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ARTICLE

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Hydrodynamic properties of mucins secreted by primary cultures of Guinea-pig tracheal epithelial cells: determination of diffusion coefficients by analytical ultracentrifugation and kinetic analysis of mucus gel hydration and dissolution

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Abstract We have used two different approaches to determine hydrodynamic parameters for mucins secreted by guinea-pig tracheal epithelial cells in primary culture. Cells were cultured under conditions that promote mucous cell differentiation. Secreted mucins were isolated as the excluded fraction from a Sepharose CL-4B gel filtration column run under strongly dissociating conditions. Biochemical analysis confirmed the identity of the high molecular weight material as mucins. Analytical ultracentrifugation was used to study the physical properties of the purified mucins. The weight average molecular mass (M_w) for three different preparations ranged from 3.3×10^6 to 4.7×10^6 g/mol (corresponding to an average structure of 1–2 subunits), and the sedimentation coefficient from 25.5 to 35 S. Diffusion coefficients ranging from 4.5×10^{-8} to 6.4×10^{-8} cm^2/s were calculated using the Svedberg equation. A polydispersity index (M_z/M_w) of ~1.4 was obtained. Diffusivity values were also determined by image analysis of mucin granule exocytosis captured by videomicroscopy. The time course of hydration and dissolution of mucin was measured and a relationship is presented which models both phases, each with first order kinetics, in terms of a maximum radius and rate constants for hydration and dissolution. A median diffusivity value of 8.05×10^{-8} cm^2/s (inter-quartile range = 1.11×10^{-7} to 6.08×10^{-8} cm^2/sec) was determined for the hydration phase. For the dissolution phase, a median diffusivity value of 6.98×10^{-9} cm^2/s (inter-quartile range = 1.47×10^{-8} to 3.25×10^{-9} cm^2/sec) was determined. These values were compared with the macromolecular diffusion coefficients ($D_{20,w}$) obtained by analytical ultracentrifugation. When differences in temperature and viscosity were taken into account, the resulting $D_{37,g}$ was within the range of diffusivity values for dissolution. Our findings show that the physicochemical properties of mucins secreted by cultured guinea-pig tracheal epithelial cells are similar to those of mucins of the single or double subunit type purified from respiratory mucus or sputum. These data also suggest that measurement of the diffusivity of dissolution may be a useful means to estimate the diffusion coefficient of mucins in mucus gel at the time of exocytosis from a secretory cell.

Key words Mucin · Mucus gel kinetics · Cell culture · Analytical ultracentrifugation

Abbreviations *GPTE* Guinea-pig tracheal epithelial cells · *IQR* Inter quartile range · *CF* Cystic fibrosis · R_{max} Maximum radius of gel secretion · k_h Rate constant of hydration · k_d Rate constant of dissolution · D Diffusivity · D_h Diffusivity of hydration · D_d Diffusivity of dissolution

Introduction

Mucins are large glycoproteins with molecular weights ranging from 1 to 40 million Da. They consist of a polypeptide backbone to which oligosaccharide side chains are attached via *O*-glycosidic linkages to serine or threonine residues. They have a linear, disulphide linked subunit structure with one subunit corresponding to a molecular weight (weight average, M_w) of 2–3 million (Harding 1989; Sheehan and Carlstedt 1989; Sheehan and Carlstedt 1984a). Mucins are the major macromolecular component of mucus gels secreted by epithelia and are largely responsible for the rheological behaviour of mucus.

Much of the information on the physical and chemical properties of mucins has come from studies of material purified from animal epithelial secretions and from human sputum. With the large quantities of sample available from these sources, techniques such as laser light scattering and analytical ultracentrifugation (Harding 1989; Sheehan and Carlstedt 1989) have been used to measure the diffusion coefficient, sedimentation coefficient, molecular weight,

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size distribution and radius of gyration of mucin macromolecules. Mucins from a single source exhibit polydispersity in their hydrodynamic properties. Moreover, similar ranges of hydrodynamic parameters are seen for mucins from different sources or even different species (Harding 1989).

The biosynthesis and secretion of mucins have been extensively studied in vitro using airway epithelial cells cultured under conditions that promote a mucous secretory phenotype (e. g. Kim and Brody 1989; Emery et al. 1995). Biochemical analyses have confirmed that mucins are expressed and secreted by such cultures (Kim et al. 1985; Adler et al. 1990). However, there are, to our knowledge, no studies reported on the measurement of hydrodynamic properties of mucins obtained from cultured airway epithelial cells.

Cultured airway epithelial cells have also been used to study mucus gel expansion and hydration following exocytosis (Verdugo et al. 1993; Verdugo 1984). On the surface of mucous secretory cells, protrusions are observed which expand rapidly and within 5 to 20 s form globules of hydrated mucus gel of up to 25 μm diameter (Verdugo 1984).

In the present study, we have purified high molecular weight glycoproteins secreted by primary cultures of guinea-pig tracheal epithelial (GPTE) cells and confirmed, biochemically, their identity as mucins. Analytical ultracentrifugation was used to obtain values of molecular weight and sedimentation coefficient, enabling diffusion coefficients and polydispersity indices to be calculated. The data indicate that mucins secreted by cultured GPTE cells have molecular weights and polydispersities typical of mucins from other sources.

Analysis of mucus gel hydration from secretory cells present in primary cultures of GPTE cells enabled us to measure the kinetics of the hydration of mucus gel and calculate values for the diffusivity of the gel network expansion. Mucus gels formed in cell culture gradually dissolve in the excess of cell culture fluid. This phenomenon allowed us to extend the kinetic analysis to determine diffusivity values for the dissolution of mucus gel networks.

After correction for differences in temperature and viscosity, the value for the macromolecular diffusion coefficient measured by hydrodynamic analysis of mucins in dilute solution was similar to the value determined for the diffusivity of dissolution of mucus gel. By contrast, the diffusivity determined for the hydration phase was an order of magnitude greater than the hydrodynamic value. We suggest that the diffusivity of dissolution provides a means to estimate the diffusion coefficient of mucins in mucus gel at the point of exocytosis from a secretory cell.

Materials and methods

Materials

Ham's F12 nutrient medium (Ham 1965), L-glutamine and penicillin/streptomycin were obtained from Life Technologies, Paisley, U.K. Vitrogen 100 was purchased from Imperial Laboratories, Andover, Hants, U. K. D-[6-^{3}H]-Glucosamine hydrochloride (22 – 28 Ci/mmol) was supplied by Amersham International plc, Amersham, Bucks., U.K., Sepharose CL-4B was purchased from Pharmacia Ltd., Milton Keynes, U.K. All other reagents were from Sigma, Poole, U.K.

Cell culture

GPTE cells were isolated from male Dunkin-Hartley guinea-pigs (300 – 500 g) by protease digestion (Wu 1986). Primary cultures were initiated by seeding cells at a density of 1×10^4 cells/cm^2 on to Falcon Primaria® 6-well plates (35 mm diameter wells) coated with 0.7 ml of 0.25% (w/v) collagen gel per 10 cm^2 (Lee et al. 1984). Twelve 6-well plates were seeded with cells isolated from 5 guinea-pig tracheae. Cells cultured for video microscopy were seeded on collagen gel-coated 35 mm Primaria® dishes or glass Lab-Tek™ Tissue Culture Chamber Slides™ (Nunc products, Life Technologies Ltd, Paisley, U.K.).

The cells were cultured using 1.5 ml/well of serum-free hormone-supplemented medium (Wu et al. 1985). Cells were cultured at 37 °C in a humidified incubator in an atmosphere of 5% CO_2, 95%air. Culture media were changed every third or fourth day. For glycoconjugate preparation, secreted products were radiolabelled from day five with D-[6-^{3}H]-glucosamine hydrochloride (4 μCi/ml). Conditioned media were collected from day 8 to either day 28 or day 35 in culture, centrifuged (3000 *g* for 5 min) to remove cell debris, and stored at –20 °C.

Preparation of high molecular weight glycoconjugates

Conditioned media from each preparation of cells (12×6-well plates) were pooled (total volume approx. 1.0 l) and the following protease inhibitors were added: PMSF (0.1 mM), N-ethylmaleimide (5 mM) and EDTA (5 mM). The media were concentrated (final volume ~50 ml) under N_2 (45 psi) using a YM10 Diaflo® membrane (molar mass exclusion limit 10 kDa) in an Amicon® pressure cell. Concentrated media were extensively dialysed (6×2 l) at 4 °C against 10 mM NH_4HCO_3 containing the same concentrations of protease inhibitors as above. Concentrated material was treated successively with chondroitinase ABC (0.2 units/ml at 37 °C for 18 h in 0.1 M Tris/acetate, pH 7.3), hyaluronidase (type IV-S, 2 units/ml at 37 °C for 24 h, in 0.1 M sodium acetate, pH 6.0) and DNase I (20 units/ml at 25 °C for 18 h, in 0.15 M NaCl, 5 mM $MgSO_4$, 75 mM KH_2PO_4, pH 7.2) after dialysis against the appropriate buffer. Samples (100 μl) were examined for digestion by gel filtration on a TSK G4000SW column (0.75×30 cm; see below). After final enzymatic treatment the material was freeze-dried and stored at –20 °C.

Freeze-dried material was reconstituted in 10 ml of 6 M guanidine hydrochloride (GuHCl) containing 10 mM NaH_2PO_4 (pH 6.5) and fractionated on a Sepharose

CL-4B gel filtration column (2 cm diameter×50 cm, flow rate 10 ml/h). Radioactivity in the collected fractions (2 ml) was assayed using liquid scintillation counting. Void volume fractions were pooled and dialysed at 4 °C against 10 mM NH_4HCO_3; the remaining fractions were discarded. The dialysed fractions were concentrated (final volume 1 to 2 ml) under N_2 pressure (45 psi) using a YM10 Diaflo® membrane. An aliquot of each preparation was freeze-dried to obtain a dry weight measurement. The preparations were dialysed against phosphate chloride buffer (33 mM NaCl, 16.7 mM $NaHPO_4$, 16.7 mM NaH_2PO_4, pH 6.8, I 0.10) and stored at –20 °C prior to further processing.

Enzymic digestion and β-elimination

Samples of the purified material were digested with the following enzymes: chondroitinase ABC (0.5 units/ml) at 37 °C for 18 h in 0.1 M Tris acetate buffer (pH 7.3); heparinase type III (2 units/ml) at 43 °C for 18 h in 0.1 M sodium acetate buffer (pH 7.0) containing 1 mM $CaCl_2$; hyaluronidase type IV-S (60 units/ml) at 37 °C for 24 h in 0.1 M sodium acetate buffer (pH 6.0); bovine pancreatic trypsin (1 mg/ml) at 37 °C for 24 h in 50 mM Tris-HCl (pH 8.0) containing 50 mM $CaCl_2$; *Pseudomonas* species keratanase (10 units/ml) at 37 °C for 24 h in 0.2 M Tris-HCl buffer (pH 7.4). They were also subjected to β-elimination in 50 mM NaOH, 1 M sodium borohydride at 45 °C for 16 h (Carlson, 1968). After β-elimination, the pH of the samples was neutralized by addition of acetic acid (final concentration 5%). Control samples were incubated in buffer alone.

Digests were filtered (0.45 μm pore size) and 50 μl chromatographed on a TSK G4000SW gel filtration column (0.75×30 cm). The running buffer was 0.03 M potassium phosphate, pH 6.8, and the flow rate was 0.5 ml/min. Collected fractions (1 ml) were assayed using liquid scintillation counting.

Analytical ultracentrifugation

Sedimentation equilibrium and sedimentation velocity measurements were performed with a Beckman (Palo Alto, U.S.A.) Optima XL-A analytical ultracentrifuge. Solute distributions were recorded using absorption optics at 230 nm. Sedimentation equilibrium measurements were of the "low" or "intermediate" type (Creeth and Harding 1982) where the concentration at the meniscus remains finite. These were performed at 20 °C and 2000 rpm. Phosphate chloride buffer dialysate (pH 6.8, $I = 0.10$) was used as a reference blank. It was assumed that equilibrium had been reached when two identical scans were recorded several hours apart; this occurred after 46 h. The final solute distribution ASCII data were analysed using the MSTARA program (Harding et al. 1992).

Owing to the scarcity of material, the partial specific volume ($\bar{v}$) previously measured for pig gastric mucin (0.64 ml/g) was used in the calculations: previous studies (Sheehan and Carlstedt 1984 b; Carlstedt et al. 1983 b) give $\bar{v}$ for mucins in the range 0.62 – 0.66 ml/g. For the same reason, only one loading concentration was used (0.6 mg/ml). Non-ideality effects were assumed small at this concentration and M_w taken as $\approx M_{w,\mathrm{app}}$.

Sedimentation velocity measurements were performed at 20 °C and 35,000 rpm, with dialysis buffer as the reference blank. Consecutive scans were recorded at 20 min intervals. Sedimentation coefficient values were determined by measuring the rate of movement of the boundary per unit centrifugal field. The values obtained were normalized to standard conditions of solvent density and viscosity (that of water at 20 °C) to give $s_{20,w}$ (van Holde 1985). Again, owing to scarcity of material, a single low concentration was used for each analysis (0.6 mg/ml). As with sedimentation equilibrium, concentration effects were assumed to be negligible at low concentration.

Light microscope observation and recording of mucus gel secretion

Videomicroscopy was performed using cells that had been cultured for at least 15 days. Mucus gel secretion was observed using an Olympus IMT-2 inverted microscope at 600× magnification under phase contrast or Normaski optics using objective lenses with a long depth of field. A heated stage was used to maintain temperature at 37 °C. The pH was maintained at 7.4 throughout the observation period by addition of HEPES buffer (final concentration 20 mM) to the culture medium. Cells secreting globules of mucus gel were recorded via a video camera, monitor and S-VHS video recorder.

Mucus gel secretion was observed as the appearance on the cell surface of semicircular protrusions. These expanded rapidly, reaching a maximum size in 5 – 15 s. Preliminary studies showed that after this initial expansion phase ("hydration" phase) the area of the mucin secretions began to decrease as they dissipated into the surrounding culture medium ("dissolution" phase). The dissolution phase occurred at a slower rate than the hydration phase, and the secretions took 1 – 2 min to disappear completely. We studied both phases.

Data collection

A total of 172 individual secretory events were collected for analysis, from five different sets of cultures.

Three cultures were examined to assess the effect of time in culture on secretion kinetics. Cells were video-recorded on days 15, 21, 25 and 29 in culture. Secretory events from six cells were recorded from each culture per day. One secretory event was selected from each video-recorded cell (total of 72 secretions).

Two cultures were analysed to evaluate differences between individual cells. Five cells were video-recorded

after 15 or 16 days in culture. Twenty secretory events were chosen, at random, 5 or 6 minutes apart, from each of the five cells recorded (total of 100 secretions).

Image analysis

In order to study kinetics, individual secretory events were captured from the video tape as a series of 31 frames over 60 s. The area of the mucus gel in each captured frame was measured using semi-automated computer software (M. E. Electronics Ltd., Reading, U.K.), calibrated using the image of a 10 μm graticule. The first image in each series was loaded into the image analysis software and the outline of the cell (the baseline) was drawn to the image using a touch screen and stylus. Each image in the series was then viewed consecutively and the cell outline adjusted as necessary. The image series was reexamined, with the baseline blacked out by the computer, and the outline of the mucus gel globule was drawn on each image. The area of the mucus globule in each image was calculated.

Time and area data were recorded for each image in a series. Each image was analysed 3 times to reduce drawing errors, and the averaged data were used in further analysis.

Data analysis

To study both phases seen during a secretory event, we extended the model of mucus gel swelling proposed by Verdugo (1984). The Verdugo equation models mucus gel swelling as a first order kinetic relationship between radius and time. We extended Verdugo's model, to account for the dissolution of mucus gel. This is based on a model for dissolution of solids where, for an approximately hemispherical gel the radius decreases with first order kinetics with respect to time. The resulting expression that we used to model mucus gel hydration and dissolution is:

$$r = R_{\max}\,(e^{-k_d t} - e^{-k_h t}) \qquad (1)$$

Where r is the radius (μm) at time t (s); $R_{\max}$ is the maximum radius (μm); k_h is the rate constant of mucus gel hydration (s^{-1}) and k_d is the rate constant of mucus gel dissolution (s^{-1}).

For each image the radius of the mucus gel globule was calculated from its area, by assuming that the secretion was hemispherical. The change in gel radius over time was analysed by fitting Eq. (1) to the data using the Marquart algorithm (Ultrafit nonlinear curve fitting package, Biosoft, Cambridge, U.K.). Best fit values were obtained for $R_{\max}$, k_h and k_d. Goodness of fit was assessed by χ^2 (Armitage, 1971).

Calculation of diffusivity values

Frontal diffusivity values were calculted using the equation:

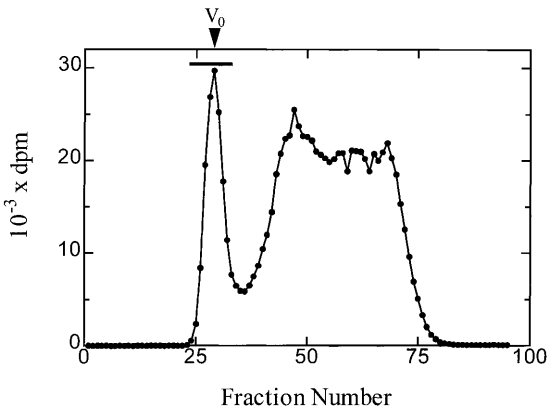

Fig. 1 Gel filtration elution profile of GPTE cell conditioned media on Sepharose CL-4B gel filtration column. Media from GPTE cells, cultured as described in Methods, were collected over 5 weeks and pooled. After concentrating, pretreatment with chondroitinase ABC and hyaluronidase (see Methods) and extensive dialysis against 10 mM NH_4HCO_3, the sample was freeze dried, reconstituted and chromatographed in 6 M GuHCl on a Sepharose CL-4B gel filtration column (2 cm diameter×50 cm, flow rate 10 ml/h). Radioactivity in the fractions (2 ml) was assayed by liquid scintillation counting. The fractions which were pooled and subjected to further processing and analysis are indicated by the *bar*

$$D = (R_{\max})^2\,K \qquad (2)$$

based on Verdugo (1984) and Tanaka and Fillmore (1979) where K can be k_h or k_d. Therefore, two values were calculated for each secretion: diffusivity of hydration (D_h) and diffusivity of dissolution (D_d).

Diffusivity values were analysed by one-way and two-way analysis of variance to detect differences between cell culture preparations, age of culture and individual cells. The components of variance were also calculated to determine the sources of variation.

Results and discussion

Biochemical characterisation of high molecular weight glycoconjugates secreted by GPTE cells

Numerous studies have shown that protease-dissociated airway epithelial cells adopt a mucous secretory cell phenotype when cultured on collagen gel in serum-free, hormone-supplemented medium (e.g. Wu et al. 1991; Wu et al. 1990; Adler et al. 1990). In the present study we cultured GPTE cells under similar conditions and investigated the hydrodynamic properties of mucins secreted into the medium by the cells. High molecular weight radiolabelled mucins were isolated from the cell-conditioned medium by Sepharose CL-4B gel filtration chromatography (Fig. 1). In order to eliminate the possibility that the void volume material was contaminated with DNA or glycoconjugates other than mucins, the cell conditioned medium was pretreated with chondroitinase ABC, hyaluronidase and DNaseI before chromatography.

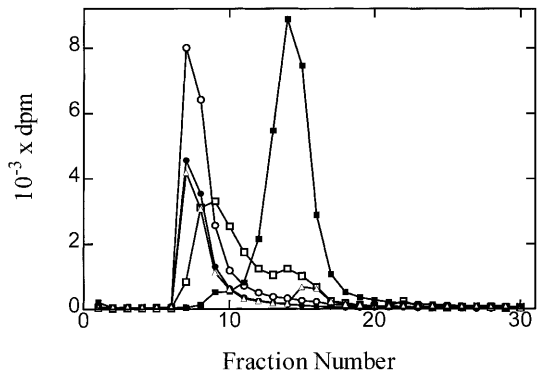

Fig. 2 Enzymatic digestion and β-elimination of mucin samples from GPTE cells. Gel filtration on TSK G4000SW column of purified mucin sample after treatment with the following enzymes; ● untreated; △ keratanase (10 units/ml, 37 °C for 24 h in 0.2 M Tris-HCl, pH 7.4) and □ bovine pancreatic trypsin (1 mg/ml, 37 °C for 24 h in 50 mM Tris-HCl, pH 8.0) and β-elimination (■) of samples of 50 mM NaOH and 1 M sodium borohydride for 16 h at 45 °C; samples were neutralised with acetic acid (final concentration 5%) after incubation (○ = untreated β-elimination sample). Following digestion, aliquots were filtered (0.45 µm pore size) and 50 µl were chromatographed. Running buffer was 0.03 M potassium phosphate (pH 6.8) and the flow rate was 0.5 ml/min. Collected fractions (1 ml) were assayed for radioactivity by scintillation counting

Table 1 Summary of physical biochemical analysis of GPTE mucin samples

Sample	$10^{-6}\times M_w$ (g/mol) [a]	$10^{-6}\times$range of M_w(r) values	$s_{20,w}$ (S)	$10^{8}\times D_{20,w}$ (cm^2/s) [b]
1	4.7 ± 0.3	1.0 – 6.0	28.5 ± 2.4	4.5 ± 0.5
2	3.3 ± 0.2	0.5 – 6.0	28.5 ± 3.0	6.4 ± 1.0
3	4.5 ± 0.4	1.0 – 6.5	35.0 ± 7.0	5.8 ± 1.2

[a] From extrapolation of $M^*(r)$ function to the cell base (as in Fig. 3 b)
[b] $D_{20,w}$ calculated using the Svedberg equation (Svedberg and Pedersen, 1940) and the results for M_w and $s_{20,w}$

Approximately 20% of the total radiolabelled material eluted in the void volume of the column. The void volume fractions were pooled for further processing, the other fractions were not analysed further. Dry weight measurements revealed that in three separate experiments 1.7, 0.8 and 0.4 mg of high molecular mass glycoconjugates were purified from about 1.0 l of conditioned medium. The largest amount of material was obtained from the cells which had been cultured for the longest time but the differences in the yield most likely reflect experimental variation (e.g. tissue from different animals, different lots of cell culture components).

To confirm that the material isolated in the void volume fractions was authentic mucin, samples were treated with trypsin or various glycosidases and analysed by gel filtration (Fig. 2). The untreated samples contained only a single void volume peak. Chondroitinase ABC and hyaluronidase did not affect the samples as expected since the original cell-conditioned medium had already been digested with these enzymes. Heparinase had little effect on the samples (~4% digested; data not shown), although keratanase digested ~10% of the void volume material (Fig. 2). The results show that glycosaminoglycans were not major components of the purified samples. The keratanase result may be anticipated since some glycoproteins act as substrates for this enzyme (Fukuda and Matsumura 1976). A similar pattern of resistance to proteoglycan-digesting enzymes has been previously reported in high molecular weight glycoconjugates secreted from cultured GPTE cells (Adler et al. 1990).

A large decrease in size was observed after tryptic digestion (Fig. 2). This is consistent with a model of mucin structure in which regions of naked protein are present between heavily glycosylated domains (Harding 1989; Sheehan and Carlstedt 1989). The "naked" peptide regions would be susceptible to proteinase attack generating lower molecular weight oligosaccharide-rich glycopeptides. After alkaline borohydride treatment more than 80% of the radiolabel eluted in the included volume of the column (Fig. 2); this would be expected as the removal of *O*-linked carbohydrates and the breakdown of the peptide core will reduce the size of mucin molecules. A similar pattern of digestion and β-elimination was observed for all three samples.

These biochemical analyses provide strong evidence that the high molecular weight glycoconjugates purified from GPTE cell-conditioned media are authentic mucins. These data are consistent with reports on the secretory products of human (Wu et al. 1990), hamster (Wu et al. 1991) and guinea-pig airway epithelial cells (Adler et al. 1990).

Analytical ultracentrifugation of purified mucins

Sedimentation velocity. As a result of the low concentration used, insufficient signal was obtained at 280 nm. However, a measurable signal, albeit noisy, could be obtained in the far u.v., at 230 nm where detection of the peptide bond was possible. Despite the noise, the broadness of the boundary clearly indicated considerable polydispersity in the samples. An estimate for the sedimentation coefficient $s_{20,w}$ was possible and the values obtained for the three preparations (Table 1) were between those obtained for mucin "subunits" (~19 S) and those for cervical (40 S) and respiratory (50 S) mucins (Sheehan and Carlstedt 1984 a).

Sedimentation equilibrium. Plots of ln A_{230} against radial displacement squared (Fig. 3 a) had strong upwards curvature indicating heterogeneity; this is typical for bronchial mucins (Harding, 1984; Creeth, 1980). Extrapolation of the $M^*(r)$ function (Fig. 3 b) to the cell base, i.e. $\xi(r) = 1$ gave the whole cell (i.e. "whole distribution") M_w (Creeth and Harding, 1982) for the 3 samples analysed (Table 1). The molecular weights obtained are, like the sedimentation coefficient, between values obtained for "mucin subunits" ($M_w \approx 2-3\times10^6$) and respiratory mucins ($M_w \approx 5-18\times10^6$) (Sheehan and Carlstedt 1989; Sheehan and Carlstedt 1984 a; Harding 1984; Creeth and

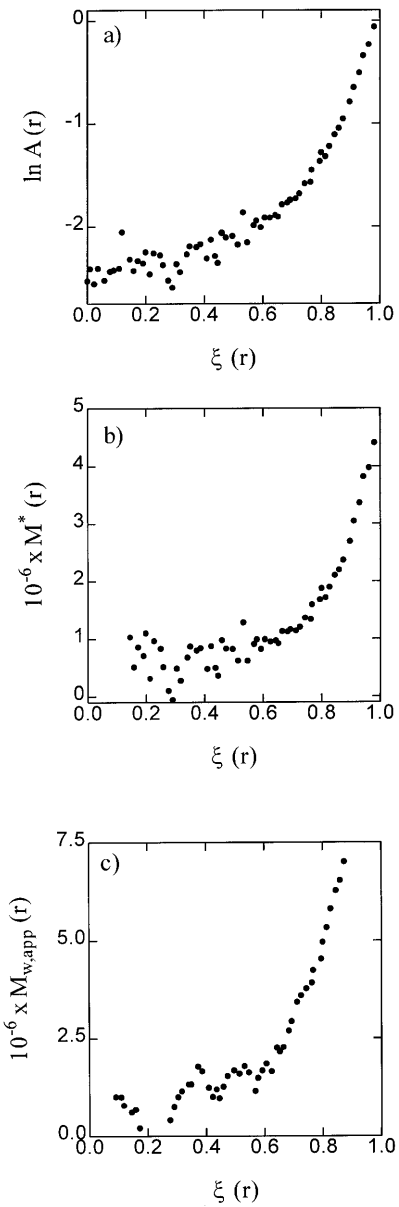

Fig. 3 a–c Sedimentation equilibrium of GPTE mucin. **a** Plot of log concentration (absorbance at 230 nm) versus the normalised radial displacement squared parameter ξ from a low speed sedimentation equilibrium experiment on GPTE culture mucin sample 1, prepared as described in Methods. $\xi = 0$ corresponds to the air/solution meniscus and $\xi = 1$ the cell base. Rotor speed = 2000 rev/min, temp = 20 °C; ultracentrifuge loading concentration was 0.6 mg/ml. **b** Corresponding "M^*" extraction of the whole cell distribution weight average molecular weight $M_{w,app}$ for GPTE cell culture mucin sample. This is obtained from extrapolation of the M^* function to the cell base ($\xi = 1$). **c** Corresponding plot of (apparent) point average molecular weight ($M_{w,app}(r)$) versus ξ

Cooper 1984).

Translational diffusion coefficient. The diffusion coefficient was estimated, from the values obtained for $s_{20,w}$ and M_w, using the classical Svedberg equation (Svedberg and Pedersen 1940). The results for the three preparations are shown in Table 1. The data are similar to values published previously for mucin subunits, measured directly using dynamic light scattering ($D_{20,w} = 4.7 \times 10^{-8}$ cm^2/s) (Sheehan and Carlstedt 1984 a). Although we have determined $D_{20,w}$ in an indirect way, we have avoided the classical problems associated with dynamic light scattering measurements on these materials (Harding 1989; Harper et al. 1985), largely surrounding sample clarity.

The diffusion coefficients, along with the sedimentation coefficients and molecular weights from which they were calculated, would appear to suggest that mucins secreted by cultured GPTE cells are of the single or double subunit type (Harding 1989; Sheehan and Carlstedt 1989; Sheehan and Carlstedt 1984).

Table 2 Polydispersity of GPTE cell mucin samples

Sample	$10^{-6} \times M_z$ [a]	Polydispersity index [b] (M_z/M_w)	$10^{-6} \times$SD of weight distribution
1	6.4 ± 1.5	1.4	2.8
2	6.8 ± 1.5	2.0	3.4
3	7.0 ± 1.5	1.6	3.3

[a] Calculated from results obtained in the sedimentation equilibrium experiments (Creeth and Pain 1967)
[b] Calculated from M_z and the M_w estimated by extrapolation of the M^* function to the cell base (as in Fig. 3)

Polydispersity. We have analysed the data to make a more quantitative comment on the polydispersity evident from the sedimentation velocity and equilibrium data records (Fig. 3 c). For this we used the (whole distribution) *z*-average molecular mass, M_z: this enabled us to represent the polydispersity as a "polydispersity index", M_z/M_w (Method I of Harding 1994). To obtain M_z, the relationship described by Creeth and Pain (1967) was used. The point mass average molecular masses at the meniscus and base ($M_w(a)$ and $M_w(b)$ respectively) were estimated from Fig. 3 c and their respective concentrations ($C(a)$ and $C(b)$ respectively) from Fig. 3 a. We obtained a value for M_z of ~6.4×10^6 g/mol. If an extrapolated value of $(4.7 \pm 0.3) \times 10^6$ (from Fig. 3 b) is used for M_w, then a polydispersity index (M_z/M_w) of ~1.4 is obtained, confirming the high polydispersity of the mucin produced by these cultures. This value is actually likely to be an underestimate because we have assumed non-ideality effects as negligible. In addition, using the Herdan relations (Creeth and Pain 1967; Herdan 1949) and assuming a log-normal distribution (Creeth 1980), a weight average standard deviation of ~2.8×10^6 g/mol is obtained. This result, and the corresponding results for the two other preparations, are shown in Table 2.

Mucus gel secretion from cultured GPTE cells

GPTE cells were cultured on Primaria® dishes or glass Labtek™ slides as described. After seeding, the cells proliferated and formed confluent sheets in the centre of the dishes with smaller colonies in spaces at the periphery. Cil-

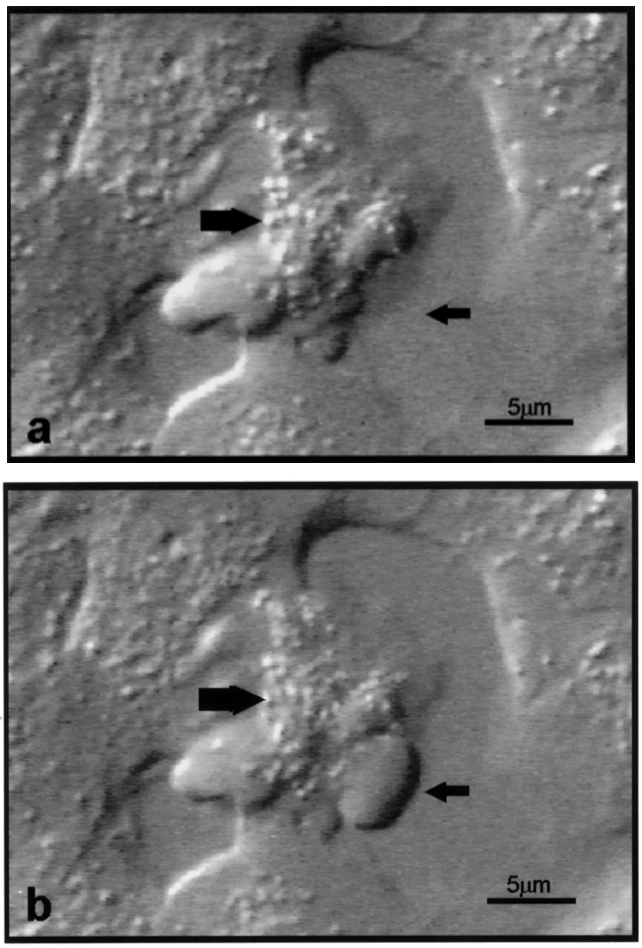

Fig. 4 a, b Secretory cells can be seen in confluent areas and also singly on the edge of colonies. Isolated GPTE cells were cultured in serum-free, hormone supplemented medium on glass Lab-Tek™ chambers coated with collagen gel (0.25% w/v; 0.7 ml/10 cm^2). Cells were observed using an Olympus IMT-2 inverted microscope. Photographs a and b show cells under Normaski optics (mag×600) after 11 d in culture. A secretory cell (*large arrow*) can be seen in the centre of the field of view and a secretion (*small arrow*) can be seen on its edge. Photographs **a** and **b** were taken 10 s apart

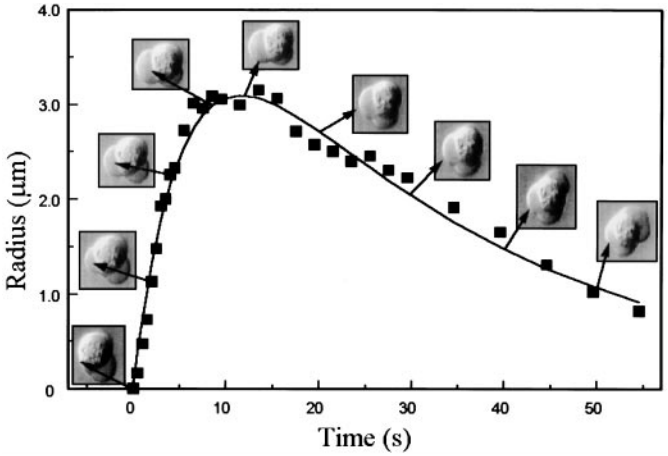

Fig. 5 Time course of mucus gel secretion from a cultured GPTE cell. Plot of mucus gel radius over a 1 min period, and showing the corresponding measured images. The curve (*solid line*) was generated by fitting equation 1 to the data (■) (see Methods). R_{max} = 3.74 μm, $k_h = 0.372\ s^{-1}$, $k_d = 0.013\ s^{-1}$ and $\chi^2 = 0.11$. GPTE cells were cultured, as described in Methods, for 15 d prior to video recording. The cell was viewed under Normaski optics at 600× magnification. The cell cytoplasm is granular in appearance and the secretion appears as a clear gel protruding from the cell perimeter (*arrows*)

iated cells were seen throughout the culture period. Secretory cells were seen from day 7 onwards in both the confluent areas (Figs. 4 a and 4 b) and singly in spaces on the edge of colonies (Fig. 5).

A time course of mucus gel secretion is illustrated in Fig. 5. After swelling rapidly post-exocytosis, the mucus gel globules gradually dissolve in the excess fluid surrounding the cell. This is in contrast to the in vivo situation where the mucus granules swell into an environment, in the airways, where fluid volume is limited.

Mucus gel globules that achieved a regular hemispherical appearance were selected for analysis of secretion kinetics. Only secretions from single cells were used, since secretions from secretory cells in confluent areas rarely achieved a regular hemispherical shape. Also, only secretions that were well separated from other secretions from the same cell were selected for analysis, because overlapping secretions were difficult to analyse since the edges of the individual secretions merged and were not clearly visible.

Data analysis

A plot of data from a single secretion is shown in Fig. 5. The maximum radius achieved by the 172 secretions ranged from 2.4 to 7.5 μm. Equation (1) was fitted to the data generated from each secretion to obtain values for R_{max}, k_h and k_d. Goodness of fit was assessed by the χ^2 test which indicated that 97% of the fits were acceptable at the 5% level (i.e. $\chi^2 < 41.3$). However, ~2% of the good χ^2 fits were obviously poor when the plots were examined by eye. These data were also excluded from the final statistical analysis, leaving a total of 162 secretions. Two other curve fitting routines were employed. Both of these minimised the sums of squares of residuals. Neither of these routines gave R_{max}, k_h or k_d results that were significantly different to those given by Ultrafit.

Equation (1) was also expanded to include variables for separate maxima for the hydration and dissolution phases, or a variable for time taken to reach maximum radius. No significant improvements in the fits were produced by increasing the number of variables (S. Dodd, unpublished data). Therefore, Eq. (1) was considered to be the simplest expression that gave an acceptable fit to the data, and was used in all the analyses.

Diffusivity of secreted mucin gels

Frequency distributions of Log diffusivity (D) values calculated from Eq. (2), for 162 secretions, are shown in Figs. 6 a (D_h) and 6 b (D_d). Two-way analysis of variance indicated that there was no significant difference between

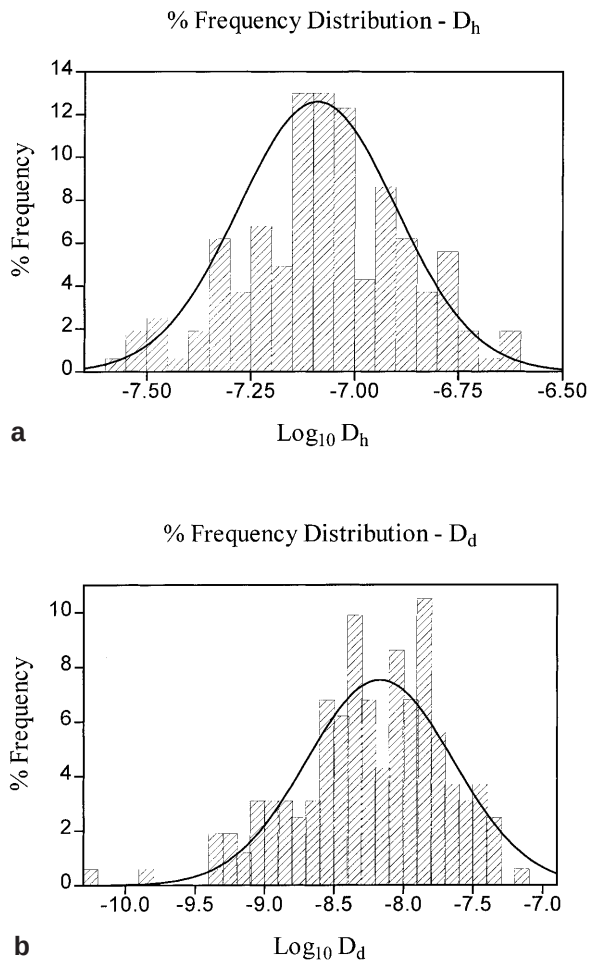

Fig. 6 a, b Frequency distributions of Log_{10} diffusivity values. **a** % Frequency distribution of Log_{10} D_h calculated from equation 2. R_{max} and k_h were obtained by fitting Eq. (1) to data from the 162 secretions analysed. The *solid line* represents the Gaussian distribution calculated from the mean and SD of the Log_{10} D_h data. **b** % Frequency distribution of Log_{10} D_d calculated from equation 2. R_{max} and k_d were obtained by fitting Eq. (1) to data from the 162 secretions analysed. The *solid line* represents the Gaussian distribution calculated from the mean and SD of the Log_{10} D_d data

cell culture and time in culture for the Log D_h values ($p > 0.05$). There was a significant difference in Log D_h between the five single cells when analysed by one-way analysis of variance ($p \leq 0.0001$).

In contrast, when the Log D_d values were analysed by two-way analysis of variance, there were significant differences for time in culture ($p = 0.002$) and for individual cultures ($p = 0.003$). The general trend, in the three cultures used, was an increase in Log D_d from day 15 to day 29 in culture. There was also a significant difference in Log D_d between the individual cells when analysed by one-way analysis of variance ($p \leq 0.0001$) over the same period.

During the experiments to assess the effect of time in culture, one secretory event was selected from each of 72 cells. Data from this part of the study were more variable than the data from five single cells. The components of variance were calculated for the data from the five single cells (Armitage 1971). Of the total variance, 63.8% and 57.9% was attributable to differences between cells, for D_h and D_d respectively. This test showed that the variance was mainly due to differences between cells rather than within cells. Therefore, evidence from the statistical analysis suggests that secretions from the same cell have similar secretion kinetics. Secretions from separate cells, however, have different kinetics. These results agree with previous studies (Aitken and Verdugo 1989).

The variability in the data may be a consequence of cells synthesising mucin molecules of different size. Diffusivity of a polymer is known to decrease relative to the square of its length (Edwards 1986). The observed decrease in D_d during time in culture may be a result of the synthesis of mucins with an increased number of subunits. Alternatively different MUC genes encoding larger core polypeptides may be expressed at different times in culture. MUC5AC and MUC5B have been identified as two secretory mucin gene products in human respiratory tract mucus (Hovenberg et al. 1996; Thornton et al. 1997), but there is no information available on the expression of different MUC genes in guinea-pig airway cells. Variability in diffusivity may also be related to variation in glycosylation and in ionic properties resulting from differential expression of glycosyl transferases (Emery et al. 1997). It is reasonable to conclude that the variability in the values for D_h and D_d reflect the high polydispersity of molecular size of guinea pig mucins measured in free solution.

The median diffusivity values calculated, from the 162 secretions analysed, were: $D_h = 8.05\times10^{-8}$ $cm^2 \cdot s^{-1}$ ($IQR = 1.11\times10^{-7}$ to 6.08×10^{-8}) and $D_d = 6.98\times10^{-9}$ $cm^2 \cdot s^{-1}$ ($IQR = 1.47\times10^{-8}$ to 3.25×10^{-9}). The median D_h for guinea pig secretions is lower than D_h results previously reported for secretions from rabbit tracheal epithelial organ cultures analysed by a similar method (3×10^{-6} to 5×10^{-5} $cm^2 \cdot s^{-1}$; Verdugo et al. 1987; Aitken and Verdugo 1989; Verdugo, 1984). However, D_h values obtained using goblet cells from mucosal explants from CF patients (3×10^{-8} $cm^2 \cdot s^{-1}$) and normal subjects (7.8×10^{-7} $cm^2 \cdot s^{-1}$; Verdugo et al. 1993) are similar to those calculated in this study.

Comparison of mucin diffusion coefficient measured by solution hydrodynamic methods and diffusivity values measured in secretion of mucus gel

The macromolecular diffusion coefficient, $D_{20,w}$, can be compared with the frontal diffusivity values, D_h and D_d. However, to make a comparison, it is first necessary to estimate $D_{37,g}$ by adjusting $D_{20,w}$ to take into account differences in temperature and viscosity. $D_{37,g}$ represents the diffusion coefficient at 37 °C of mucin macromolecules in a gel of equivalent viscosity to the secreted mucus gel. Direct comparison can be made between $D_{37,g}$ and the diffusivity values, D_h and D_d.

$D_{37,g}$ can be estimated by using the following relationship (see, e.g. van Holde 1985):

$$D_{37,g} = D_{20,w} \cdot \left[\frac{310.15}{293.15} \cdot \frac{\eta_{20,w}}{\eta_{37,g}} \right] \quad (3)$$

The mean $D_{20,w}$ from Table 1 is 5.6×10^{-8} cm$^2 \cdot$ s^{-1}. The viscosity of water at 20 °C, $\eta_{20,w}$, is 1.002 centipoise (cP). An estimate of $\eta_{37,g}$, the viscosity at 37 °C of the secreted mucus gel, can be made as follows using literature data:

1. The reduced viscosity (η_{red}) of the mucus gel is estimated at ~1160 ml/g by using the following equation (Huggins, 1942):

$$\eta_{red} \approx [\eta]\,(1 + K_\eta\,[\eta]\,c) \quad (4)$$

a) Intrinsic viscosity $[\eta]$ for a mucin of molecular weight $\sim5\times10^3$ kDa is taken to be 240 ml/g (Fogg et al. 1996). b) Huggins constant K_η (which describes the concentration dependence of η_{red}): ~0.4 (Creeth and Knight, 1967). c) The mucin concentration at the expanding edge of the secreted gel is estimated to be ~4% (0.04 g/ml (Allen et al. 1976).

2. $\eta_{37,g}$ is calculated to be ~33.2 cP using Eqs. (5) and (6):

$$\eta_r = (\eta_{red} \cdot c) + 1 \quad (5)$$

$$\eta_{37,g} = \eta_r \cdot \eta_{37,w} \quad (6)$$

where, η_r is the relative viscosity; and $\eta_{37,w}$ is the viscosity of water at 37 °C (~0.7 cP). This value for mucus gel viscosity is within the range, 10 to 1000 cP, of previously reported values (Marriott et al. 1982; Marriott 1982).

From the above $D_{37,g}$ can be estimated to be $\sim1.8\times10^{-9}$ cm$^2 \cdot$ s^{-1}. The range of possible $D_{20,w}$ values from Table 1 corresponds to a range of $D_{37,g}$ values of 1.3 to 2.4×10^{-9} cm$^2 \cdot$ s^{-1}. Notwithstanding the assumptions involved with Eq. (4) these values are still over an order of magnitude less than D_h (for the hydration process) measured by image analysis (Fig. 6 a). This supports the view that the diffusivity occurring during the hydration phase of mucus gel globule secretion is an active process, i.e. it is energetically driven. This conclusion is consistent with the results of Verdugo and colleagues. For mucus secretions from rabbit tracheal organ cultures (Aitken and Verdugo 1989; Verdugo et al. 1987; Verdugo 1984), diffusivity of hydration values were an order of magnitude greater than diffusion of human cervical and respiratory mucins measured, in solution, by laser photon correlation spectroscopy (Verdugo et al. 1983; Lee et al. 1977).

By contrast, the $D_{37,g}$ value of $\sim1.8\times10^{-9}$ cm$^2 \cdot$ s^{-1} falls within the range of the observed D_d (for the dissolution process) values (Fig. 6 b). This implies that the movement of mucins during the dissolution phase is not energetically driven. In comparing the macromolecular with the frontal diffusivities in this way we have, of course, assumed that concentration dependent non-ideality effects are comparable.

In this study we have prepared high molecular weight glycoconjugates from primary cultures of GPTE cells. These molecules were shown to possess properties typical of mucins purified from other sources when analysed by both biochemical and hydrodynamic techniques. The estimated diffusion coefficients were similar to those obtained for mucins by dynamic light scattering (Sheehan and Carlstedt 1989). Our biochemical data are consistent with a previous study that examined the biochemical properties of mucins secreted by GPTE cells in culture (Adler et al. 1990). However, to our knowledge, this is the first study of the hydrodynamic properties of mucins collected from cultured airway cells. Further studies are necessary (e.g. thiol reduction) to establish the subunit composition of these molecules. The weight average molecular weight of $4-5\times10^6$ for these samples corresponds to an average structure of one or two of the "subunits" (the entity left after thiol reduction) defined by Sheehan and Carlstedt (1989). The results do nonetheless show that these cells secrete mucins with properties comparable to those found in mucus from other species.

We have also described a technique to study and characterise the behaviour and physical properties of mucins in secreted mucus gel globules using video microscopy. Comparison of the diffusion coefficient from hydrodynamic analysis to the D_d value obtained from the kinetics study showed that the two functions are closely related. This comparison also demonstrated that measurement of D_d provides a means in an in vitro biological system to estimate the diffusion coefficient of mucins in mucus gel at the time of exocytosis from a secretory cell.

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