# Biosynthesis of the MUC2 mucin: evidence for a slow assembly of fully glycosylated units

John K. SHEEHAN*||, David J. THORNTON*, Marj HOWARD*, Ingemar CARLSTEDT†, Anthony P. CORFIELD‡ and Christos PARASKEVA§

*Wellcome Centre for Cell-Matrix Research, School of Biological Sciences, University of Manchester, 2.205 Stopford Building, Manchester M13 9PT, U.K., †Department of Cell and Molecular Biology, Section for Molecular Pathogenesis, Lund University, P. O. Box 94, S-22100, Lund, Sweden, ‡University Department of Medicine Laboratories, Bristol Royal Infirmary, Marlborough Street, Bristol BS2 8HW, U.K. and §CRC Colorectal Tumour Biology Group, Department of Pathology and Microbiology, University of Bristol, School of Medical Sciences, University Walk, Bristol BS8 1TD, U.K.

The human colonic cell line PC/AA was grown to near confluency over 24 days and labelled with [$^{14}$C]proline and [$^{3}$H]glucose over the last 48 h in culture. The cell layer was extracted with 6 M guanidinium chloride and the mature fully glycosylated mucins were isolated at a density of 1.45 g/ml by using density-gradient centrifugation in CsCl/4 M guanidinium chloride. These mucins were identified as MUC2 with an anti-peptide antibody. The macromolecules were fragmented by reduction into two distinct populations of MUC2 subunits as assessed by agarose electrophoresis. The MUC2 mucin was polydisperse in length, ranging from 500 nm to many microns and its molecular-mass distribution, assessed by rate-zonal centrifugation, ranged from $5 \times 10^6$ to $40 \times 10^6$ Da. However, the metabolically labelled MUC2 mucins, though found throughout the whole distribution, were of much smaller average size. Since the entire distribution is not uniformly radiolabelled over 48 h, the formation of the largest species must be preceded by glycosylation and occur slowly, over several days, via the assembly of fully glycosylated units which are likely to be at least dimers [Asker, Baeckstrom, Axelsson, Carlstedt, and Hansson (1995) Biochem. J. **308**, 873–880].

## INTRODUCTION

Mucins are high-molecular-mass glycoproteins that may contain up to 90 % by weight carbohydrate [1–3]. A number of different mucins have been distinguished, some of which are located at the epithelial cell surface whereas others are secreted to form mucus. Gel-forming mucins from the respiratory tract, stomach and cervix form disulphide-bond-linked linear multimers via end-to-end linkage of subunits to give macromolecules up to and exceeding 10 $\mu$m in length [4–6]. Since this assembly process determines the size of the mucins it is likely to be a key event in the control of the properties of most mucus gels. However, intestinal mucus remains insoluble regardless of the solvent conditions, suggesting that this linear assembly process alone might not be sufficient to explain its behaviour [7,8].

More than nine human genes, termed *MUC* genes, have been identified coding for mucin apoproteins [9] and at least two of these, MUC2 and MUC3, are expressed in normal human colorectal tissue [10]. In addition, MUC4 [11] and MUC5b [12] have been reported as normal components. Biochemical investigations suggest that MUC2 is the major gel-forming mucin in human colon [8,13].

The process of assembly of mucin subunits into large polydisperse macromolecules is not defined. Biosynthetic studies suggest a rapid (over a period of hours) disulphide-bond-mediated oligomerization of the mucin apoprotein in the endoplasmic reticulum followed by O-linked glycosylation to yield the mature mucin [14,15]. In the presence of tunicamycin this oligomerization is abolished, suggesting a vital role for N-linked glycans in this process [14,15]. The extent of oligomerization ranges from dimers, trimers and tetramers for rat gastric mucins [14], trimers and tetramers for human gastric mucins [15] and dimers for human MUC2 from an intestinal cell culture [16]. It has been demonstrated that MUC2 and other mucins show homology to the D-domains of von Willebrand factor that are essential for the oligomerization of the latter glycoprotein which dimerizes via a C-C terminus assembly followed by the formation of tetramers via an N-N assembly in the endoplasmic reticulum [17,18]. In the case of von Willebrand factor it has been demonstrated that highly multimeric forms (10–30 monomers) are found as granule-stored forms that undergo regulated secretion. A similar situation may pertain to MUC2 and other mucins.

Initial studies carried out on the PC/AA cell line demonstrated the presence of a secreted gel layer made up of mucins [19]. These mucins are sialylated, but differ from normal secreted colonic mucins in a lack of sialic acid O-acetylation and a low expression of sialyl-Le$^x$ {Gal$\beta$1 $\rightarrow$ 4[Fuc$\alpha$1 $\rightarrow$ 3]GlcNAc$\beta$1-R}, while normal sulphation is retained [20,21]. Here we demonstrate that these mucins are the products of the *MUC*2 gene and that this glycoprotein is assembled from subunits into very large polydisperse macromolecules of up to at least decamers that are stored within the cell for days. The dimers, trimers and tetramers reported in the literature could not account for molecules of this size and a further assembly process must thus be postulated. Our metabolic labelling studies suggest that the formation of the mature mucins may occur by the assembly of glycosylated units over many days.

## EXPERIMENTAL

### Cell culture and metabolic labelling

The PC/AA adenoma cell line was derived from a single, large, colonic tubular adenoma of 3–4 cm diameter that exhibited only mild dysplasia. The cells were continuously passaged *in vitro* at 37 °C in 5 % $CO_2$ in air and were grown in the presence of 3T3 feeder layers on collagen type IV-coated T25 flasks [22]. The

Abbreviations used: PAS, periodate-Schiff; VNTR, variable number tandem repeat; TFMSA, trifluoromethanesulphonic acid.

|| To whom correspondence should be addressed.

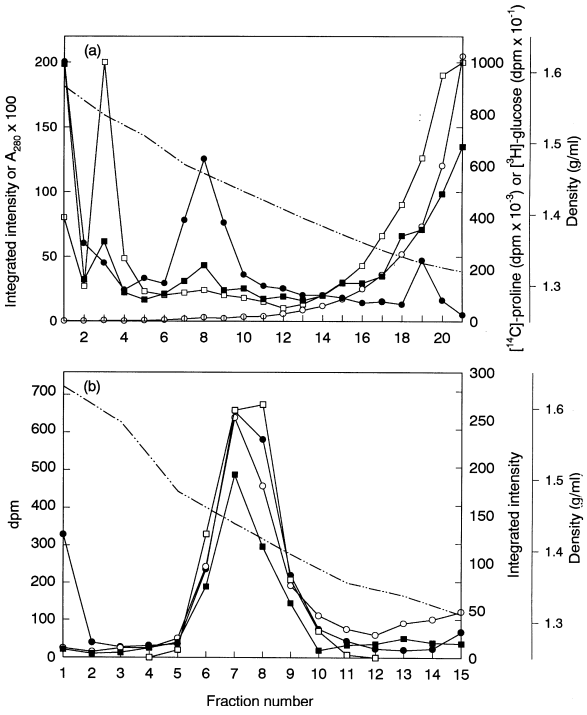


**Figure 1 Isopycnic density-gradient centrifugation of cell layer extract**

(**a**) The cell layer extract in 6 M guanidinium chloride was brought to 4 M guanidinium chloride and CsCl added to a density of 1.4 g/ml and centrifuged at 40 000 rev./min for 65 h at 15 °C. The gradient was emptied from the bottom of the tube and fractions (1.9 ml) were assayed for $A_{280}$ (□), density (— · · —), [$^3$H]glucose (■) and [$^{14}$C]proline (○) radioactivity and with the PAS assay (●). (**b**) The mucin band recovered between densities 1.35 and 1.50 g/ml was re-run twice under the same conditions and data for the second centrifugation are shown. Tubes were unloaded as above and fractions (0.9 ml) analysed for [$^3$H]glucose (■), [$^{14}$C]proline (○) radioactivity, MUC2 reactivity (□), density (— · · —) and with the PAS assay (●). The integrated intensity is defined as in Thornton et al. [25].

PC/AA cells at passages 13–19 used in this study retain a normal diploid karyotype, are non-tumorigenic in nude mice, have an ultrastructure characteristic of colonic cells showing the presence of mucus-containing vesicles, and secrete a gel layer which accumulates during culture. Cells were harvested at near confluency (70–80 % confluent) and incubated with 370 kBq L-[U-$^{14}$C]proline (9.84 GBq/mmol) and 740 kBq D-[6-$^3$H]glucose (925 GBq/mmol) (Amersham International plc., Amersham, Bucks., U.K.) for 48 h. Cells from 2–3 flasks [approx. (2–3) × $10^7$ cells] were used to isolate mucins.

## Isolation of mucins

The cell layer was washed with PBS and then extracted with 6 M guanidinium chloride/PBS containing 1 mM PMSF, 5 mM EDTA, 10 mM benzamidine hydrochloride, 0.1 mg/ml soybean trypsin inhibitor and 10 mM *N*-ethylmaleimide for 24 h, after which time the total cell layer was solubilized. The extract was diluted to adjust the guanidinium chloride concentration to 4 M and CsCl added to a density of 1.4 g/ml. The samples were centrifuged in a Beckman 70Ti rotor at 40 000 rev./min for 65 h at 15 °C. The major mucin population (density 1.4 g/ml) was pooled and re-run twice as above before the final mucin preparation was pooled and dialysed into 4 M guanidinium chloride.

## Rate-zonal centrifugation

Samples (100–200 μl) in 4 M guanidinium chloride were layered on to preformed, guanidinium chloride gradients (6–8 M; total volume 12 ml) and centrifuged in a Beckman SW 40 swing-out rotor at 40 000 rev./min and 20 °C for 2.5 h for intact and 8.5 h for reduced mucins [23]. Tubes were unloaded from the top into 500 μl fractions. The concentration of guanidinium chloride in each fraction was determined by measurement of the refractive index [23].

## Agarose gel electrophoresis

Reduced mucins were electrophoresed in 40 mM Tris-acetate/1 mM EDTA, pH 8.0, containing 0.1 % SDS in 1.0 % (w/v) agarose gels and blotted on to nitrocellulose prior to detection with various antisera [24]. Radioactivity on the nitrocellulose membrane was determined using a Fujix Bas 2000 Bioimager.

## Electron microscopy

Mucins and reduced mucin subunits were spread in a monolayer of benzyldimethylalkylammonium chloride (BAC) from a hypophase of 50 mM magnesium acetate. The molecules were diluted directly into the hypophase from a solution of 4 M guanidinium chloride. Molecules were visualized using a Phillips 400 electron microscope at 100 kV and contour lengths were measured with a Planix digital planimiter (Hall & Watts, Links Trading Estate, Yeovil, U.K.) and the number-average ($l_n$) and weight-average ($l_w$) lengths calculated [5].

## Analytical methods

Analysis of mucins was performed by using the periodate-Schiff (PAS) assay on molecules deposited on nitrocellulose by slot blotting [25]. Direct-binding ELISA were performed using nitrocellulose as the solid phase as previously described [26]. Radioactivity was determined by scintillation counting and Fujix Bas 2000 phosphoimager.

## Deglycosylation

Mucins or reduced mucin subunits were immobilized on to polyvinylidene difluoride (PVDF) membranes by slot or Western blotting and O-glycans removed by treatment with trifluoromethanesulphonic acid (TFMSA) [24]. Blots were probed with antibodies and antisera raised against or reactive with synthetic peptides corresponding to amino acid sequences identified from cDNA cloning of the mucins MUC1 to MUC5.

## Antisera

The following antisera were used in this study. Monoclonal and polyclonal antibodies raised against sequences in the variable number tandem repeat (VNTR) of MUC1–MUC5ac were a kind gift from Dr. Peter Devine (University of Queensland). The specificities of the antibodies to MUC1 [BC3] and MUC2 [4F1] are described [27,28]. Murine antibodies were raised to keyhole limpet haemocyanin conjugates of peptides from the VNTRs of MUC3 (SHSTPSFTSSITTTETTSHSTP), MUC4 (TSSSASTGHATPLPVTDTSS) and MUC5ac (SAPTTSTTSAPTTSTTSAP). Also employed were the polyclonal antisera LUM2-3 and LUM5-1 raised to peptides in the non-VNTR of MUC2 (NGLQPVRVEDPDGC) and MUC5ac (RNQDQQGPFKMC) respectively. LUM2-3 has been shown to react with the major gel-forming mucin from human colon [8], which is believed to be the product of the *MUC2* gene [8,13]. LUM5-1 has been shown to react with a population of mucins present in human gastric

secretions [8] where the *MUC5ac* gene is known to be expressed. Monoclonal antibody AM3, whose epitope was the carbohydrate structure sialyl-Le$^x$ [29], was a kind gift from Dr. Chris Hanski (Klinikum Steglitz der Frein Universitat).

## RESULTS

The PC/AA adenoma cell line was grown to near confluency and then metabolically labelled with [$^{14}$C]proline and [$^3$H]glucose to introduce a radiolabel into newly synthesized mucins. The medium was discarded and the cell layer was solubilized with 6 M guanidinium chloride. There was no insoluble material apparent using this procedure which was not the case for mucins isolated from rat small intestine and human colon [7,8]. Mucins were then isolated from the cell layer by isopycnic density-gradient centrifugation in CsCl/4 M guanidinium chloride.

The distribution of glycoconjugates in the density gradient was assessed by a PAS assay and [$^3$H]glucose radioactivity, whereas the protein distribution was monitored by absorbance at 280 nm, and [$^{14}$C]proline radioactivity (Figure 1a). Absorbance at 280 nm also detects nucleic acids and it is clear that the DNA peak (density 1.52 g/ml) is distinct from the major mucin band (1.45 g/ml). The proportion of radiolabel in the major PAS-rich mucin band is very low, representing about 1% of the total radioactivity incorporated into proteins. The major PAS-rich mucin was subjected to two further rounds of isopycnic density-gradient centrifugation in CsCl/4 M guanidinium chloride and the result of the third density gradient is shown in Figure 1(b). The preparation was probed with mucin apoprotein-specific

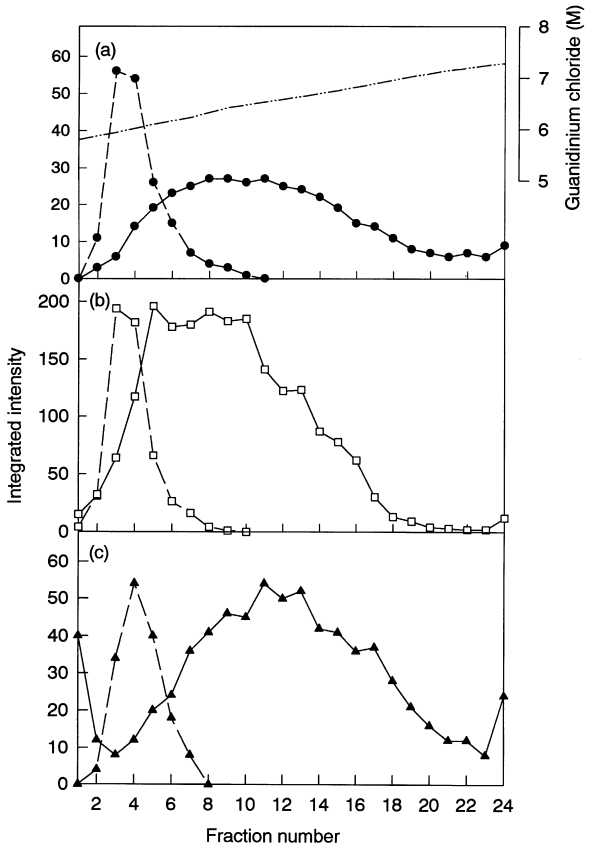


**Figure 2 Rate-zonal centrifugation of mucins**

A sample (200 $\mu$l) of the purified mucins before (solid line) and after reduction (dashed line) was layered on to a preformed 6–8 M guanidinium chloride gradient and centrifuged for 2.5 h at 40 000 rev./min and 20 °C. Tubes were emptied from the top and fractions (0.5 ml) analysed (**a**) with PAS, (**b**) for MUC2 reactivity and (**c**) for sialyl-Le$^x$ reactivity. Also shown in (**a**) is the concentration of guanidinium chloride (— · · —). The integrated intensity is defined as in Thornton et al. [25].

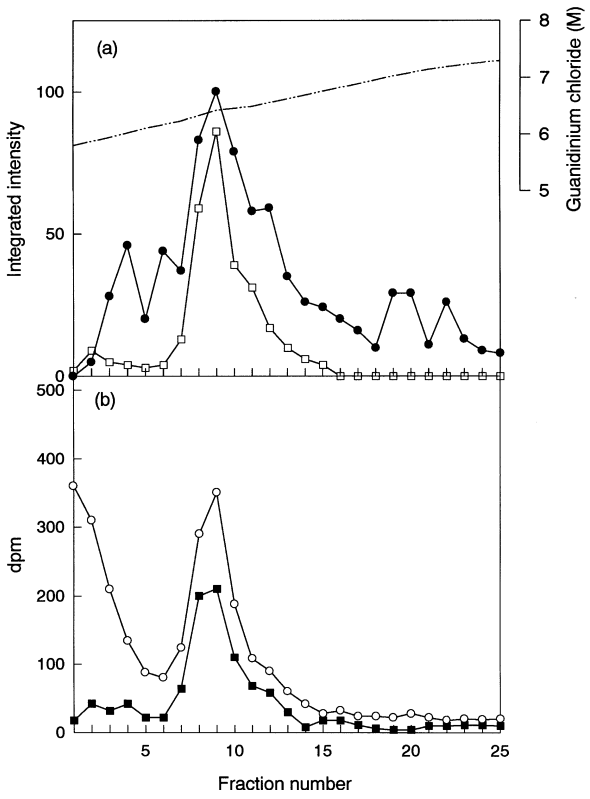


**Figure 3 Rate-zonal centrifugation of reduced mucin subunits**

A sample (200 $\mu$l) of reduced and alkylated mucins was layered on to a preformed 6–8 M guanidinium chloride gradient and centrifuged for 8.5 h at 40 000 rev./min and 20 °C. Tubes were emptied from the top and fractions (0.5 ml) assayed (**a**) for carbohydrate with PAS (●), MUC2 reactivity (□) and the concentration of guanidinium chloride (— · · —) and (**b**) for [$^{14}$C]proline (○) and [$^3$H]glucose (■) radioactivity. The integrated intensity is defined as in Thornton et al. [25].

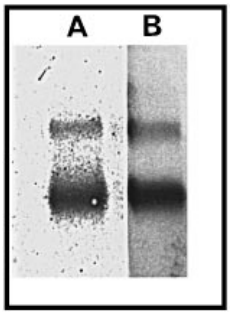


**Figure 4 Agarose gel electrophoresis of reduced mucin subunits**

Reduced mucin subunits were electrophoresed in a 1% (w/v) agarose gel and subsequently blotted on to nitrocellulose as described in the text. Radioactivity on the blot (lane A) was quantified using a Fujix Bas 2000 Bioimager and the blot was then probed (lane B) with the non-VNTR MUC2 antiserum (LUM 2-3).

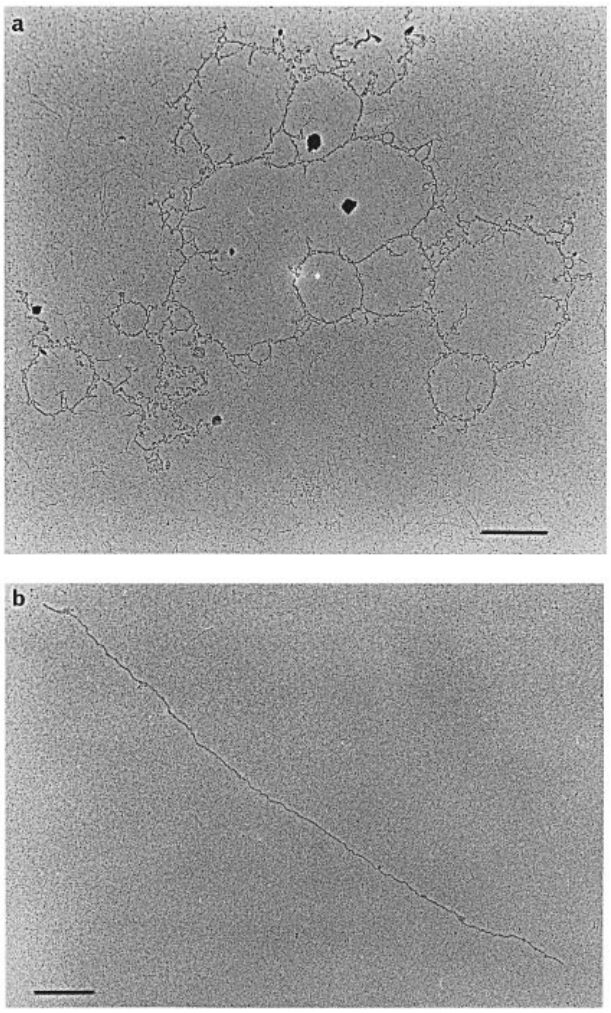


**Figure 5 Electron microscopy of the mucins**

Electron micrographs of intact mucins show some molecules as coiled and interacting webs of molecules (**a**) or as extended filamentous structures (**b**), sometimes many $\mu$m in length. The bar represents 500 nm in (**a**) and 200 nm in (**b**).

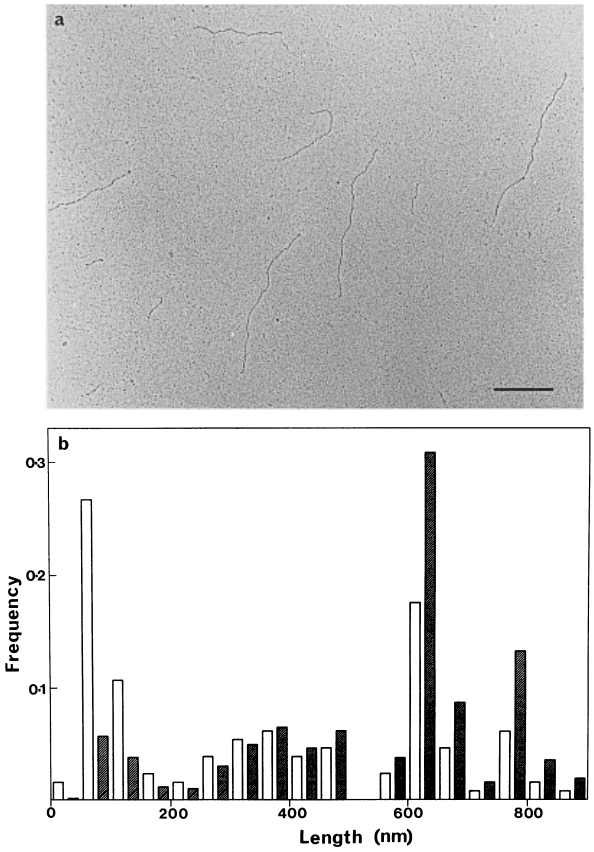


**Figure 6 Electron microscopy of reduced mucin subunits**

(**a**) The molecules appear as extended structures shorter than those observed for the unreduced macromolecules (Figure 5). The bar represents 200 nm. (**b**) The length distribution (150 molecules), as number-average (open boxes) and weight-average (filled boxes) lengths, indicates a wide range of sizes. The weight distribution is dominated by distinctive components at around 625 nm and 775 nm. The number average distribution is dominated by a component around 75 nm.

antisera and the mucin distribution was reactive only with the non-VNTR MUC2 antiserum (LUM 2-3). No reactivity was observed with probes for MUC1 (VNTR), MUC3 (VNTR), MUC4 (VNTR) and MUC5ac (both VNTR and non-VNTR) or with a MUC2 VNTR-specific antiserum even after deglycosylation with TFMSA.

The apparent mass distribution of the mucins was assessed by rate-zonal centrifugation and indicated that the molecules were polydisperse as assayed for both carbohydrate, with PAS and sialyl-$Le^x$ reactivity (Figures 2a and 2c), and MUC2 reactivity (Figure 2b). Reduction of disulphide bonds cleaved the large polydisperse mucins into smaller and more uniform fragment/s (Figures 2a–2c), providing clear evidence for a subunit structure. The mass distributions of both the intact and reduced molecules as assayed with PAS and the anti-(non-VNTR MUC2) serum were similar (Figure 2b).

The apparent mass distribution of the reduced mucin subunits was studied by rate-zonal centrifugation performed with a longer run time (Figure 3a). A major PAS-rich subunit population was identified but there was also evidence for a number of minor populations. Virtually all MUC2 reactivity was confined to the major population. Both metabolic labels were associated with the major peak but the [$^{14}$C]proline radiolabel highlighted a slowly sedimenting component at the top of the tube (Figure 3b). No evidence for such a component was found after centrifugation of the intact mucins and this material was apparently released from the oligomeric mucins by reduction. It is not possible to say from our data whether this is a fragment of the mucin apoprotein or if it represents an attached 'non-mucin' protein. A similar observation was made upon reduction of respiratory mucins [30].

Agarose gel electrophoresis was performed on the reduced mucin preparation (Figure 4). Two major subunit bands were observed accounting for some 90 % and 10 % of the [$^{14}$C]proline radioactivity respectively (Figure 4, lane A). These bands were observed in a similar proportion with the non-VNTR MUC2 probe (Figure 4, lane B).

The size and polydispersity of the mucins was confirmed by electron microscopy. Two forms of the molecules were noted, a minor proportion was visualized as convoluted threads (Figure 5a), whereas the major one appeared as filaments 0.5–10 mm in length (Figure 5b). In contrast, reduced mucin subunits were visualized as shorter filaments between 50 nm and 900 $\mu$m in length (Figure 6a). The number and weight distributions for 150

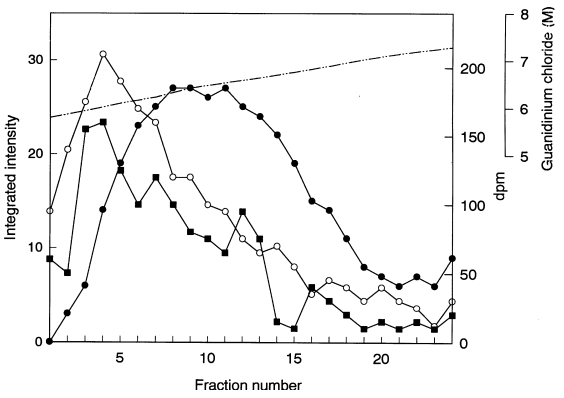


**Figure 7 Rate-zonal centrifugation of the mucins**

A sample (200 $\mu$l) of the purified mucins was layered on to a preformed 6–8 M guanidinium chloride gradient and centrifuged for 2.5 h at 40000 rev./min and 20 °C. Tubes were emptied from the top and fractions (0.5 ml) were analysed for [$^3$H]glucose (■) and [$^{14}$C]proline (○) radioactivity, the concentration of guanidinium chloride (— · · —) and with the PAS assay (●). The integrated intensity is defined as in Thornton et al. [25].

reduced subunits are shown in Figure 6(b) and it is clear that the weight distribution is dominated by two distinct components approx. 625 nm and 775 nm in length.

The size distribution of the metabolically labelled mucins, as compared with that for the entire population (assayed with PAS), was assessed with rate-zonal centrifugation (Figure 7). The distributions of [$^{14}$C]proline and [$^3$H]glucose were distinct from the PAS/MUC2 profile in that the radiolabel predominated in the smaller MUC2 species.

## DISCUSSION

The cell line PC/AA is the first premalignant human colorectal cell line to be studied with regard to mucin production and a previous investigation identified a major large sialic acid-rich mucin both in the cell layer and in the medium [19]. Here we have further analysed the cell layer form of this mucin. Purification was achieved by density-gradient centrifugation to yield mucins with a similar density range to that observed for mucins from other human mucus secretions [6,31,32]. The sialyl-Le$^X$ structure, which is formed late in the biosynthesis of the oligosaccharides, is present on mucins throughout the entire size distribution. In the light of these two observations we conclude that we have isolated an intracellular population of mucins that are 'mature' regarding their glycosylation.

Mucins over the entire density and mass distributions reacted strongly with a polyclonal non-VNTR MUC2 antiserum whereas attempts to identify the *MUC*1, 3, 4, and 5*ac* gene products were unsuccessful. The mucins were reduced into subunits which were fractionated on the basis of mass (rate-zonal centrifugation) and charge/size (agarose gel electrophoresis). Two major subunit populations were identified with the metabolic labels and/or an assay for carbohydrate and both were shown to contain the MUC2 apoprotein. Thus, MUC2 appears to be the major mucin stored in the PC/AA cell.

Rate-zonal centrifugation of the oligomeric MUC2 mucin indicated a significant polydispersity in mass similar to that previously observed for both cervical and respiratory mucins [6,23,31] and we estimate that the MUC2 mucins have an approximate range of relative molecular mass from $5 \times 10^6$ to $40 \times 10^6$. Electron microscopy visualizes the molecules as very long threads, polydisperse in length, similar to those we have previously observed for other mucins [5,6,31]. A small number of molecules are of extreme size and exhibit a highly convoluted and entangled form. The reduced MUC2 subunits were much smaller and were visualized as extended filaments. The weight-average length distribution indicates that the major molecular size is between 600 and 900 nm with distinct species at approx. 625 nm and 775 nm.

The electron microscopy data indicate the presence of MUC2 mucins that must be at least 12 subunits in length and the rate-zonal centrifugation data are consistent with this observation. Thus, the extent of oligomerization (dimers to tetramers) noted in previous biosynthetic studies [14–16] cannot explain the presence of the much larger mucins observed here. Rate-zonal centrifugation shows that the size of intact MUC2 mucins as viewed by the metabolic labels and carbohydrate assay are different. Although the radiolabelled molecules occur over the entire size range, the smaller ones are preferentially labelled. In contrast, there is no evidence for differences between labelled and unlabelled subunits. The coincidence in size distribution of [$^3$H]glucose (labels mainly carbohydrate) and [$^{14}$C]proline (labels protein) within the intact molecules suggests that most of the carbohydrate precursor is added on to the newly synthesized apoproteins and not at a later stage on mucins stored in the cell. If oligomerization is completed within the endoplasmic reticulum before glycosylation, as suggested in previous studies [14–16], it would be expected that carbohydrate assays (showing the final distribution) and radiolabel (showing the size of the newly synthesized ones) would coincide. Since this is clearly not the case, we conclude that final oligomerization must take place downstream to the trans-Golgi and we postulate that mucin assembly takes place in two stages, i.e. before and after O-glycosylation. Possibly, this assembly process takes place in secretory vesicles as proposed for the von Willebrand factor [17].

The fact that the radiolabel is not representative for the whole distribution even after 48 h has important consequences for metabolic labelling studies on mucins and subsequent investigations on the mechanisms of their secretion and assembly. The radiolabelled molecules are presumably representative of the most recently synthesized ones, but if a significant proportion of the mucins were present in the cells before the metabolic labels were introduced the latter would be excluded from analysis. Short labelling periods followed by experiments relying solely on radioactivity to detect the mucins might give misleading information about their quantity and size. Our data indicate that mucins may be stored in the cell over periods in excess of 2 days and very long labelling times might be needed to achieve a homogenous and representative labelling.

### Conclusions

The *MUC*2 gene product appears to be the major mucin synthesized and stored by PC/AA cells under the culture conditions described here. The molecules are present as long filaments up to 10 $\mu$m in length that after reduction are cleaved into two distinct subunit populations (625 and 775 nm). The 'mature' mucins may be stored in the cell for more than 2 days and over a period of time, possibly exceeding this, undergo a thiol-mediated assembly process resulting in the very polydisperse distribution of molecular sizes present. This final assembly may take place after O-glycosylation, in the Golgi or post-Golgi compartment.

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