Biosynthesis of two distinct types of mucin in HM3 human colon cancer cells

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Mucins, high- M_r glycoproteins with a large amount of O-glycosidically linked carbohydrate, protect the colonic epithelial surface and are altered in ulcerative colitis and colon cancer. At least two mucin genes, *MUC2* and *MUC3*, are expressed at high levels in the human intestine. As an experimental model for studying the biosynthesis of human intestinal mucins, we used HM3 colon cancer cells. When mature mucins labelled with [^3H]glucosamine or [^3H]threonine were analysed by gel filtration, it was found that secreted mucins ($M_r > 10^8$) were larger than soluble cellular mucins (M_r approx. 5×10^6). Only secreted mucin was sensitive to reduction. Both *MUC2* and *MUC3* proteins, identified by labelling with [^3H]threonine or [^{35}S]cysteine and immunoprecipitation with antibodies to synthetic mucin pep-

tides, were already of large size ($M_r > 180000$) by the earliest labelling time (5 min). The *MUC3* precursor was completely degraded by trypsin, but the *MUC2* precursor had a trypsin-resistant fragment of M_r approx. 240000 containing threonine and cysteine. The trypsin-resistant *MUC2* fragment contained N-linked carbohydrate, as indicated by a decrease in size as a result of peptidyl N-glycosidase digestion or tunicamycin treatment of HM3 cells. These results show that HM3 colon cancer cells produce at least two distinct human intestinal mucins. They also indicate that the mechanisms of biosynthesis of intestinal mucins differ from those of other mucin-like glycoproteins that have been studied.

INTRODUCTION

Mucins are high- M_r glycoproteins with a large amount of O-glycosidically linked carbohydrate. They are secreted by the epithelia of a number of organs. In the lower gastrointestinal tract, mucins, which are the main component of the viscous mucus layer that lines the intestine, are thought to protect the epithelial surface from mechanical and enzymic damage, and may also influence diffusion of ions and nutrients [1]. Pathological alterations in the composition of colonic mucins occur in colon cancer and inflammatory bowel disease [2,3]. In colon cancer, there is evidence that elevated mucin production is correlated with increased metastatic potential of tumours [4,5].

Biochemical and immunochemical studies have shown that there are several different types of mucin. Recently, several human mucin genes have been identified [6–10]. At least two mucin genes, *MUC2* and *MUC3*, are expressed at high levels in the intestine [11]. Both *MUC2* and *MUC3* proteins have tandem repeat sequences of amino acids that are thought to be heavily glycosylated [6,7]. The *MUC2* gene encodes a 23-amino-acid repeat peptide with a threonine- and proline-rich consensus sequence, PTTTPITTTTVPPTPTGTQT. The *MUC3* gene encodes a 17-amino-acid repeat peptide with a threonine- and serine-rich consensus sequence, HSTPSFTSSITTETTS. Because the *MUC2* and *MUC3* apomucin polypeptides have not yet been identified in cells or tissues, we studied the biosynthesis of these two distinct human intestinal mucins in HM3 human colon cancer cells [12], using antibodies against synthetic repeat peptides.

In the present study, we first compare properties of the mature mucins in the medium, cytosol and membrane fractions of HM3 colon cancer cells. Second, we study the kinetics of the biosynthesis of the mucin proteins. Third, we compare the

MUC2 and *MUC3* proteins with regard to their susceptibility to trypsin and chymotrypsin. Finally we show that *MUC2* mucin precursors have N-linked carbohydrate.

EXPERIMENTAL

Materials

The preparation of anti-MRP, an antiserum against the *MUC2* tandem repeat synthetic peptide [6], has been described, as has the preparation of human small intestine mucin, designated SIM [7]. The extensive deglycosylation of SIM using anhydrous HF has been described previously as well [7]. This extensively deglycosylated mucin is referred to as SIB and antiserum against this material is designated anti-SIB [7]. [^3H]Glucosamine (30 Ci/mmol) was from ICN (Irvine, CA, U.S.A.). [^3H]Threonine (16 Ci/mmol) was from Amersham (Arlington Heights, IL, U.S.A.). L-[^{35}S]Cysteine (1000 Ci/mmol) was from NEN (Boston, MA, U.S.A.). Endoglycosidase H was from Genzyme (Boston, MA, U.S.A.). Peptidyl N-glycosidase was from Oxford Glycosystems (Abingdon, Oxfordshire, U.K.). Fixed *Staphylococcus aureus* (Zysorbin) was from Zymed Labs (South San Francisco, CA, U.S.A.). Trypsin, chymotrypsin, dithiothreitol, iodoacetamide, phenylmethanesulphonyl fluoride (PMSF), tunicamycin, leupeptin, pepstatin, Sepharose CL-4B, Sepharose 2B, keyhole limpet haemocyanin and other chemicals were from Sigma (St. Louis, MO, U.S.A.). A synthetic peptide with the *MUC3* sequence, KTTSNSTPSFTSSITTETTS, was synthesized by the Biomolecular Resource Center of the University of California, San Francisco. This peptide represents one complete tandem repeat unit plus five amino acids of the next unit. The lysine was added to allow glutaraldehyde conjugation as described in the text.

Cell culture and radiolabelling of cells

HM3, a high-mucin variant of human colon cancer cell line LS174T established and characterized in our laboratory [12], was grown in Dulbecco's modified Eagle's medium H-16 (DMEM) with 10% fetal calf serum.

For radiolabelling with glucosamine, subconfluent cells, grown in 25 cm² culture flasks, were cultured in low-glucose DMEM containing 10% fetal calf serum. After a 24 h starvation period, the cells were washed once in glucose-free DMEM, and subsequently labelled for 24 h with 50 μ Ci/dish [³H]glucosamine in 3 ml of glucose-free DMEM containing 10% fetal calf serum.

For long-term radiolabelling with threonine, cells were pre-incubated for 1 h at 37 °C in threonine-free DMEM and then labelled for 24 h with 250 μ Ci/dish [³H]threonine in 3 ml of low-threonine DMEM (threonine-free DMEM/DMEM, 9:1) containing 10% fetal calf serum.

For short-term radiolabelling with [³⁵S]cysteine or [³H]threonine, subconfluent cells, grown in 35 mm Petri dishes, were preincubated for 1 h at 37 °C in either DMEM lacking cysteine and methionine or threonine-free DMEM. The cells were then labelled for 5–60 min with 250 μ Ci of L-[³⁵S]cysteine (1000 Ci/mmol) or L-[³H]threonine (16 Ci/mmol).

Tunicamycin treatment was performed as follows. Subconfluent cells, grown in 35 mm Petri dishes, were preincubated with 20 μ g/ml tunicamycin for 3 h in DMEM containing 10% fetal calf serum and then for 1 h in cysteine- and methionine-free DMEM containing 10% fetal calf serum and 20 μ g/ml tunicamycin. The cells were then labelled for 1 h with [³⁵S]cysteine in cysteine- and methionine-free DMEM containing 10% fetal calf serum and 20 μ g/ml tunicamycin.

Harvesting and homogenization of labelled cells

After radiolabelling, flasks were chilled on ice and the medium was quickly removed. The cell layers were rinsed twice with 10 mM sodium phosphate, pH 7.4, buffer containing 150 mM NaCl and a protease inhibitor cocktail (0.5 μ g/ml leupeptin, 0.7 μ g/ml pepstatin, 0.2 mM PMSF and 1 mM EDTA), and the washes were combined with medium. The supernatant after centrifugation for 10 min at 170 g was designated 'medium fraction'. The cell layers were scraped into 10 mM Tris/HCl, pH 8.0, containing protease inhibitors as above, then sonicated for 2 min on ice. The cell homogenates were centrifuged (100 000 g, 1 h) and the supernatant was designated 'cytosol fraction'. The pellet was dissolved in the above buffer with 1% Triton X-100, sonicated, and centrifuged (100 000 g, 1 h). The supernatant was designated 'membrane fraction'. These three fractions and the cell homogenates were stored at –70 °C until use.

Gel filtration

All gel-filtration columns were run at 4 °C. Medium, cytosol and membrane fractions were chromatographed at a flow rate of 36 ml/h on a Sepharose CL-4B column (3 cm $\times$ 50 cm), in 10 mM Tris/HCl buffer, pH 8.0, containing protease inhibitors. For chromatography of membrane fractions, the column buffer also contained 0.1% Triton X-100. The void-volume fractions were collected, dialysed against distilled water and lyophilized. The void volume was designated the high- M_r glycoprotein (HMG) fraction.

For analysis of the HMG fractions on Sepharose 2B, the samples were redissolved in 10 mM Tris/HCl, pH 8.0, buffer containing 1 M NaCl, 0.1% Triton X-100 and protease inhibitors

and applied to a Sepharose CL-2B column (1 cm $\times$ 50 cm), which was eluted with the same buffer.

Reduction and protease digestion of HMGs

[³H]Glucosamine- and [³H]threonine-labelled HMGs were dissolved and reduced for 5 h at 37 °C with 10 mM dithiothreitol in 100 mM Tris/HCl, pH 8.0, buffer containing 6 M guanidinium chloride and 5 mM EDTA. The samples were then alkylated with 40 mM iodoacetamide in the dark overnight at room temperature. [³H]Glucosamine- or [³H]threonine-labelled HMGs were treated with trypsin (20 μ g/ml) at 37 °C for 24 h in 50 mM Tris/HCl, pH 8.0.

Immunoprecipitation

Portions of cell homogenates were suspended in 1 ml of immunoprecipitation buffer (1% Nonidet P40, 1 mg/ml BSA, 0.5 M NaCl, 1 mM EDTA, 0.5 μ g/ml leupeptin, 0.7 μ g/ml pepstatin, 1 mM PMSF, 10 mM Tris/HCl, pH 8.0), preincubated with 10 μ l of normal rabbit serum for 30 min at 30 °C, chilled on ice for 15 min under gentle agitation and mixed with 100 μ l of washed 10% Zysorbin for 1 h at 4 °C under gentle agitation. The non-specific complexes were removed by centrifugation at 12 000 g for 2 min. The supernatant was incubated with 20 μ l of anti-apomucin rabbit serum, either anti-MRP (antiserum against MUC2 synthetic peptide) or anti-M3P (antiserum against MUC3 synthetic peptide), for 30 min at 30 °C, and chilled on ice for 15 min under gentle agitation. Then 100 μ l of washed 10% Zysorbin was added and the mixture was incubated overnight at 4 °C under gentle agitation. The specific immunocomplexes were washed three times with immunoprecipitation buffer, then stored at –70 °C until use.

SDS/PAGE analysis

Samples were dissolved in 80 μ l of 0.125 M Tris/HCl, pH 6.8, containing 1.56% SDS, 7% 2-mercaptoethanol, 10% glycerol and 0.05% Bromophenol Blue (sample buffer). After being boiled for 5 min, the samples were clarified by centrifugation (12 000 g, 10 min) and analysed on SDS/5% polyacrylamide gels with a 3% stacking gel [13]. Prestained M_r standards (Bio-Rad) were used as reference. After electrophoresis, the gels were fixed in 40% methanol/10% acetic acid/1% glycerol. For fluorography, the gels were soaked in distilled water for 15 min and immersed in Autofluor (National Diagnostics, Manville, NJ, U.S.A.) for 1 h with gentle shaking. The gels were dried and exposed to Kodak X-Omat AR films at –70 °C.

Enzyme digestion of immunoprecipitates

Immunoprecipitates were washed with 1 ml of 0.1 M NH₄HCO₃, pH 8.0, and the precipitates were suspended in 35 μ l of 0.45% SDS, and boiled for 5 min. After being cooled, the precipitates were digested at 37 °C for 30 min with 2 μ l/ml trypsin or chymotrypsin. The digestion products were analysed by SDS/PAGE.

For peptidyl *N*-glycosidase and endoglycosidase H treatment of immunoprecipitates, cells were labelled with [³H]threonine or [³⁵S]cysteine, precipitated with anti-MRP, solubilized in 0.45% SDS, treated with trypsin (2 μ g/ml) for 30 min at 37 °C, and then boiled for 5 min. After the addition of the same volume of 100 mM sodium phosphate (pH 8.6) containing 2% *n*-octyl- β -glucoside, 2 mg/ml BSA, 0.4 mM PMSF, 2 mM EDTA, 1 μ g/ml leupeptin, 1.4 μ g/ml pepstatin and 0.04% NaN₃, 10 munits of

peptidyl *N*-glycosidase was added, and samples were incubated for 17 h at 37 °C. Alternatively, the trypsin-treated immunoprecipitates were incubated for 17 h at 37 °C with 10 munits of endoglycosidase H in 100 mM sodium phosphate (pH 5.5) containing 2 mg/ml BSA, 0.4 mM PMSF, 2 mM EDTA, 1 µg/ml leupeptin, 1.4 µg/ml pepstatin and 0.04 % NaN₃.

RESULTS

Characterization of mature mucins

The HMGs synthesized and secreted by the HM3 variant of LS174T colon cancer cells have been shown to have properties characteristic of mucins [12]. However, the sizes of these products, which are excluded on gel filtration on Sepharose CL-4B, have not been established. Furthermore, although membrane-bound mucin-like glycoproteins have been described in several cell lines [14–16], HM3 colon cancer cells have not been investigated for the presence of membrane-bound mucin.

Mucins were metabolically labelled and the HMG fraction was isolated by gel filtration on Sepharose CL-4B as in previous studies in this laboratory [4,12,17–20]. Table 1 demonstrates that HMG in the medium, cytosol and membrane fractions can be

Table 1 Labelling of HMG with [³H]glucosamine and [³H]threonine

Results are given as 10⁻⁵ × c.p.m./mg of protein recovered in the void volume after Sepharose 4B chromatography. They are means (± range) (*n* = 2).

	Medium	Cytosol	Membrane
[³ H]GlcN labelling	8.6 ± 1.9	1.4 ± 0.3	0.5 ± 0.2
[³ H]Thr labelling	8.2 ± 0.4	3.7 ± 0.4	8.1 ± 0.9
[³ H]GlcN/[³ H]Thr ratio	1.05	0.38	0.06

Figure 1 Autoradiogram of SDS/PAGE of [³H]glucosamine-labelled mucin from membrane, cytosol and medium fractions

The void-volume fractions from Sepharose CL-4B columns of membrane (lanes A, D), cytosol (lanes B, E), and medium (lanes C, F) fractions were subjected to SDS/PAGE with (lanes A–C) or without (lanes D–F) reduction with 2-mercaptoethanol. The arrowhead indicates the interface between the stacking and separating gels.

Figure 2 Chemical and enzymic degradation of [³H]glucosamine-labelled mucin

Portions of [³H]glucosamine-labelled HMGs from the membrane (A), cytosol (B) and medium (C) fractions were chromatographed on Sepharose CL-2B. Results shown for membrane, cytosol and medium fractions correspond to 1 mg, 0.2 mg and 0.05 mg of cellular protein respectively. Arrows, left to right, show the elution positions of Blue Dextran 2000 (*M_r* ≈ 2 × 10⁶), dextran (*M_r* ≈ 4.8 × 10⁵) and Phenol Red as an included-volume marker. (a) Control HMG fractions without treatment; (b) HMG digested for 24 h at 37 °C with 20 µg/ml trypsin; (c) reduced and alkylated HMG.

labelled with [³H]glucosamine and with [³H]threonine. HMG labelled with [³H]glucosamine is present in all three fractions, but most of it is secreted during the course of 24 h labelling. The lower ratio of [³H]glucosamine to [³H]threonine in the membrane HMG could be because either it is less glycosylated or this fraction contains more non-glycosylated (non-mucin) proteins.

The [³H]glucosamine-labelled HMGs were further examined by SDS/PAGE and autoradiography (Figure 1). The HMG of all three fractions contained mucin of very large size. Although estimation of the size of these products is uncertain, all appear to be considerably larger than the 180000-*M_r* standard. There are differences in the apparent sizes of the mucin in the three fractions, with the secreted mucin being largest. Because the membrane mucin is smaller than that in the medium or cytosol, it is unlikely to have been adsorbed to the membranes during cell

Figure 3 Chemical and enzymic degradation of [^3H]threonine-labelled mucin

Portions of [^3H]threonine-labelled HMGs from the membrane (A), cytosol (B) and medium (C) fractions were chromatographed on Sepharose CL-2B. (a) Control HMG fractions without treatment; (b) HMG digested for 24 h at 37 °C with 20 $\mu\text{g}/\text{ml}$ trypsin; (c) Reduced and alkylated HMG. Results shown for membrane, cytosol and medium fractions correspond to 0.1 mg, 0.2 mg and 0.05 mg of cellular protein respectively.

harvesting. The membrane HMG has some radioactivity at the dyefront, suggesting that some of the ^3H label in this fraction may be in mucin-associated glycoprotein or glycolipid rather than a part of the mucin molecule.

To test for the presence of disulphide-linked aggregates, the products were also examined by SDS/PAGE in the presence of 2-mercaptoethanol (Figure 1, lanes D–F). Here we observed that the difference in size of the mucins in the three fractions remained even after reduction. The medium HMG, with reduction-sensitive labelled products in the stacking gel, was the only fraction that was greatly affected by 2-mercaptoethanol treatment. Unlike secreted mucin, there appear to be no disulphide-linked aggregates in cellular mucin.

The mucins present in the medium, cytosol and membrane fractions were further examined by gel filtration on Sepharose 2B. For medium HMG, most of the [^3H]glucosamine label

(Figure 2a) and the [^3H]threonine label (Figure 3a) was at or near the void volume of Sepharose 2B. These are of median apparent $M_r > 1 \times 10^6$ (Table 2). HMG from the cytosol was smaller, with a median M_r approx. 5×10^5 . Membrane-bound HMG was smaller than the Blue Dextran 2000 marker. Even though SDS/PAGE indicated that much of the [^3H]glucosamine in the membrane HMG is in low- M_r molecules, no separation was evident on gel-filtration analysis. One possible explanation for this is that the low- M_r product is very tightly bound to mucin.

When medium HMG was digested with trypsin, most of the [^3H]glucosamine (Figure 2b) and [^3H]threonine (Figure 3b) were still near the void volume, consistent with clustering of the O-linked oligosaccharides in threonine-rich regions of the mucin molecule. However, there was a portion of the [^3H]threonine label (about 31%) and the [^3H]glucosamine label (about 19%) degraded to products of $M_r < 2 \times 10^6$ (Table 2). This suggests that the trypsin-sensitive regions are more sparsely glycosylated. In the cytosol HMG, the [^3H]glucosamine-labelled mucin was resistant to trypsin, but about 40% of the [^3H]threonine label was shifted to a size of $M_r < 2 \times 10^6$. The HMG from the membrane fraction was already of smaller size than that from the medium or from cytosol. For membrane-bound mucin, trypsin digestion produced a peak of both [^3H]threonine and [^3H]glucosamine near the included volume ($M_r < 1.4 \times 10^6$).

When medium HMG, labelled with either [^3H]glucosamine or [^3H]threonine, was reduced and alkylated, the sharp peak at the void volume of Sepharose 2B was shifted to a broader peak (Figures 2c and 3c). However, the reduced medium mucin was still relatively large, and only 17% was of $M_r < 2 \times 10^6$. No low- M_r product, e.g. 'link' protein of M_r approx. 118000, was detected [21]. For cytosol HMG, reduction and alkylation had little effect on the size of the glucosamine-labelled glycoprotein, but there was release of a threonine-labelled fragment of M_r approx. 430000. This indicates that there is non-glycosylated protein disulphide-linked to the glycosylated portion of soluble cellular mucin. For membrane HMG, on the other hand, reduction had little effect on the size distribution of [^3H]threonine-labelled molecules. However, the glucosamine-labelled molecules were shifted to a smaller size (to M_r approx. 7.5×10^5 from M_r approx. 1.5×10^6).

Preparation and characterization of antisera against the MUC3 tandem repeat synthetic peptide, anti-M3P

We previously prepared antiserum to a synthetic peptide based on the MUC2 sequence (anti-MRP) [6]. Antibodies were similarly prepared against a MUC3 synthetic peptide here. A 1:1 weight ratio of the synthetic peptide KTTSNSTPSFTSSITTTETTSHS was linked to keyhole limpet haemocyanin using glutaraldehyde as described [22]. This conjugate (1 mg) was then injected into a rabbit at multiple sites using complete Freund's adjuvant. After 1 month the rabbit was boosted using incomplete Freund's adjuvant. The rabbit was bled after 2 weeks and serum was prepared and analysed for binding activity against deglycosylated human small intestine mucin (SIB). As shown in Figure 4(a), this antiserum, designated anti-M3P, bound to SIB, as did anti-SIB. The more avid binding of anti-SIB to SIB is to be expected as SIB contains epitopes from both MUC2 and MUC3. Both anti-SIB and anti-M3P also bound to a conjugate of M3P and BSA (Figure 4b). This indicates that both antisera recognize the synthetic peptide. This latter point is important as antibodies against keyhole limpet haemocyanin only could have been produced, but if so, anti-M3P would be expected to lack activity against the M3P-BSA conjugate. The lack of cross-reactivity of

Table 2 Gel filtration of HMG on Sepharose 2B

	Medium		Cytosol		Membrane	
	GlcN	Thr	GlcN	Thr	GlcN	Thr
$10^{-6} \times$ Median M_r	120	170	4.4	5.4	1.5	0.5
Percentage of label at $M_r < 2000000$						
Control	8	8	36	35	54	81
Trypsin-treated	27	39	36	75	81	96
Reduced/alkylated	17	16	30	61	68	89

Figure 4 E.I.I.s.a. of anti-M3P

The wells of a microtitre dish were coated overnight with 25 ng of SIB (a) or 25 ng of an M3P-BSA conjugate (b). Assays were conducted as described previously [23]. Anti-SIB ($\square$), anti-M3P ($\blacktriangle$) or preimmune serum from the anti-M3P rabbit ($\bullet$) was used at the dilutions indicated.

anti-M3P with the MUC2 synthetic peptide, MRP, is demonstrated in Figure 5.

Kinetics of mucin synthesis

To study the biosynthesis of individual mucin proteins, we labelled HM3 cells and immunoprecipitated mucin precursors with either anti-MRP or anti-M3P. No mature mucin, labelled with [^3H]glucosamine, was immunoprecipitated with either anti-MRP or anti-M3P (Figure 6). This is the expected result, as the

Figure 5 Immunoblot using anti-M3P

Proteins were electrophoresed through 7% polyacrylamide gels, transferred to nitrocellulose, and probed using a 1:75 dilution of anti-M3P as described [6]. Samples were: lane A, SIM; lane B, SIB; lane C, BSA; lane D, M3P-BSA conjugate; lane E, MRP-BSA conjugate. Each lane contained 2.5 μg of protein. The polydispersity of the signal in lane D is due to cross-linking of the BSA monomers and irregular conjugation of the synthetic peptide. The small size of SIB is due to random peptide-bond cleavage during deglycosylation [6,23].

Figure 6 Apomucins in HM3 cells labelled with [^3H]threonine, [^3H]glucosamine and [^{35}S]cysteine

HM3 cells were labelled with [^3H]threonine for 24 h, [^3H]glucosamine for 24 h and [^{35}S]cysteine for 5–60 min. Cell homogenates were subjected to immunoprecipitation with anti-MRP or anti-M3P. The immunoprecipitates were analysed on a 5% polyacrylamide gel.

apomucin peptide antigens in mature mucin are cryptic because of glycosylation [23]. In cells labelled with [^3H]threonine for 24 h, the only product immunoprecipitated by the two anti-apomucin sera was of very high M_r , at the top of the separating gel on SDS/PAGE. Because of the large size of these products, it was considered possible that they could be aggregates formed late in the biosynthetic process rather than apomucins. Therefore we examined earlier time points using [^{35}S]cysteine. By the earliest time point, the mucin precursors were already of very high M_r . The earliest products labelled were much larger than those found

for human MUC1 mucin [24,25]. Thus there are apparently no small precursors for MUC2 or MUC3 mucin.

Differences in MUC2 compared with MUC3 precursors

Because the products immunoprecipitated by both anti-MRP and anti-M3P barely migrated on SDS/PAGE, it was not possible to distinguish them on the basis of size. Hence, we sought to determine whether specific mucin precursors or mixed aggregates were immunoprecipitated under these conditions. In a sequential immunoprecipitation experiment (Figure 7), labelled homogenates were pretreated with anti-M3P, then precipitated with anti-MRP. Conversely, labelled homogenates were pretreated with anti-MRP and then precipitated with anti-M3P. For both [^3H]threonine-labelled mucin precursors and [^{35}S]cysteine-labelled mucin precursors, there was little or no decrease in the amount of MUC2 mucin precursor by anti-M3P pretreatment and little or no decrease in the amount of MUC3 mucin precursor by anti-MRP pretreatment. This applies to both the material at the stacking/separating gel interface and the material entering the gel. Thus there is very little cross-reaction between the two apomucin-specific antisera. Furthermore, these results indicate that there are no (cellular) mixed aggregates of MUC2 and MUC3 mucin precursors. Interestingly, a somewhat different population of [^{35}S]cysteine-labelled bands appears to have entered the separating gel in the experiment shown in Figure 7 from that shown in Figure 6, at least for MUC3. This may be the result of slightly different conditions of labelling in the two experiments or perhaps slightly different electrophoretic conditions affecting the appearance of the two autoradiograms. Also, there may have been some difference in the efficiency of the removal of impurities in the two sets of immunoprecipitations. In any event, each of the two antisera is clearly capable of immunoprecipitating its specific mucin antigen without immunoprecipitating the other mucin.

To characterize the MUC2 and MUC3 mucin precursors further, we investigated their susceptibility to digestion with trypsin (Figure 8). We found that MUC2 apomucin is much more resistant to trypsin digestion than MUC3 apomucin, which is further evidence of the distinctness of the two mucins. Although the MUC3 mucin precursors are completely degraded by 0.2 $\mu\text{g}/\text{ml}$ trypsin, there are trypsin-resistant fragments in MUC2 precursors, even when digested with a 100-fold higher concentration of trypsin.

The proteolytic susceptibility of the MUC2 and MUC3 precursors was further investigated using both [^3H]threonine and [^{35}S]cysteine as labels (Figure 9). Trypsin digestion of MUC2 gave a fragment with an approximate M_r of 240 000 which is labelled by both threonine and cysteine. This may represent one of the cysteine-rich domains of the MUC2 mucin molecule, whereas larger tryptic products lacking cysteine could be either the tandem repeat region or other threonine-rich regions of the molecule [26,27]. Chymotrypsin was less useful for characterization of the MUC2 product, as only high- M_r products were formed from the [^3H]threonine-labelled immunoprecipitate, whereas a wide range of products was formed from [^{35}S]cysteine-labelled immunoprecipitate. Chymotrypsin digestion of [^3H]threonine-labelled MUC3 immunoprecipitates gave a fragment of M_r approx. 219 000. The same product, as well as larger and smaller fragments, was also present in [^{35}S]cysteine MUC3 immunoprecipitates. As for [^3H]threonine-labelled MUC3 (Figure 8), trypsin digestion of [^{35}S]cysteine-labelled MUC3 gave complete degradation.

Figure 7 Sequential immunoprecipitation with anti-MRP and anti-M3P

Homogenates of HM3 cells labelled for 60 min with either [^3H]threonine or [^{35}S]cysteine were pretreated with normal rabbit serum or with anti-M3P; then residual MUC2 apomucin was immunoprecipitated with anti-MRP. Homogenates were also pretreated with normal rabbit serum (NRS) or with anti-MRP; then residual MUC3 apomucin was immunoprecipitated with anti-M3P. The mobility of molecular-size standards is indicated by arrows.

Figure 8 Trypsin treatment of MUC2 and MUC3 apomucins

Cells were labelled for 60 min with [^3H]threonine, and immunoprecipitated with anti-MRP (lanes A–D) or anti-M3P (lanes E–H). Samples of the immunoprecipitates were solubilized in 0.45% SDS, and either analysed directly (lanes A and E) or incubated for 30 min at 37 °C with trypsin at concentrations of 0.2 $\mu\text{g}/\text{ml}$ (lanes B and F), 2 $\mu\text{g}/\text{ml}$ (lanes C and G) or 20 $\mu\text{g}/\text{ml}$ (lanes D and H).

Presence of N-linked oligosaccharides in MUC2 mucin precursors

From the sequence of the MUC2 gene [6,26,27], it is known that there are several potential N-glycosylation sites (Asn-Xaa-Thr/Ser) in the mucin molecule, but it has not been established whether N-linked oligosaccharides are added to these sites. To test for the presence of N-linked carbohydrate in MUC2 mucin, trypsin-treated anti-MRP immunoprecipitates were digested with peptidyl N-glycosidase and endoglycosidase H (Figure 10). The

Figure 9 Proteolytic degradation of MUC2 and MUC3 apomucins

Homogenates of cells labelled for 60 min with [^3H]threonine (lanes A, B, E, F) or [^{35}S]cysteine (lanes C, D, G, H) were immunoprecipitated with anti-MRP (A–D) or anti-M3P (E–H), then digested with either 2 $\mu\text{g}/\text{ml}$ chymotrypsin (A, C, E, G) or 2 $\mu\text{g}/\text{ml}$ trypsin (B, D, F, H), and analysed by SDS/PAGE. The solid arrowhead marks the location of a trypsin-resistant fragment of MUC2; the open arrowhead marks the location of a chymotrypsin-resistant fragment of MUC3.

Figure 10 Peptidyl *N*-glycosidase and endoglycosidase H treatment of tryptic fragments of MUC2 apomucin

Cells were labelled for 60 min with [^3H]threonine or [^{35}S]cysteine, precipitated with anti-MRP, solubilized in 0.45% SDS, treated with trypsin (2 $\mu\text{g}/\text{ml}$) for 30 min at 37 $^{\circ}\text{C}$, and then boiled for 5 min. After the addition of protease inhibitors, the samples were incubated for 17 h at 37 $^{\circ}\text{C}$ with 10 munits of endoglycosidase H or peptidyl *N*-glycosidase. The arrowhead on the left marks the interface between the stacking and separating gels. The arrowheads on the right mark the mobility of control and *N*-glycanase-treated segments.

trypsin-resistant fragment of MUC2 mucin precursor is sensitive to digestion with peptidyl *N*-glycosidase, with a decrease in M_r of about 20000. The presence of aggregates at the top of the separating gel is unexplained.

Figure 11 Effect of tunicamycin on biosynthesis of MUC2 mucin precursors

Cells were labelled with [^{35}S]cysteine for 60 min in the presence (lanes C, D) or absence (lanes A, B) of 20 $\mu\text{g}/\text{ml}$ tunicamycin and immunoprecipitated with anti-MRP. Samples of the immunoprecipitates were solubilized in 0.45% SDS, and analysed by SDS/PAGE with (lanes B, D) or without (lanes A, C) prior trypsin digestion. The open and closed arrowheads mark the mobility of trypsin-resistant bands observed from cells labelled in the absence and presence of tunicamycin respectively.

The presence of N-linked carbohydrates in MUC2 mucin was further demonstrated by testing the effect of tunicamycin, which specifically inhibits addition of N-linked oligosaccharides (Figure 11). Tunicamycin treatment of HM3 cells decreased incorporation of [^{35}S]cysteine into mucin precursors precipitable by anti-MRP. Furthermore, the tryptic fragment is reduced in tunicamycin-treated cells by M_r 26000 compared with the control. This is in substantial agreement with the results of peptidyl *N*-glycosidase treatment. The decrease seen in treated cells in total [^{35}S]cysteine labelling suggests that N-glycosylation, although accounting for a minor proportion of the total size of mature MUC2 mucin, may be critical for the stability or processing of MUC2 precursors.

DISCUSSION

Although a previous study from this laboratory showed that HM3 cells express mRNAs for both the *MUC2* and the *MUC3* genes [7], it was not known whether these cells made both types of apomucin protein. We find that both proteins are synthesized, with similar kinetics. We also find that the mature mucins secreted by HM3 cells differ from the soluble intracellular mucin and from membrane-bound HMG. However, it is not yet known whether the cellular mucins are immature forms of the secreted mucin or if they represent distinct proteins.

The much greater sensitivity to degradation by trypsin of MUC3 precursors compared with MUC2 precursors confirms that the two are distinct. However, the physiological significance of the trypsin sensitivity of MUC3 protein is unclear, as it is mature intestinal mucin, rather than underglycosylated intracellular precursor, that would normally be exposed to pancreatic proteases. The relative protease resistance of MUC2 protein might be of practical value, as it suggests that antibodies to MUC2 apomucin, which preferentially stain some gastro-intestinal tumours in immunohistochemical studies [11,28,29], might be expected to detect cancer-associated faecal antigens also.

Although predicted from DNA sequences [6,26,27], this is the first direct demonstration of N-linked carbohydrate in MUC2 mucin. For MUC1 mucin, the presence of N-linked oligosaccharides has been confirmed by demonstrating shifts in the mobility of metabolically labelled mucin precursors after digestion with endoglycosidase H and peptidyl *N*-glycosidase [24,25].

We find that the [³⁵S]cysteine-labelled tryptic fragment, which probably corresponds to either the C-terminal or N-terminal region of the MUC2 molecule, is decreased in *M_r* by about 20 000 by peptidyl *N*-glycosidase digestion or by tunicamycin treatment of the cells. This is consistent with the enrichment of potential N-glycosylation sites in the cysteine-rich domains of MUC2 [26,27]. The lack of effect of endoglycosidase H digestion on the mobility of the trypsin-resistant fragment may reflect the relatively long labelling time (60 min) used in these experiments. It should be noted that other N-linked oligosaccharides, present in trypsin-sensitive regions of the molecule, would not be detected in our experiments. However, the content of N-linked carbohydrate in MUC2 mucin must be low, as mannose was not detectable by chemical analysis in mucin purified from xenografts of LS174T, the parental cell line for HM3 [30]. Because the MUC3 mucin precursor is much more sensitive to trypsin, the possible presence of N-linked oligosaccharides in MUC3 mucin was not investigated.

The MUC2 and MUC3 mucins are quite different from MUC1 mucin. No low-*M_r* precursors are detected in MUC2 or MUC3, even at 5 min labelling time. Recent DNA-sequencing results [27] indicate that MUC2 mucin, with about 5000 amino acids, is much larger than MUC1 mucin. MUC2 mucin also differs from MUC1 mucin in that it has two cysteine-rich domains, at the C-terminus and the N-terminus, which may be important in oligomerization of mucin monomers. Furthermore, our tunicamycin experiments suggest that, for MUC2, the N-linked oligosaccharide, although small in amount, could be functionally important for the folding and stability of the cysteine-rich domains [26,27].

Two other mucin-like glycoproteins, rat gastric mucin [31] and the ASGP-1 glycoprotein from rat mammary adenocarcinoma cells [16], also have biosynthetic mechanisms distinct from human MUC1 mucin. Biosynthetic maturation of ASGP-1 involves slow recycling of cell-surface mucin through intracellular compartments where additional carbohydrate is added [16]. Biosynthesis of rat gastric mucin involves an early intracellular oligomerization, a process that requires prior N-glycosylation [31]. As we find disulphide-linked aggregates in mature secreted mucin but not in soluble cellular mucin or mucin precursors, the oligomerization of human intestinal mucins must occur at a later stage. The diversity of biosynthetic mechanisms for the post-translation processing of different mucins may provide cells with another opportunity, besides regulation of mucin gene transcription, to control the amount and type of mucin produced.

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