Physical characterization of a low-charge glycoform of the MUC5B mucin comprising the gel-phase of an asthmatic respiratory mucous plug

John K. SHEEHAN¹, Marjorie HOWARD*, Paul S. RICHARDSON†, Toby LONGWILL† and David J. THORNTON*

*Wellcome Trust Centre for Cell-Matrix Research, University of Manchester, School of Biological Sciences, 2.205, Stopford Building, Manchester M13 9PT, U.K., and

†St. George's Hospital Medical School, Cranmer Terrace, London SW17 0RE, U.K.

We have previously noted that sequential extraction of an asthmatic mucous exudate with 6 M guanidinium chloride yielded a fraction of the mucins that were most resistant to solubilization and of high M_r [Sheehan, Richardson, Fung, Howard and Thornton (1995) *Am. J. Respir. Cell Mol. Biol.* **13**, 748–756]. Here we show that this mucin fraction is dominated (at least 96% of the total) by the low-charge glycoform of the MUC5B gene product. Seen in the electron microscope the mucins appeared mainly as compact 'island' structures composed of linear threads often emanating from globular 'nodes' rather than the discrete linear threads more typical of mucins that we have previously described. The effect of reducing agents was as expected for other gel-forming mucins, i.e. reduced subunits or monomers of M_r 3×10^6 were produced within 15 min of treatment. Kinetic experiments on the cleavage of the intact mucins with the proteinase trypsin indicated two clear regimes of

fragmentation. An initial rapid cleavage generated mucins ranging from $M_r = 4 \times 10^6$ to 30×10^6 that in the electron microscope appeared as polydisperse threads (500–3000 nm in length), similar to normal and other respiratory mucins that we have previously characterized. A subsequent slower fragmentation over many hours yielded a major fragment of M_r 3×10^6 and length 200–600 nm, very similar in size and M_r to the subunits obtained by reduction. The results suggest that the MUC5B mucin is assembled, first into polydisperse linear threads, which are then linked together via a protein-mediated process. This might involve part of the mucin polypeptide or an as yet unidentified protein(s). The high proteinase susceptibility of the linkage suggests that it might be a point of control for mucin size and thus mucus rheology.

Key words: asthma, gel-forming mucins, mucus.

INTRODUCTION

The physical properties of mucus are dominated by large secreted mucins that are characterized by their extreme density of O-linked oligosaccharide substitution. As a consequence of the glycosylation the mucin polypeptide has limited sites of access for proteinases. The respiratory gel-forming mucins are assembled from a variable number of subunits of M_r $(2-3) \times 10^6$, organized via the agency of disulphide bonds [1–8], which appear in the electron microscope typically as long, linear flexible threads [4,5,9,10]. The mechanisms by which mucins are oligomerized is still unclear but recent cDNA sequencing data [11–16] on a number of mucins (MUC2, MUC5AC, MUC5B and MUC6) have identified domains at the C- and/or N-termini of these mucin polypeptides that have homology with domains within von Willebrand factor that are involved in oligomerization [17,18]. By analogy with the von Willebrand factor it seems reasonable to propose that mucin subunits assemble by a similar mechanism, although this has not been demonstrated unambiguously to date.

In a study on the viscid mucous exudate from a patient who died in *status asthmaticus*, mucins were identified as the major macromolecular component of the gel [1]. More recently we demonstrated that three distinct oligomeric, gel-forming mucins are present in this mucus exudate, all of which are composed of subunits of similar mass and size; all seem, at least superficially, to be assembled in a similar fashion [19]. One of these has been shown to be the product of the *MUC5AC* gene [19] and the other two are differently charged glycoforms of the *MUC5B* gene product [20]. These two forms of the *MUC5B* mucin differ with

respect to their elution positions on anion-exchange chromatography and their electrophoretic mobilities in agarose-gel electrophoresis [19,20]. Wickstrom et al. [21] have also noted differently charged populations of the *MUC5B* mucin in respiratory tract secretions. In addition, similar *MUC5B* glycoforms have been observed in saliva [21,22].

In our original study [1] we noted that sequential extraction of the viscid mucous exudate with 6 M guanidinium chloride yielded a fraction of the mucins (22% of the total) that were more resistant to solubilization and of high M_r . Here we demonstrate that the low-charge glycoform of the *MUC5B* mucin gene product predominates in this fraction. We also present a more detailed physical description of these mucins in an attempt to identify specific properties that might explain their unusual mass, size and shape.

EXPERIMENTAL

Preparation of mucins

The sample employed for the physical characterization experiments reported here was a high- M_r mucin fraction isolated from an individual who died in *status asthmaticus* as described previously [1]. In summary, 3 g of viscid gel exudate was taken from the airways *post mortem* and three preliminary extractions were made, each in 50 ml of 6 M guanidinium chloride containing proteinase inhibitors. The residual gel was finally extracted in a large excess of 6 M guanidinium chloride containing proteinase inhibitors; the mucins were purified by isopycnic centrifugation. The mucins in this fraction represented 22% of the total gel-

Abbreviation used: PAS, periodate-Schiff.

¹ To whom correspondence should be addressed (e-mail jsheehan@fs1.scg.man.ac.uk).

Figure 1 Anion-exchange chromatography of reduced mucin subunits

Reduced mucin subunits were chromatographed on a Mono Q HR5/5 column as described in the Experimental section. Fractions (0.5 ml) were assayed after immobilization on nitrocellulose with (a) the PAS reagent (solid line) or (b) with antisera specific for mucins MUC5B (○) and MUC5AC (●). The nominal elution gradient is shown (broken line). The inset in (b) shows the electrophoresis of aliquots of selected fractions on a 1% (w/v) agarose gel that was then Western blotted to nitrocellulose (as described in the Experimental section) and the blot was probed with the MUC5B-mucin specific antiserum. The arrow shows the expected position of migration of the highly charged glycoform of the MUC5B mucin.

forming pool and are those designated Extract III in the original study [1].

Preparation of reduced mucin subunits

Reduced mucin subunits were obtained by treatment of the purified mucins in 6 M guanidinium chloride/0.1 M Tris/HCl (pH 8.0) with 10 mM dithiothreitol for 5 h at 37 °C. Iodoacetamide was then added to a final concentration of 25 mM and the mixture was left in the dark overnight at room temperature.

Analytical methods

Total carbohydrate was determined with a periodate-Schiff (PAS) assay after slot-blotting of mucins to nitrocellulose [23]. Mucins on blots were also detected with the polyclonal antisera raised against synthetic peptides from specific sequences within the mucins MUC5AC and MUC5B. The two antisera used were MAN-5ACI, a polyclonal antiserum raised against the same peptide domain as used as the immunogen for LUM5-1 [19], and MAN-5BI, as described previously [20].

Anion-exchange chromatography

Reduced mucins were transferred into 6 M urea/10 mM piperazine (pH 5.0) containing 0.02% CHAPS by chromatography

on a Pharmacia PD-10 column before separation by anion-exchange chromatography. Reduced mucins were then chromatographed on a Pharmacia Mono Q HR 5/5 column eluted with a linear gradient of 0–0.4 M lithium perchlorate/10 mM piperazine (pH 5.0) in 6 M urea containing 0.02% CHAPS [24].

Agarose-gel electrophoresis

Reduced mucin subunits were subjected to electrophoresis in 1% (w/v) agarose gels in 40 mM Tris/acetate/1 mM EDTA (pH 8.0) containing 0.1% SDS, at 30 V for 18 h at room temperature. After electrophoresis, subunits were transferred to nitrocellulose by vacuum blotting before detection of mucins with antibodies [24]. Bands were detected with horseradish peroxidase-labelled secondary antibodies in conjunction with an enhanced chemiluminescence Western detection kit.

Light scattering

Absolute intensity measurements were performed in 6 M guanidinium chloride as described previously [5]. The concentration of the stock solution employed was measured by freeze-drying an aliquot after extensive dialysis. Assessment of the water content of whole mucins in the dried material was done as described previously and the values for dn/dc previously obtained for cervical mucins were employed in this study [25]. Light-scattering measurements were performed in a Malvern 4700C spectrometer in glass cells employing 200–300 μ l of sample in 6 M guanidinium chloride; the data were analysed by the method of Zimm [26]. Samples were clarified by filtration with Millipore 5 μ m filters, followed by centrifugation at 15000 g at 20 °C for 1 h.

Alternatively, light scattering was performed after chromatography on either a Sephacryl S-1000 column or a Superose 6 column (each of total volume 25 ml) in 0.2 M NaCl employing an in-line Dawn DSP laser photometer and a Wyatt 903 interferometric monitor to measure light scattering and sample concentration respectively. Light-scattering data were collected simultaneously at 18 angles between 10° and 151° and the data were analysed by the method of Zimm (see Figure 3a).

The kinetic measurements were performed with the Malvern 4700C spectrometer. Mucins were treated in the light-scattering cell by introducing dithiothreitol or trypsin at relatively high concentrations via long-stemmed pipette tips, which were also employed for mixing, the whole process taking 5 s. Typically an addition of 5 μ l was made to 200–300 μ l of solution, the final concentration of dithiothreitol being 10 mM or that of trypsin 10 μ g/ml. The digital signal from the photomultiplier was passed to a digital-to-analogue converter and plotted with a chart recorder. Measurements were made at an angle of 20°, giving a good direct measure of the change of apparent M_r at the concentration employed.

Electron microscopy

Mucins were prepared for electron microscopy by using the mica sandwich technique [27]. A drop (40 μ l) of solution containing 0.01 μ g/ml mucin in 0.2 M ammonium acetate was put on the surface of a freshly cleaved piece of mica. The other piece was placed on top to form the sandwich, which was left for 2 min to allow adsorption. The sandwich was put in 0.2 M ammonium acetate and opened, then left to wash in the solution for 5 min. The mica was then removed, put in liquid nitrogen and thereafter on a liquid-nitrogen-cooled copper block, then placed in a coating unit where it was freeze-dried. The mica was first coated with platinum at an angle of 7°, then coated with carbon as for the normal rotary shadowing technique. The replica was removed

on a water surface and placed on 600-mesh copper grids, then examined in a Jeol 1200C electron microscope.

RESULTS

We have shown that the products of two mucin genes, *MUC5B* and to a smaller extent *MUC5AC*, comprise the bulk of the gel-forming molecules in the exudate of an individual who died in *status asthmaticus* [19,20]. Before that, from the same sample of mucus, we identified a fraction of mucins of unusually high M_r

that were difficult to solubilize (designated Extract III in [1]); it is these molecules that we have studied in more detail here.

Reduced mucin subunits prepared from this mucin preparation were analysed by anion-exchange chromatography (Figures 1a and 1b). The reduced MUC5B mucin subunits were eluted earlier than the MUC5AC-reactive material, and most of the PAS response (approx. 96% of the total) was associated with the MUC5B distribution. The elution position of the MUC5B mucin subunits from the anion exchange column indicated the absence of the more highly charged glycoform of this mucin observed

Figure 2 Electron microscopy of intact mucins

The samples were prepared for electron microscopy as described in the Experimental section. (a) The molecules are generally found as condensed 'islands' which often (b, c) have clear domains ('nodes') from which many chains seem to emanate. Scale bars: 300 nm (a), 100 nm (b), 200 nm (c).

Figure 3 Light scattering of intact mucins

The mucins (200 μ l at 500 μ g/ml) were chromatographed on a Sephacryl S-1000 column at a flow rate of 0.4 ml/min in 0.2 M NaCl and the effluent was monitored for light scattering and refractive index (see the Experimental section). The outputs from both the light-scattering and refractive index detectors are recorded in volts. (a) Light scattering at 90°; the inset shows a typical Zimm plot achieved at each point across the chromatogram. (b) The associated refractive index profile is shown together with the calculated values for M_r (Δ) and R_g ($\circ$) across the distribution.

previously in the total unfractionated sample of mucus and in other respiratory secretions [19,21]. This was confirmed by agarose-gel electrophoresis (Figure 1b, inset) because no bands were observed in the expected position for this more highly charged MUC5B species [20].

The intact, unreduced mucins appeared most often as heavily entangled filamentous structures (Figure 2a) forming distinct 'islands' in which it was difficult to find ends. These 'islands' in many cases had large globular 'nodes' in the middle (Figure 2b) and in a few cases the 'nodes' seemed to have numerous strands running from them (Figure 2c).

The unreduced MUC5B mucin preparation was subjected to gel chromatography on Sephacryl S-1000 (Figure 3) and the absolute size (radius of gyration, R_g) and M_r distributions were determined across the chromatogram by in-line light-scattering measurements (Figure 3). The results showed that the molecules are polydisperse in both size (150–450 nm) and M_r [(10–100) $\times 10^6$]. Reduction caused a decrease in M_r of the mucins to 3×10^6 and subsequent trypsin treatment produced high- M_r mucin glycopeptides (average $M_r = 330 \times 10^3$) (results not shown). This fragmentation pattern is consistent with previous data on both cervical and respiratory mucins [4–6,28]. The kinetics of fragmentation of the mucins by these two treatments was monitored by light scattering (Figure 4). Both were single processes; reduction was complete within 15 min (Figure 4a), whereas subsequent trypsin-treatment was faster and complete within

Figure 4 Kinetics of reduction and subsequent treatment of intact mucins with trypsin

The losses of light scattering with time during the reduction of intact mucins (a) and their subsequent treatment with trypsin (b) were monitored at an angle of 20° (see the Experimental section).

2 min (Figure 4b). In contrast, the kinetics of trypsin-induced fragmentation of the whole mucins had three phases (Figure 5): an extremely rapid process taking place over a period of seconds followed by a slower process over tens of minutes and finally an extremely slow degradation over many hours. The M_r distributions of the mucins after treatment with trypsin (0, 1 and 1000 min) were measured by in-line light scattering after gel

Figure 5 Kinetics of digestion of intact mucins with trypsin

The loss of light scattering with time during digestion of intact mucins with trypsin at 25 °C was monitored at an angle of 20° (see the Experimental section).

Figure 6 Effect of time of treatment with trypsin on the mass distribution of the mucins

Mucins were treated with trypsin for 0 min (dotted line), 1 min (broken line) and 1000 min (solid line), then chromatographed on Sephacryl S-1000 (0 and 1 min traces) or Superose 6 (1000 min trace) at a flow rate of 0.4 ml/min in 0.2 M NaCl. The effluent was monitored for light scattering and refractive index (see the Experimental section). Trypsin digestion was stopped by the addition of an equal volume of 8 M guanidinium chloride. The M_r of the mucins was determined across the distribution and the results are presented as the weight fraction of the molecules at each M_r .

chromatography on either Sephacryl S-1000 or Superose 6 (Figure 6). After 1 min of proteolysis the elution profile changed from a homogeneous peak with an average M_r of 30×10^6 to a bimodal distribution of average M_r 12×10^6 with distinct components at approx. 6×10^6 and 16×10^6 . After 1000 min of trypsin digestion a more homogeneous fragment ($M_r = 3 \times 10^6$) was evident, similar in mass and size to the monomers prepared after reduction of the intact molecules.

Electron microscopy of the mucins after brief (1 min) treatment with trypsin showed that the compact 'island' structures (Figure 2) were disrupted and the most common particles were large linear threads up to 3000 nm in length (Figure 7a) more typical of other respiratory mucin preparations that we have studied [4,5]. More extensive trypsin digestion (1000 min) left disperse filamentous fragments between 100 and 500 nm in length (Figure 7b).

The relationship between size and shape for molecules in solution can be expressed by the Mark-Houwink parameter α , which is obtained from the relationship $R_g = AM_r^\alpha$, where A is a constant relating to the mass per unit length of the molecule. The parameter α can be used as an index for the stiffness of the molecules; for example, for rod-like molecules α would equal 1, for random coils α would equal 0.5–0.6 and for compact spheres α would equal 0.33. In a previous study on respiratory mucins the value of α was determined as 0.66, which is consistent with the molecules' being stiff random coils [25]. However, for the asthmatic MUC5B mucin preparation a plot of $\log R_g$ against $\log M_r$ across the gel-filtration chromatogram gave a straight line whose slope yielded a value for α of 0.41 (Figure 8). This is below the value expected for a random coil (0.5–0.6) and is more consistent with a compact or branched structure, in agreement with the appearance of the mucins in the electron microscope (Figure 2). After only 1 min of digestion with trypsin the plot of $\log R_g$ against $\log M_r$ plot yielded a value for α of 0.62, which is

consistent with a stiff random coil, similar to the respiratory mucins studied previously [25].

DISCUSSION

We have previously demonstrated that the total gel-forming mucin pool in an asthmatic exudate [1] is composed of three mucin species, namely the MUC5AC mucin and two glycoforms of MUC5B mucin [19,20]. From these two studies we estimate that the MUC5B and MUC5AC mucins accounted for approx. 86% and 14% of the gel-forming mucins respectively. Furthermore the low-charge population of the MUC5B mucin is by far the predominant species (approx. 79% of the total gel-forming mucins). The results presented here demonstrate that the fraction of mucins most difficult to solubilize from this mucus with a highly chaotropic agent (6 M guanidinium chloride) was composed almost entirely of the low-charge glycoform of the MUC5B mucin. We estimate from the anion-exchange chromatography profiles of PAS and antibody reactivity that this accounts for at least 96% of the preparation. The mucins are polydisperse in M_r , ranging from 10×10^6 to 100×10^6 with an average of 30×10^6 . These mucins are larger than the total population of normal respiratory mucins [average $M_r = (14\text{--}16) \times 10^6$] [5]. However, results obtained on the mucin preparation from normal individuals indicate that the MUC5AC mucin might predominate [19]. Thus the differences in M_r might reflect the different mucin compositions of the preparations.

The MUC5B mucin preparation studied here appears in the electron microscope mainly as tangled masses often condensed around 'nodes' from which many chains emanate. Interestingly, this morphology was observed previously for asthmatic mucins [29], although at that time it was not possible to determine which MUC gene product(s) was under investigation. The morphology of the mucins reported in the present study is unlike that previously observed for other airway mucins that appear as linear threads in the electron microscope [4,5]. However, even in these other studies a small proportion of the mucins had the same appearance as those described here.

The value derived for the shape-dependent Mark-Houwink parameter ($\alpha = 0.41$) is consistent with the condensed or branched morphology observed in the electron microscope. However, although the intact mucins appear unlike most of the molecules in other respiratory mucin preparations, reduction of disulphide bonds generates reduced mucin subunits of M_r approx. 3×10^6 that are similar to those previously observed for normal and other respiratory mucin preparations [4,5]. Thus it is apparent that disulphide bonds are involved, either directly in the organization of this unusual mucin architecture or in the stabilization of the tertiary structure of one or more non-mucin proteins essential to its organization. In support of this latter contention is the demonstration that a protein-rich fraction is released by reduction of the total population of mucins purified from the sample of asthmatic mucus studied here [6]. Furthermore other workers have also demonstrated that protein components are released from purified respiratory mucin preparations by reduction [3,30]. Heterotypic complexes of proteins with mucins have been reported; for example, studies on salivary secretions demonstrate that several proteins are involved in heterotypic complexes with the large MG1 mucins [31]. This finding is of particular interest because we have recently demonstrated that MG1 mucin population is predominantly composed of the MUC5B mucin [22].

The fragmentation of the intact MUC5B mucins by trypsin suggests the presence of at least two different proteinase-susceptible domains. Cleavage at the most proteinase-sensitive

Figure 7 Electron microscopy of mucins after treatment with trypsin

Mucins were prepared for electron microscopy as described in the Experimental section; the micrographs show intact mucins after 1 min (a) and 1000 min (b) of treatment with trypsin. Scale bars: 500 nm (a), 100 nm (b). Digestion with trypsin was stopped by the addition of an equal volume of 8 M guanidinium chloride.

Figure 8 Relationship between M_r and R_g of intact and trypsin-treated (1 min) mucins

The mucins before and after trypsin treatment were chromatographed on Sephacryl S-1000 and the column effluent was monitored for light scattering and refractive index as described in the Experimental section. The slope (α) of the plot of $\log M_r$ against $\log R_g$ yields for the intact mucins (●, broken line) a value of 0.41 and for the trypsin-treated mucins (○, solid line) a value of 0.62.

domain causes a rapid decrease in M_r of the mucins and a change in their morphology to yield linear thread-like molecules more typical of normal and other respiratory mucin preparations that we have studied [4,5,7]. In addition, their molecular shape in solution, as assessed by the Mark-Houwink parameter ($\alpha = 0.62$), is consistent with a change from a branched or condensed structure to a stiff random coil, as previously determined for respiratory mucins [32]. Therefore the sensitivity of the unusual mucin architecture to both proteolysis and reduction suggests that the very high M_r and morphology are due to some as yet undescribed protein linkage; whether this involves only the mucin polypeptide or some other protein(s) is unknown.

In conclusion, we have identified a distinct morphology for a subpopulation of MUC5B mucins from the mucus that obstructed the airways of an individual who died in *status asthmaticus*. This morphology might be a feature of normal mucins that is over-represented in this sample of mucus. The mucin architecture seems to be dependent on the presence of 'nodes' around which the mucin chains condense. These 'nodes' might contain associated non-mucin proteins whose level might determine the mucin morphology. Alternatively these 'nodes' might be a site that is the target for rapid proteolytic modification of the mucins and this might be a normal processing event that is less efficient in people with asthma. A loss of efficiency in such a modulation process, e.g. lack of specific proteases or elevated levels of an

inhibitor, might yield abnormally massive mucins and thus a rheologically compromised and difficult mucus. A more detailed evaluation of the morphology of the mucins from a range of normal and asthmatic individuals will have to be undertaken to clarify this hypothesis.

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