MUC5B is a major gel-forming, oligomeric mucin from human salivary gland, respiratory tract and endocervix: identification of glycoforms and C-terminal cleavage

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INTRODUCTION

The large gel-forming glycoproteins which are the major macro-molecular components of mucus are members of the mucin superfamily. Biochemical and physicochemical studies, as well as electron microscopy, have shown respiratory and cervical mucins to be large structures (M, 10–30 million) composed of subunits linked end-to-end by disulphide bonds [1–5]. Within the mucin apoproteins, regions rich in oligosaccharide side chains alternate with ‘naked’ domains along the protein core. The oligosaccharide-rich domains of the apoprotein are enriched in serine, threonine and proline, whereas the ‘naked’ regions contain relatively high levels of cysteine (for a review, see [6]).

At least nine mucin genes (MUC1–MUC4, MUC5AC, MUC5B and MUC6–MUC8) have been identified (for reviews, see [7,8]). Although only MUC1, MUC2 and MUC7 have been fully sequenced [9–11], large stretches of MUC5AC [12–15] and MUC5B [16], as well as the C-terminal sequences of MUC3 [17], MUC5B [18–20] and MUC6 [21], are now known. MUC5AC has been shown to encode a large gel-forming mucin produced by the goblet cells of the respiratory-tract epithelium [22,23], and the MUC2 mucin is a component of the ‘insoluble’ glycoprotein complex from human intestine [24]. The presence of MUC5B and MUC6 within the same mucin gene cluster as MUC5AC and MUC2 [25] has led to the suggestion that all these glycoproteins are large, oligomeric gel-forming mucins.

Two major mucin populations have been identified in whole saliva; a large one (MG1) and a smaller, monomeric, one (MG2) [26,27]. Both the MUC7 gene, which encodes MG2, and MUC5B are expressed in sublingual, submandibular, palate and labial glands [11,28,29]. In addition, MUC4 has been shown to be expressed at low levels in submandibular glands [30]. Human respiratory-tract secretions contain three major large, secreted mucin populations [31], one of which has been identified as MUC5AC [22,32] and shown to be a product of the epithelial goblet cells [23]. Two other mucin populations have recently been identified as MUC5B, suggesting that this mucin occurs as ‘glycoforms’, i.e. molecular variants of the same apoprotein differing in their glycosylation [33]. In cervix, the MUC2, MUC3, MUC4, MUC5AC, MUC5B and MUC6 mucin genes are expressed [34], but the genetic identity of the predominant mucins in the secretion remains unclear.

We have developed antisera against peptide sequences present in the non-glycosylated regions of the MUC5B mucin apoprotein and used them to show that MUC5B is a large oligomeric mucin present in the gel phase of chronic bronchitic sputum and saliva as well as cervical-tract secretions. Our data confirm and extend the observations that the MUC5B apoprotein may occur as glycoforms and show that these mucins may be proteolytically cleaved in the C-terminal domain.

EXPERIMENTAL

Materials

Nitrophenyl phosphate and BSA (Fraction V; pH 7.0) were purchased from Serva, 3,3′-diaminobenzidine tetrahydro-
chloride, N-ethylmaleimide (NEM) and alkaline phosphatase-conjugated anti-mouse IgG from Sigma Chemical Co., dithiotheritol (DTT) from Merck and di-isopropyl phosphofluoridate (DFP) from Fluka. Guanidinium chloride (GdmCl; practical grade) and iodoacetamide were from ICN Biochemicals. Stock solutions of GdmCl (approx. 8 M) were treated with activated charcoal and filtered through a PM10 filter (Amicon) before use. Alkaline phosphatase-conjugated swine anti-rabbit serum, the Biotin Blocking System and the StreptABComplex/HRP kit were from Dako. The F2 monoclonal antibody recognizing the antigen (DFP) from Fluka. Guanidinium chloride (GdmCl; practical grade) and iodoacetamide were from ICN Biochemicals. Stock solutions of GdmCl (approx. 8 M) were treated with activated charcoal and filtered through a PM10 filter (Amicon) before use.

Preparation of the LUM5B-2, LUM5B-3 and LUM5B-4 antisera

Synthetic peptides with sequences RNREQV GKFFKM C (LUM5B-2) and AQAPGPVLRLGQVVEC (LUM5B-3) present in the cysteine-rich domains of the super-repeats within the central exon of the MUCSB apoprotein [16] and the sequence PQGFEYKRVAGQC (LUM5B-4) from the C-terminal domain of MUC5B [18–20] were conjugated to keyhole-limpet haemocyanin and used to raise antibodies in rabbits. An initial injection of 100 µg of peptide conjugate in Freund’s complete adjuvant given intracutaneously was followed 4 weeks later by a booster injection of 100 µg of peptide conjugate in Freund’s incomplete adjuvant. Rabbits were bled 2–3 weeks later, and the antisera were named LUM5B-2, LUM5B-3 and LUM5B-4 respectively.

Isolation of salivary, respiratory and cervical mucins and mucin subunits

For the collection of whole saliva, subjects rinsed their mouths with 0.9% NaCl solution and secretions were subsequently induced by chewing Parafilm. Secretions obtained during the first 0.5 min were discarded, and samples were then collected into a container placed on ice over the following 10 min. Samples were centrifuged in a Beckman L-70 Optima centrifuge (Beckman 70.1 Ti rotor, 4 °C, 45 min, 36000 rev./min) to separate the gel and the sol phases. The gel phase was twice rinsed with PBS (0.15 M NaCl/10 mM sodium phosphate buffer, pH 7.4) containing 1 mM DFP, 5 mM EDTA and 5 mM NEM and centrifuged as described above. The gel phase was extracted using 6 M GdmCl/10 mM sodium phosphate buffer, pH 6.5, containing 5 mM EDTA and 5 mM NEM (extraction buffer) while stirring overnight at 4 °C, followed by centrifugation in a Beckman L-70 Optima centrifuge (Beckman 70.1 Ti rotor, 4 °C, 45 min, 36000 rev./min). The extraction procedure was repeated twice and the supernatants were pooled.

Sputum from a chronic-bronchitic patient was collected and treated as described previously [5]. After separation into a gel and a sol phase, the gel phase was extracted four times with extraction buffer essentially as described above and the supernatants pooled.

Pregnancy mucus plugs released from the cervix during labour were collected by specially instructed midwives and stored at −70 °C [36]. Samples were collected with informed consent, and the study (RE 1996/3663), which was carried out in accordance with the Declaration of Helsinki II, was approved by The Ethical Committee, Aarhus Amt, Denmark. A total of 12 samples were pooled and thawed in the presence of 0.1 mM DFP and stirred for 5 min before the addition of ice-cold extraction buffer. Samples were extracted three times with extraction buffer as described above and the supernatants pooled.

Mucins were isolated from the solubilized gel phases of the salivary and respiratory secretions as well as from cervical mucus using isopycnic density-gradient centrifugation in 4 M GdmCl/CsCl/10 mM sodium phosphate buffer, pH 6.5, containing 5 mM EDTA in a Beckman L-70 Optima centrifuge (Beckman 50.2 Ti rotor, 36 000 rev./min, 15 °C, 96 h, initial density 1.39 g/ml). Mucin-containing fractions were pooled (results not shown) and subjected to a second density-gradient-centrifugation step in 0.5 M GdmCl/CsCl/10 mM sodium phosphate buffer, pH 6.5, containing 5 mM EDTA and 0.01% CHAPS (initial density 1.50 g/ml). For preparation of reduced mucin subunits, samples were dialysed against 6 M GdmCl/0.1 M Tris/HCl buffer, pH 8.0, containing 5 mM EDTA, treated with 10 M DTT for 5 h at 37 °C, and alkylated by the addition of iodoacetamide (2.5 molar excess over dithiothreitol) for 15 h in the dark.

Rate-zonal centrifugation

Rate-zonal centrifugation of whole mucins and reduced subunits was performed as described by Sheehan and Carlstedt [37]. GdmCl gradients (6–8 M) were formed using a Hoeffer gradient maker connected to an LKB 2232 microperpump at a flow of 40 ml/h in 14 ml centrifuge tubes. Samples were layered on to the top of the gradients and tubes spun in a Beckman L-70 Optima centrifuge (Beckman SW41 Ti rotor, 2 h 45 min, 45 000 rev./min, 20 °C). Fractions (400 µl) were collected from the top of the tubes and analysed for carbohydrate and reactivity with the LUM5B-2 and, where appropriate, the LUM5-1 antiserum.

Gel chromatography and ion-exchange HPLC of reduced mucins

Gel chromatography was performed on a Sephacryl S-500HR column (1.6 cm × 51 cm) eluted with 4 M GdmCl, pH 7, at a flow rate of 0.1 ml/min. Fractions (1 ml) were analysed for carbohydrate and reactivity against the LUM5B-2 and LUM5B-4 antisera. Ion-exchange HPLC was performed on a Mono Q 5/5 HR column as outlined by Hovenberg et al. [22]. Samples were dialysed against 6 M urea/10 mM pipervanex/perchlorate buffer, pH 5.0, containing 0.1% CHAPS (starting buffer). The column was eluted with starting buffer for 10 min at a rate of 0.5 ml/min followed by a linear gradient of LiClO4 in starting buffer up to a final concentration of 0.4 M LiClO4, over 60 min. Fractions (0.5 ml) were collected and analysed for carbohydrate as well as reactivity with the LUM5B-2 antiserum and the F2 monoclonal antibody. In addition, fractions from the respiratory and cervical secretions were analysed for the presence of MUC5AC using the LUM5-1 antiserum [22].

ELISA

Initial investigations suggested that the epitopes recognized by the LUM5B-2 and LUM5B-3 antisera may be present within folded regions of the protein core and could be exposed by reduction and alkylation of the mucins (see below). Thus, for analysis with the LUM5B-2, LUM5B-3 and LUM5B-4 antisera, after appropriate dilution in 4 M GdmCl/10 mM sodium phosphate buffer, pH 7, samples (100 µl) of whole mucins were coated on to multi-well assay plates (Falcon) overnight, at room temperature in the presence of 1 mM DTT (10 µl of a 10 mM solution in 6 M GdmCl/1 M Tris/HCl buffer, pH 8, containing 5 mM EDTA, was added). After removal of the coating solution, alkylation was performed using 2.5 mM iodoacetamide in 6 M
MUC5B mucins from human salivary gland, airways and endocervix

Figure 1 Isopycnic density-gradient centrifugation in CsCl/0.5 M GdmCl of mucins from (a and b) the gel phase of whole saliva, (c and d) the gel phase of respiratory secretions and (e and f) cervical mucus

Material extracted in 6 M GdmCl/10 mM sodium phosphate buffer, pH 6.5, containing 5 mM EDTA, 5 mM N-ethylmaleimide and 1 mM DFP, was subjected to isopycnic density-gradient centrifugation in CsCl/4 M GdmCl. The mucin fractions were pooled and subjected to a second density-gradient centrifugation step in CsCl/0.5 M GdmCl containing 0.01% CHAPS (36 000 rev./min, 15 °C, 96 h, Beckman 50.2Ti rotor, Beckman Optima L-70 centrifuge). (a, c and e) fractions analysed for density (+), sialic acid (●), PAS reactivity (▲), absorbance at 280 nm (———) and (b, d and f) reactivity with the LUM5B-2 (●), LUM5B-3 (▲) and LUM5B-4 (□) antisera. Antibody reactivity was measured using ELISA and is expressed as absorbance at 405 nm. Fractions were pooled according to the horizontal bars.

Effects of reduction and alkylation of mucins upon reactivity with the LUM5B-2 antiserum

Mucins from saliva, respiratory tract and cervical secretions prepared by density-gradient centrifugation were pooled as shown in Figure 1 and used to examine how reactivity with the LUM5B-2 antiserum changed with an increasing time of reduction. Aliquots (100 µl) of mucin solutions were used to coat microtitre plates overnight. DTT (10 µl of a 10 mM solution in 6 M GdmCl/0.1 M Tris/HCl buffer, pH 8, containing 5 mM EDTA) was added to the wells and the reduction reaction stopped with alkylation immediately or after 0.5, 1, 5, 10, 20, 30, 40 and 60 min by adding 10 µl of a 25 mM solution of iodoacetamide. Reactivity with the LUM5B-2 antiserum was then assessed using ELISA as described above.

Cervical pregnancy mucins pooled as shown in Figure 1 were used to establish the optimum conditions for reactivity with the LUM5B-2 antiserum. Two dilution series (100 µl) in 6 M GdmCl/0.1 M Tris/HCl buffer, pH 8, containing 5 mM EDTA and 1 mM DTT, were coated on to microtitre plates overnight at room temperature. After discarding the coating solutions and adding 100 µl of 6 M GdmCl/1 M Tris/HCl buffer, pH 8, containing 5 mM EDTA, one of the series was alkylated by adding 10 µl of a 25 mM solution of iodoacetamide (in the same buffer) and incubating for 1 h at room temperature. Finally, one dilution series was reduced and alkylated in test tubes prior to coating and as a control, a dilution series of mucins without any treatment was used. Reactivity with the LUM5B-2 antiserum was finally assessed using ELISA as described above. In addition, a dilution series of mucins reduced and alkylated in test tubes prior to coating as well as a dilution series of mucins without any
treatment were coated on to microtitre plates overnight at room temperature and analysed for carbohydrate using a Boehringer glycan detection kit.

Immunohistochemistry

Human tissues were fixed in 10% neutral buffered formal/saline overnight, dehydrated, embedded in paraffin and 4 μm sections cut. The slides were dewaxed, rehydrated and treated with 10 mM sodium citrate buffer, pH 6, at 100 °C in a microwave oven for 10 min. Prior to treatment with the LUMSB-2, LUMSB-3 and LUMSB-4 antisera, sections were reduced with 10 mM DTT in 0.1 M Tris/HC1 buffer, pH 8.0, at room temperature for 30 min, alkylated with 25 mM iodoacetamide in 0.1 M Tris/HC1 buffer, pH 8.0, for 30 min and endogenous peroxidase activity quenched by immersion in 3% H2O2 for 30 min. Sections were blocked with goat serum for 1 h, and endogenous biotin was blocked by treatment with the Dako biotin blocking kit (avidin for 10 min followed by biotin for 10 min). After incubation with the LUMSB-2, LUMSB-3 or LUMSB-4 antisera (diluted 1:2000 in 0.15 M NaCl/0.05 M Tris/HC1 buffer, pH 7.4 (TBS)) or the F2 hybridoma supernatant (diluted 1:5 in TBS) for 1 h in Coverplate immunostaining chambers (Shandon), antibody binding was detected using the Dako StreptABComplex/HRP kit with diaminobenzidine as the substrate. Sections were finally counterstained with Mayer’s haematoxylin.

Analytical methods

Densities of the fractions from the density gradients were measured using a Carlsberg pipette as a pycnometer. Sialic acid was detected using an automated version [38] of the original procedure [39] modified as described by Davies et al. [5]. Carbohydrate was determined by blotting aliquots from the density-gradient fractions on to nitrocellulose membranes and staining with the PAS procedure [40]. Staining was evaluated using a scanning densitometer (Hoeffer) in the transmittance mode, and the results were within the linear range of response as compared with a respiratory mucin standard. Carbohydrate was also detected as periodate-oxidizable structures using a Boehringer glycan detection kit [41]. Aliquots (100 μl) from the fractions were coated on to microtitre plates overnight and, after washing with 0.15 M NaCl/5 mM sodium phosphate buffer, pH 7.4, containing 0.05% (v/v) Tween 20, were treated with 25 mM metaperiodate/0.1 M sodium acetate buffer, pH 5.5 (100 μl at room temperature for 20 min). This procedure was followed by incubation with 100 μl digoxigenin/succinyl-c-hexanoic acid hydrazide (0.6 μg/ml in 0.1 M sodium acetate buffer, pH 5.5) for 1 h at room temperature. After washing, bound digoxigenin was detected using alkaline phosphatase-conjugated Fab fragments from sheep anti-digoxigenin antibodies (100 μl; diluted 1:2000 in 0.15 M NaCl/5 mM sodium phosphate buffer, pH 7.4, containing 0.05% (v/v) Tween 20 and 0.5% (w/v) BSA) with nitrophenyl phosphate (2 mg/ml in 1 M diethanolamine/HCl buffer, pH 9.8, containing 5 mM MgCl2) as a substrate. Reactivity was expressed as absorbance at 405 nm after 1 h.

RESULTS

Mucins were prepared from the gel phases of whole saliva and respiratory-tract secretions, as well as from cervical pregnancy mucus plugs, by isopycnic density-gradient centrifugation first in CsCl/4 M GdmCl and then in CsCl/0.5 M GdmCl. In CsCl/4 M GdmCl, mucins and nucleic acids were separated from low-buoyant-density proteins (results not shown). Fractions with densities of approx. 1.51–1.37 g/ml (containing mucins and nucleic acid) were pooled and subjected to a second density-gradient centrifugation step in CsCl/0.5 M GdmCl (Figure 1). The gel phase from whole saliva gave rise to a broad mucin population between the densities of 1.43 and 1.53 g/ml as shown by both analysis for sialic acid and PAS reactivity (Figure 1a). The presence of a ‘shoulder’ on the high-density side of the distribution suggests a heterogeneity within the population. Analysis with the LUMSB-2, LUMSB-3 and LUMSB-4 antisera followed the distribution obtained with the chemical analyses and suggested the presence of at least two MUC5B populations (Figure 1b). In addition, a small peak of LUMSB-4 antisera reactivity was present on the lower-buoyant-density side of the major peak. In the gel phase of chronic-bronchitic-patient sputum, the major mucin population showed a similar broad...
Mucins from all three sources were used to examine the kinetics of reduction in relation to reactivity with the LUM5B-2 antiserum (Figure 2a). Reactivity with the antiserum increased rapidly within the first 1 min of exposure to DTT and then more slowly, to reach a plateau at about 60 min, suggesting that most of the available LUM5B-2 antibody binding sites were exposed within this time. When DTT was removed without subsequent alkylation, there was a tendency for the antibody reactivity to decrease with time, suggesting a certain degree of ‘re-folding’, but this effect varied between samples and sets of experiments (results not shown). The conditions required for optimum reactivity with the LUM5B-2 antiserum were investigated using cervical mucins. Reactivity of untreated ‘whole’ mucins was very low and increased markedly when the fractions were treated with DTT during coating overnight (Figure 2b). Alkylation of the mucins after reduction during coating led to a slight increase in reactivity with the LUM5B-2 antiserum, as compared with reduction alone, although when the time between reduction and alkylation was prolonged, the reactivity was lower than if samples were alkylated directly. When whole mucins were subjected to reduction/alkylation in test tubes or dilution plates prior to coating on to the microtitre plates, there was an additional increase in reactivity. To investigate whether the differences in reactivity with the LUM5B-2 antiserum seen between untreated and reduced mucins resulted from differences in coating efficiency, dilution series were coated before and after reduction and the amount of coated mucins estimated as carbohydrate using the glycan detection method. This showed no difference between whole mucins and reduced subunits in the efficiency with which they were coated on to the microtitre plates (Figure 2b). The data thus suggest that antibody binding to the cervical MUC5B mucins was significantly enhanced by reduction and, in
Subunits prepared by reduction/alkylation of the cognate ‘whole’ mucins were dialysed against 6 M urea/10 mM piperazine/perchlorate buffer, pH 5.0, containing 0.1% CHAPS (starting buffer), and chromatographed on a Mono Q 5/5 HR column as described in the text. The column was eluted with starting buffer for 10 min, followed by a linear gradient over 60 min to a final concentration of 0.4 M LiClO$_4$ in starting buffer. Fractions (0.5 ml) were collected and analysed for carbohydrate with the glycan-detection method (D) as well as reactivity with the LUM5B-2 (U), F2 (a) and (b and c) LUM5-1 (^) antibodies.

Parallel experiments, MUC5B mucins from saliva and the respiratory tract were shown to behave in a similar way. Although the reactivity is slightly lower under these conditions, for practical reasons data on whole mucins in the present paper were obtained after reduction in microtitre plates during coating followed by alkylation.

Mucins from the three sources were pooled as shown in Figure 1 and subjected to rate-zonal centrifugation to investigate the size and oligomeric nature of the MUC5B mucins (Figure 3). In saliva, ‘whole’ mucins showed a broad range of molecular sizes, as revealed by the carbohydrate assay and LUM5B-2 reactivity was seen over the entire distribution (Figure 3a). Since no reactivity was seen with the LUM5-1 antiserum for salivary mucins in the CsCl-4 M GdmCl gradient (results not shown), the rate-zonal distribution was not tested with this antibody. After reduction, the mucins were much smaller, indicating that MUC5B is an oligomeric mucin composed of subunits linked by disulphide bonds (Figure 3b). In the respiratory-tract secretions, mucins also showed a wide range of molecular sizes, although the relative amount of the larger mucins species was less than that in salivary secretions (Figure 3c). The reactivity with the LUM5B-2 antiserum followed largely the chemical analyses, whereas the low-molecular-mass side reacted with the LUM5-1 antiserum indicating that, as would be expected from previous investigations [22], MUC5AC was present in the secretions. Again, reduced MUC5B subunits were smaller than the whole mucins (Figure 3d) indicating that MUC5B from the airways is also an oligomeric mucin. In cervix, the LUM5B-2 antiserum reactivity was strongest over the larger species, whereas reactivity with the LUM5-1 antiserum indicated the presence of apparently smaller MUC5AC mucins (Figure 3e). After reduction, the MUC5B subunits were similar to those from the other two tissue sources (Figure 3f), and MUC5B from all three tissues thus appears to be a large, oligomeric mucin composed of subunits linked by disulphide bonds.

Ion-exchange HPLC of reduced subunits was used to investigate further the MUC5B mucin in the secretions. In saliva, analysis for carbohydrate revealed three partially resolved subunit peaks (Figure 4a), which all reacted with the LUM5B-2 antiserum, showing the presence of MUC5B populations which differ in charge density. An antibody (F2) recognizing the sulpho-Lewis C structure reacted over the whole distribution, indicating that all populations are sulphated. Analysis with the LUM5-1 antiserum was not carried out, since no reactivity was seen with
this antibody over the CsCl/4 M GdmCl gradient. In the respiratory secretions, analysis for carbohydrate again suggested the presence of three mucin populations, two of which coincided with the LUM5B-2 reactivity (Figure 4b). The F2 antiserum reacted with two populations, although these did not coincide exactly with the MUC5B populations. The LUM5-1 antiserum was used to identify the MUC5AC subunits known to be present in respiratory secretions which eluted between the two MUC5B populations. In the cervical secretions, only a single major population of MUC5B mucins was present (Figure 4c). Reactivity with the MUC5AC antiserum was present as a peak which coincided with the less-charged MUC5B species. The MUC5AC subunits from cervix and respiratory tract were eluted in the same position, suggesting that they have a similar charge density. Reactivity with the LUM5-1 relative to that with the LUM5B-2 antiserum was lower for the cervical secretions than would be expected from the rate-zonal gradients. This is partly explained by the fact that reactivity with the LUM5-1 antiserum is decreased by approx. 50% when the mucins are reduced and alkylated. In addition, differences in the coating efficiency of the two mucins (competitive coating) may also contribute to the decrease in LUM5-1 reactivity.

Reduced subunits from the three sources were subjected to gel chromatography on Sephacryl S-500. In all cases most of the material was eluted close to the void volume, and LUM5B-2 reactivity followed the pattern for carbohydrate (Figure 5).
However, the LUM5B-4 antiserum reacted both with the large subunits and with a population of small fragments which were well included. In respiratory and cervical-tract secretions, the included peak was partially separated into two, the larger of which also reacted in the carbohydrate assay (Figures 5b and 5c).

Immunohistochemistry was used to identify the cellular origins of the MUC5B mucins from the different tissue sources. In cervix, both the surface epithelium of the cervical canal and the glands reacted with the LUM5B-2 antiserum (Figure 6a). In the submandibular salivary gland (Figure 6b), reactivity with the LUM5B-2 antiserum was present at a few focal points within the tissue, whereas in the sublingual gland, almost all the secretory acini were stained (Figure 6c). In trachea, the submucosal glands were stained (Figure 6d), and staining was also evident over epithelial goblet cells (Figure 6e). The F2 antiserum stained a subpopulation of the MUC5B-containing secretory cells within the respiratory submucosal glands, as well as cells within the surface epithelium (Figure 6f). The same results were obtained using the LUM5B-3 and LUM5B-4 antisera as for LUM5B-2. Preimmune sera for each of the antisera showed no staining, except for a weak reaction over the gland ducts of the submandibular gland (results not shown). This reaction was abolished by pre-treating the sections with the biotin/avidin blocking system (Dako), suggesting the presence of endogenous biotin (results not shown).

**DISCUSSION**

Using antisera raised against sequences within the non-glycosylated regions of MUC5B, the mature MUC5B mucin was identified in the gel phase of human saliva, respiratory-tract secretions and in cervical pregnancy mucus. The LUM5B-2 and LUM5B-3 antisera were raised against sequences within the cysteine-rich domains of the super-repeats in the central exon of MUC5B [16] and the LUM5B-4 antiserum was raised against a sequence in the C-terminal domain [18-20]. In studies to establish the optimum conditions for the LUM5B-2 antiserum, reactivity increased when whole mucins were subjected to reduction followed by alkylation, either on the microtitre plates or in test tubes/dilution plates. This effect was shown not to be due to differences in the efficiency with which whole and reduced mucins coated on to the microtitre plates. Thus reduction of disulphide bonds appears to be required for the epitopes to be exposed, suggesting that they are located in folded regions within which the tertiary structure is stabilized by disulphide bonds. If thiol groups generated by reduction were not blocked, antibody reactivity tended to decrease, suggesting that, under some conditions, the structures may refold.

MUC5B from human saliva, respiratory-tract secretions and in cervical pregnancy mucus was shown to be a large oligomeric mucin composed of subunits linked by disulphide bonds. Thus three (MUC2, MUC5AC and MUC5B) of the four mucins within the gene cluster on chromosome 11p15.5 [25] have now been identified as large gel-forming species. Although the polydisperse mass distributions showed significant overlap, MUC5B mucins were apparently larger than MUC5AC mucins. Recently, the ‘smearied’ appearance of mucin mRNAs on Northern blots has been shown to be an artefact [42], and the typical size polydispersity of the large gel-forming mucins must thus be explained by factors other than differences at the level of transcription, one likely explanation being a variable number of subunits within the whole mucins.

In the gel phase of respiratory secretions and saliva, both density-gradient centrifugation and ion-exchange HPLC revealed a heterogeneity within the MUC5B mucins, suggesting the presence of glycoforms of MUC5B. In saliva, all populations reacted with the F2 antibody, whereas in respiratory secretions, one of the MUC5B populations reacted preferentially, suggesting that the later-to-be-eluted glycoform contains a larger proportion of the sulpho-Lewis C structure. The presence of glycoforms of respiratory MUC5B has been identified previously [33]. In cervical secretions, MUC5B and MUC5AC were eluted as single peaks, suggesting that neither mucin is present in more than one distinct glycoform. In secretions from all sources, the LUM5B-4 antibody recognizing the C-terminal domain of MUC5B reacted with the large subunits generated by reduction as well as with smaller fragments. It thus appears that a subpopulation of the MUC5B apoproteins undergoes a cleavage leading to release of a C-terminal fragment, a phenomenon which has been noticed previously for the human and rat MUC2 mucins [43].

Identification of one of the major mucins in cervical pregnancy mucus as MUC5B allows comparison between biochemical studies carried out previously and the more recently published molecular-biology data for this mucin. The biochemical model postulated for the large gel-forming mucins from cervix proposed that the highly glycosylated domains (T-domains) would be approx. 400 amino acids long and that each subunit would contain four or five such domains [1]. This model fits well with data for the MUC5B apoprotein, which predicts that the molecule contains five serine/threonine-rich regions separated by cysteine-rich regions [16].

Immunohistochemistry with the LUM5B-2 antiserum revealed the salivary MUC5B mucins to originate from both the submandibular and the sublingual glands, in agreement with data provided by *in situ* hybridization [28,29]. In the respiratory tract, MUC5B was mainly found in the submucosal glands although goblet cells in the surface epithelium were also stained. Previously, we have shown that airway MUC5AC is produced by the goblet cells and it thus appears that these cells may produce MUC5B as well. However, it is not possible to say from these data whether individual goblet cells can produce both mucins or whether they originate from different populations of cells. The F2 antibody stained a subpopulation of cells within the respiratory tract submucosal glands showing that only some gland cells produce this ‘glycoform’. It therefore appears that the different ‘glycoforms’ of MUC5B are produced by different populations of cells presumably expressing different repertoires of glycosyltransferases. In the cervix, MUC5B was found in the epithelium lining the cervical canal, as well as in the glands.

In conclusion, MUC5B has been identified as a large, oligomeric mucin produced by the submandibular and sublingual salivary glands, the airway submucosal glands and goblet cells as well as by the cervical epithelium. Although the apoprotein is the same, MUC5B ‘glycoforms’ with different ‘acidity’ are produced by different populations of cells. The cysteine-rich regions within the central super-repeat region of MUC5B may be folded and stabilized by disulphide bonds, and reactivity with an antibody directed to the C-terminal domain suggests that a fraction of the MUC5B mucins undergoes proteolytic cleavage in this region.

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