mucins and subunits, were subjected to gel chromatography on a Sephacryl S-500HR column eluted with 4 M guanidinium chloride, pH 7.0, at a rate of 0.15 ml/min. Fractions were analysed for radioactivity.

RESULTS

During the 96 h in culture, ciliary beating continued over the whole surface of the tissue, and haematoxylin/eosin stained sections indicated that tissue architecture was normal (results not shown). In separate experiments, lactate dehydrogenase levels in the medium were shown to be low after 96 h in culture.

Uptake of radiolabels into secretory cells

After 24 h, [³⁵S]sulphate was incorporated into some, but not all, of the PAS-stained goblet cells in the surface epithelium whereas

Figure 3 Isopycnic density-gradient centrifugation in 4 M guanidinium chloride/CsCl of mucins from the sol phase of the medium collected after 24 h of radiolabelling and (inset) the amount of the high-density material released during the 24 h of radiolabelling and the subsequent 24 h chase periods

The sol phase of the medium after 24 h radiolabelling was subjected to density-gradient centrifugation in 4 M guanidinium chloride/CsCl (80 h; 36 000 rev./min; 15 °C; 50.2 Ti rotor; Beckman L-70 ultracentrifuge). Fractions were analysed for sialic acid (●), A_{280} (---), density (■), ³⁵S (◆) and ³H (○). The amount of the high-density component (estimated as the area under the sialic acid curve between 1.54 and 1.41 g/ml) plotted for each of the time points is shown in the inset.

the majority of epithelial cells, probably ciliated cells, were not labelled. In the submucosa, all glands were radiolabelled, although the level of labelling appeared to vary (Figure 1a).

Figure 2 Isopycnic density-gradient centrifugation in 4 M guanidinium chloride/CsCl of mucins from the secreted gel collected after 24 h of radiolabelling (a) and the first (b), the second (c) and the third (d) 24 h period of chase

Mucus gel harvested from cultures after 24 h radiolabelling as well as the three following 24 h periods (24, 48 or 72 h of chase) was solubilized in extraction buffer prior to density-gradient centrifugation in 4 M guanidinium chloride/CsCl (80 h; 36 000 rev./min; 15 °C; 50.2 Ti rotor; Beckman L-70 ultracentrifuge). Fractions were analysed for sialic acid (●), A_{280} (---), density (■), ³⁵S (◆) and ³H (○).

Figure 4 Isopycnic density-gradient centrifugation in 4 M guanidinium chloride/CsCl of mucins from the surface epithelium (left panel) and the 'soluble' mucins from the submucosal tissue (right panel) after 24 h of radiolabelling followed by 72 h of chase

Material extracted from the surface epithelium and submucosa after radiolabelling and chase was subjected to density-gradient centrifugation in 4 M guanidinium chloride/CsCl (80 h; 36 000 rev./min; 15 °C; 50.2 Ti rotor; Beckman L-70 ultracentrifuge). Fractions were analysed for sialic acid (●), A_{280} (---), density (■), ^{35}S (◆) and ^3H (○).

[^3H]Proline was avidly taken up by most of the surface epithelial cells, with little uptake into the PAS-stained goblet cells (Figure 1b). Labelling of the submucosal glands with [^3H]proline was weak relative to that of the epithelium. After 24, (Figures 1c and 1d), 48 (Figures 1e and 1f) and 72 h (Figures 1g and 1h) of chase, the patterns of radiolabelling were similar to those seen directly after radiolabelling.

Radiolabelling of secreted mucins

Isopycnic density-gradient centrifugation in CsCl/4 M guanidinium chloride revealed two sialic acid-containing mucin populations, namely a high-density (1.46 g/ml) and a low-density (1.37 g/ml) one within the gel secreted during the 24 h radiolabelling period (pulse) (Figure 2a). The distribution of carbohydrate, as analysed by the PAS method, followed sialic acid

(results not shown). Material absorbing at 280 nm with a density consistent with DNA [21] was also present in the medium and probably originated from cells shed from the cut edges of the tissue pieces. [^{35}S]sulphate-labelled material appeared as two peaks at densities which were similar to those of the high- and low-density sialic acid-containing populations. Both the high-density population and the large amounts of low-buoyant-density material with absorbance at 280 nm were associated with [^3H]proline. In each of the chase samples, which represent material secreted over the previous 24 h, the pattern was repeated. The amount of sialic acid-containing mucin did not decrease significantly, although the relative amount of radioactivity incorporated fell successively (Figures 2b, 2c and 2d). After the 24 h pulse, the sol phase contained sialic acid-rich material at 1.46 g/ml, namely the same buoyant density as the high-density population from the gel phase (Figure 3). Large amounts of low-buoyant-density sialic acid-containing material trailed into the gradient, making it impossible to determine whether the low-density component was present. Low levels of radiolabelling with [^{35}S]sulphate were seen, while large amounts of [^3H]proline-labelled material were present at the top of the gradient. As in the gel phase, the amount of the high-density material (measured as the area under the sialic acid curve between 1.54 and 1.41 g/ml) declined for each following 24 h chase periods (Figure 3, inset).

Radiolabelling of intracellular mucins

The surface epithelial extract contained predominantly the high-density sialic acid-rich mucin population, although the low-density one was also present (Figure 4a). As in the secreted gel, [^{35}S]sulphate peaks were present at densities similar to those of the high- and the low-density populations. Large amounts of [^3H]proline-labelled material at the top of the gradient made it impossible to determine whether the low-density mucin had incorporated this radiolabel. In both the 'soluble' fraction from the submucosal tissue (Figure 4b) and the reduced/alkylated 'insoluble' residue (results not shown), the high-density and low-density populations were present. The amount of [^{35}S]sulphate relative to sialic acid associated with the low-density population

Figure 5 Isopycnic density-gradient centrifugation in 0.5 M guanidinium chloride/CsCl of mucins from the gel phase of the medium collected after 24 h of radiolabelling (a) and the first (b), the second (c), and the third (d) 24 h period of chase

Mucins from the gel phase of the medium harvested after 24 h of radiolabelling (a) as well as the three following 24 h periods (24 h (b), 48 h (c) or 72 h (d) of chase) were pooled as shown in Figure 2 and subjected to density-gradient centrifugation in 0.5 M guanidinium chloride/CsCl (65 h; 40 000 rev./min; 15 °C; 70.1Ti rotor; Beckman L-70 ultracentrifuge). Fractions were analysed for sialic acid (●), A_{280} (---), density (■), ^{35}S (◆) and ^3H (○).

in the submucosal tissue extract was greater than in either the secreted gel or the extract from the surface epithelium.

Comparison of the high-density mucins and the [^{35}S]sulphate-labelled molecules

Density-gradient centrifugation in $\text{CsCl}/0.5\text{ M}$ guanidinium chloride of the high-density populations from the $\text{CsCl}/4\text{ M}$ guanidinium chloride gradients separated the sialic acid-rich population from DNA with absorbance at 280 nm (Figures 5a–5d). Under these conditions, a [^{35}S]sulphate-labelled material at an intermediate buoyant density was partially separated from DNA and the sialic acid-rich mucins. [^3H]Proline was associated with both the [^{35}S]sulphate-rich component and the sialic acid-rich high-density mucin population. Although the total level of

Figure 6 Rate-zonal centrifugation of whole high-density mucins from the secreted gel after radiolabelling (a) and the same mucins after reduction/alkylation (b), chondroitin ABC lyase (c) and heparan sulphate lyase (d) treatment

High-density mucins pooled as shown in Figure 5(a) were subjected to reduction/alkylation or treatment with chondroitin ABC lyase or heparan sulphate lyase. Samples were then subjected to rate-zonal centrifugation in a 6–8 M guanidinium chloride gradient in a Beckman SW 40.1Ti rotor at 40000 rev./min at 20 °C for 2 h 45 min. Gradients were unloaded from the top of the tubes and fractions analysed for carbohydrate by the PAS method ($\blacktriangle$), for ^{35}S ($\blacklozenge$) and for ^3H ($\circ$).

Figure 7 Rate-zonal centrifugation of whole high-density mucins isolated from the medium after 24 h of radiolabelling and the first, second and third 24 h periods of chase

High-density mucins from secreted gel after the 24 h radiolabelling period ($\blacklozenge$) as well as the three following 24 h periods [24 ($\diamond$), 48 ($\square$) or 72 ($\triangle$) h of chase] were subjected to rate-zonal centrifugation. Gradients were unloaded from the top of the tubes and fractions analysed for ^{35}S .

radiolabelling fell successively, the relative amount of [^{35}S]sulphate under the sialic acid distribution increased after each of the 24 h chase periods, suggesting that the sialic acid-containing mucins may have become radiolabelled. Rate-zonal centrifugation revealed a clear separation between the broad distribution of mucins as identified by the PAS method and the smaller, less polydisperse radiolabelled molecules (Figure 6a). After reduction and alkylation, the radiolabelled molecules appeared, if anything, to move towards the high-molecular-mass side of the distribution, suggesting that the size of the material did not decrease (Figure 6b). Reduced mucin subunits (PAS) were much smaller than the native mucins and appeared as a peak coinciding with the radiolabelled molecules. After treatment with either chondroitin ABC lyase (Figure 6c) or heparan sulphate lyase (Figure 6d), the position of the molecules was unchanged and no small ‘fragments’ were seen at the top of the gradient, suggesting that the material was not affected by either of the enzymes.

Plotting the size distribution of the radiolabelled molecules collected directly after radiolabelling as well as after each of the successive 24 h chase periods showed that the amount of radiolabel in the molecules on the high- M_r side of the distribution increased with chase time (Figure 7). This suggests that the radiolabels are indeed slowly incorporated into the largest mucins. However, even after 24 h of radiolabelling followed by 72 h of chase, most of the radiolabel was still present in smaller molecules and the bulk of the large mucin species had not become radiolabelled.

Characterization of the [^{35}S]sulphate-labelled molecules

As expected from the rate-zonal-centrifugation data, both the ‘native’ (results not shown) and the reduced radiolabelled molecules from the radiolabelling period were eluted in the void volume of a Sephacryl S-500HR column (Figure 8a). Upon trypsin digestion, the [^{35}S]sulphate-labelled material gave rise to two populations of high- M_r glycopeptides (Figure 8b). After treatment with keratanase or endo- β -galactosidase, the major peaks were eluted slightly later than the two high- M_r glycopeptide

Figure 8 Gel chromatography on a Sephacryl S-500HR column of the radiolabelled high-density component after reduction/alkylation (a), reduction/alkylation and trypsin digestion (b) and reduction/alkylation and trypsin digestion followed by treatment with keratanase (c), endo- β -galactosidase (d) or alkaline borohydride (e)

High-density mucins from the secreted gel were reduced and alkylated and then digested with trypsin to prepare high- M_r glycopeptides. High- M_r glycopeptides were then treated with keratanase, endo- β -galactosidase or alkaline borohydride and the samples subjected to gel chromatography on a Sephacryl S-500HR column eluted with 4 M guanidinium chloride, pH 7.0, at a rate of 0.15 ml/min. Fractions (1 ml) were analysed for ^{35}S ($\blacklozenge$) and ^3H ($\circ$).

populations obtained after trypsin digestion. In addition, a peak of [^{35}S]sulphate-labelled material appeared close to the total volume (Figures 8c and 8d). After treatment of the high- M_r glycopeptides with alkaline borohydride, however, all the material was eluted close to the total volume, and [^{35}S]sulphate- and [^3H]proline-labelled material were partially separated with the main peak of [^{35}S]sulphate being eluted in front of that of ^3H -proline (Figure 8e). The peak of [^{35}S]sulphate-containing material obtained after alkaline elimination was eluted just before that released by keratanase or endo- β -galactosidase digestion.

DISCUSSION

Two mucin populations, a high-density and a low-density species, predominate in the gel phase of secretions collected from bovine trachea by lavage [17]. The high-density mucin originates from the surface epithelium, whereas the low-density one is enriched in the submucosal tissue and is probably produced by the glands [17]. In the secreted gel from organ cultures, two mucin populations similar to those identified in the tracheal lavage [17] were present, suggesting that mucins were released from both the

surface epithelium and the submucosa. [^{35}S]Sulphate was found at a density similar to the high-density mucin population, whereas most of the [^3H]proline was found at the top of the gradient in low-buoyant-density material. This is in keeping with autoradiography, which showed that [^{35}S]sulphate was taken up mostly by the surface epithelial goblet cells and the submucosal glands, while [^3H]proline predominated in the epithelium, probably in the ciliated cells which are thought to produce low-buoyant-density proteins rather than mucins. The sol phase of the medium contained large amounts of low-buoyant-density material, although a high-density species was also present. Since the sol probably contained a complex mixture of degraded mucins, non-gel-forming species and gel-forming species in equilibrium with the gel phase, and the level of radiolabelling with [^{35}S]sulphate was low, this fraction was not studied further.

In CsCl/0.5 M guanidinium chloride the high-density population from the gel phase was separated from DNA [21], and a partial separation was seen between the major sialic acid containing peak and the major [^{35}S]sulphate-rich component. This suggests either that two components with overlapping buoyant densities are present or that the radiolabelled molecules comprise a subpopulation of the high-density mucins. Rate-zonal centrifugation of the high-density species subsequently revealed a clear separation between a population of high-density mucins with a polydisperse distribution similar to that seen for the high-density mucin from bovine tracheal lavage [17] and the radiolabelled molecules which were present as a peak at the low- M_r end of the distribution. The radiolabelled population is thus smaller than the large polydisperse mucins identified with chemical techniques. Reduction of disulphide bonds caused fragmentation of the larger mucins, indicating that they are oligomers composed of subunits linked by disulphide bonds. The size of the radiolabelled species did not, however, change upon reduction and appeared, if anything, to increase, suggesting that this component is not oligomeric. The radiolabelled molecules were similar in size to reduced subunits from the oligomeric mucins, making it difficult to determine whether they represent unpolymerized subunits or a completely different component. However, it is clear that the predominant radiolabelled species found in the secreted gel phase after 24 h of radiolabelling is not representative of the major oligomeric mucins released from the tissue or of those identified previously in samples of lavage from bovine trachea.

Studies in cell lines have suggested that the large mucin species are formed slowly [16], possibly owing to slow synthesis and assembly of subunits or the fact that, when mucins are present in intracellular stores, further production continues only at a low level. The latter idea is supported by studies in a cat model in which metabolic radiolabelling of mucins was greatly enhanced by the stimulation of secretion, causing the emptying of intracellular stores prior to the administration of the precursors [24]. Rate-zonal centrifugation of the high-density population from the gel phase showed that the relative amount of radioactivity on the high- M_r side of the distribution increased in successive 24 h chase periods, suggesting that the large secreted mucins actually became radiolabelled, although over a much longer time period than the monomeric ones. The two mucin populations identified previously in the secreted gel phase were also found in tissue extracts of the surface epithelium and the submucosal tissue after 96 h. The level of radiolabelling, although low, was higher than in the secreted gel phase at the same time point, suggesting that radiolabelled large oligomeric mucins may have been retained within the secretory cells. This could not be confirmed with rate-zonal centrifugation due to the low levels of radiolabel.

The [^{35}S]sulphate-labelled molecules from the secreted gel were unaffected by chondroitin ABC lyase and heparan sulphate lyase and are thus unlikely to be proteoglycans containing chondroitin/dermatan sulphate or heparan sulphate side chains. Trypsin digestion yielded high- M_r glycopeptides, suggesting that the molecules are mucin-like and contain relatively long highly glycosylated stretches within the protein core. Keratanase and endo- β -galactosidase digestion caused partial degradation of the material, indicating the presence of polylactosamine structures within the oligosaccharide side chains. The elution position of the high- M_r glycopeptides was also shifted slightly after such treatment and, since gel chromatography reflects the hydrodynamic volume of the molecules which, for mucins, does not change significantly until the core *N*-acetylgalactosamine is removed [23], this slight shift is consistent with the presence of the protein core with truncated oligosaccharides. After alkaline elimination, the material was completely fragmented, indicating that the oligosaccharides within the molecules are predominantly O-linked. [^{35}S]Sulphate-containing fragments obtained after alkaline elimination were slightly larger than those produced by keratanase or endo- β -galactosidase digestion, as would be expected for whole oligosaccharide chains as compared with partially degraded ones.

Highly glycosylated domains from the radiolabelled molecules were somewhat larger than those generated previously from the high-density mucin from the bovine lavage samples, suggesting that they differ in length [17]. The radiolabelled molecules thus appear to represent a unique monomeric mucin species rather than a subpopulation of unpolymerized subunits from the oligomeric ones. The radiolabelled molecules were found in the secreted gel phase, but it is not known whether they are gel-forming or whether they are associated with the large gel-forming mucins. A radiolabelled mucin-like molecule similar to that identified here was present in secretions from cat [24,25] and canine trachea [26]. In cat trachea *in vivo*, a high-density component was present after radiolabelling with [^3H]glucose for 3 h. The molecules were not disulphide-bond-linked oligomers, were partially degraded by keratanase and endo- β -galactosidase, contained O-linked glycans and had a monosaccharide composition in keeping with that for mucins. Thus a similar apparently monomeric mucin has been identified in bovine, canine and feline airways, but whether such a mucin is produced in human airways is not yet known.

The radiolabelled mucins are similar in size to reduced subunits from the oligomeric species and would therefore be eluted in the void volume of Sepharose CL-4B, Sepharose CL-6B and Sephacryl S-500 columns. Many studies have been carried out in which short periods of radiolabelling have been used and the output of mucins subsequently estimated from the release of radioactivity in high- M_r material (see, for example, [27–29]). In some cases samples have been chromatographed after digestion with chondroitin ABC lyase and heparan sulphate lyase and the amount of material in the excluded fractions taken as a measure of mucus-forming mucins. This study clearly shows that such an approach may give misleading results, since most of the [^{35}S]sulphate is incorporated into large macromolecules which are not representative of the oligomeric mucins isolated from the mucus gel.

In conclusion, we have shown, in organ cultures of bovine trachea, that radiolabelled precursors are poorly incorporated into the large oligomeric gel-forming species which are released into the medium, whereas a novel monomeric mucin-like mol-

ecule is heavily labelled. It is thus unlikely that data obtained in studies where the release of radiolabelled macromolecules is used as a marker for mucin secretion are applicable to the large, oligomeric gel-forming mucin species and care should be taken in extrapolating such data to mucus secretion.

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