Human MUC5AC mucin dimerizes in the rough endoplasmic reticulum, similarly to the MUC2 mucin

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Biosynthetic studies on the human MUC5AC mucin were performed by immunoprecipitations with antisera recognizing only the non-O-glycosylated apomucin in the colon adenocarcinoma cell line LS 174T. Pulse–chase studies and subcellular fractionations showed that MUC5AC formed dimers in the rough endoplasmic reticulum within 15 min of the initiation of biosynthesis. No non-O-glycosylated species larger than dimers were identified. The dimerization was N-glycosylation-dependent, because tunicamycin treatment significantly lowered the rate of dimerization. When the biosynthesis of MUC5AC apomucin was compared with that of MUC2 apomucin, also produced in the LS 174T cell line, both apomucins were assembled in similar ways with respect to their rates of dimerization with and without inhibition of N-glycosylation. No heterodimerization was observed between the human MUC5AC and the MUC2 apomucins despite the extensive sequence similarities in the positions of the cysteine residues in the C-termini proposed to be involved in mucin dimerization.

INTRODUCTION

Mucins are highly glycosylated proteins with large masses composed of more than 50 % carbohydrates, O-linked to serine and threonine residues [1,2]. These amino acids are concentrated in domains (mucin domains), often with sequences of tandemly repeated amino acids. Mucins are the main macromolecular component in the mucus layer protecting and lubricating the mucosa. The gel-forming mucins are produced by specialized cells (goblet cells) within the surface epithelium or in glands. Today nine human mucins are known, of which the membrane-bound MUC1 mucin is the best studied [3]. The secreted mucins, one of the major constituents in the viscous gel covering the mucosal surface, are proposed to form disulphide bond-stabilized oligomers. The human MUC2, MUC5AC, MUC5B and MUC6 mucins are all secreted. These four mucins genes are clustered on chromosome 11p15.5 [4] and their C-termini show sequence similarities in the positions of the cysteines.

MUC5AC is one of the major mucins expressed in the lung and stomach. The full sequence is not known but several partial sequences, not fully compatible, have been published by several groups [5–8]. Meerzaman et al. [5] cloned a partial mucin on the basis of the sequence of tryptic peptides. This sequence is only partly compatible with the C-terminus of MUC5AC as deduced by Lesuffleur et al. [8], showing large sequence similarities to the MUC2 sequence [9]. In contrast with MUC2, the middle portion of MUC5AC is made up of several mucin domains interspaced with cysteine-rich domains [6,8]. The complete N-terminus has recently been sequenced from clones isolated from stomach [7]. The C-terminus of MUC5AC [8] apomucin is similar, in the number and positions of the cysteine residues, with the C-termini of both MUC2 mucin and von Willebrand factor (vWF); vWF and human MUC2 mucin have both been fully sequenced and show sequence similarities in both the N-terminus and the C-terminus with respect to the positions of the cysteine residues [9,10]. vWF is known to form disulphide bond-stabilized dimers by linking two C-termini. Further oligomerization of vWF takes place by the formation of disulphide bonds between N-termini [11]. We have previously studied the biosynthesis of human MUC2 mucin in the colon carcinoma cell line LS 174T by immunoprecipitation and analysis by both SDS/PAGE and SDS/agarose gel electrophoresis [12]. MUC2 mucin shows N-glycosylation-dependent dimerization in the endoplasmic reticulum. Both the monomer and the dimer are transferred into the Golgi complex and become O-glycosylated. We now show that MUC5AC apomucin forms dimers in the endoplasmic reticulum in a similar N-glycosylation-dependent way to MUC2 apomucin and that no non-O-glycosylated oligomers larger than dimers are formed. Despite the similarities in the C-termini of MUC2 and MUC5AC, no heterodimerization was observed between the apomucins.

The major mucin from the small intestine of rat has been shown to be insoluble in guanidinium chloride and to be encoded by the *Muc2* gene [13–15]. This mucin contains two highly glycosylated domains, named glycopeptide A (gpdA) and B (gpdB), that can be isolated after trypsin digestion. An antiserum made against the HF-deglycosylated gpdA has been shown also to react with the human non-O-glycosylated MUC2 mucin [12]. This antiserum is now shown to react also with non-O-glycosylated human MUC5AC mucin.

METHODS

Antibodies

The rabbit antiserum anti-(glycopeptide A) (α-gpdA; PH497) was obtained by immunization with HF-deglycosylated gpdA from rat as described [14]. The antiserum anti-(MUC2 tandem repeat) (α-MUC2TR; PH897) against a synthetic peptide based on the tandem repeat region of the human MUC2 apoprotein has been described previously [12]. The antisera anti-(MUC2 N-terminal region) (α-MUC2N3; PH1491) [16] and anti-(MUC2 C-terminal region) (α-MUC2C2; PH1417) [17] were raised against synthetic peptides based on unique regions in the MUC2

Abbreviations used: α-gpdA, anti-(glycopeptide A); α-MUC2TR, anti-(MUC2 tandem repeat); α-MUC5ACCR, anti-(MUC5AC cysteine-rich regions); α-MUC5ACTR, anti-(MUC5AC threonine-rich regions); α-MUC2N3, anti-(MUC2 N-terminal region); α-MUC2C2, anti-(MUC2 C-terminal region); gpd, glycopeptide; NEM, *N*-ethylmaleimide; vWF, von Willebrand factor.

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mucin N-terminus and C-terminus respectively. Two rabbit antisera were prepared against synthetic peptides, anti-(MUC5AC threonine-rich regions) (α-MUC5ACTR; PH1428), based on a repeated threonine-rich sequence in the human MUC5AC apomucin (CHRPHPTPTTVGPTTVGS), and anti-(MUC5AC cysteine-rich regions) (α-MUC5ACCR; PH1489), based on a repeated cysteine-rich sequence in the human MUC5AC apoprotein (RNQDQQGPFKMC) were prepared as follows. New Zealand White rabbits were immunized once with 375 μg of peptide conjugated to 300 μg of keyhole-limpet haemocyanin in Freund's complete adjuvant and then twice with 250 μg of peptide conjugated to 200 μg of keyhole-limpet haemocyanin in Freund's incomplete adjuvant.

Tissue culture

The colon adenocarcinoma cell lines LS 174T (ATCC CL 188) and HT-29 (ATCC HTB 38) were cultured in Iscove's modified Dulbecco's medium (Gibco, Paisley, U.K.) containing 10% (v/v) fetal calf serum and supplemented with 110 mg/l sodium pyruvate, 36 mg/l L-asparagine, 116 mg/l L-arginine, 290 mg/l L-glutamine and 10 mg/l folic acid. Cells were grown at 37 °C under air/CO_2 (19:1) at 95% humidity.

Metabolic labelling

Cells were seeded at a concentration of approx. 5×10^7 cells per 28 cm² Petri dish the day before metabolic labelling. Cells were preincubated in methionine-free medium for 1 h followed by radiolabelling with 150 μCi of labelling mix per dish (Redivue Promix [^{35}S] labelling mix; Amersham Pharmacia Biotech). In pulse–chase experiments, cells were chased with culture medium supplemented with 15 μg of methionine/ml of medium and 25 μg of cysteine/ml of medium. Cells were washed and lysed as described [12] but with the addition of 5 mM *N*-ethylmaleimide (NEM) to the lysis buffer in some of the experiments. Inhibition of N-glycosylation was performed by incubating cells with 20 μg of tunicamycin (Calbiochem, La Jolla, CA, U.S.A.) per ml of methionine-free medium 1 h before labelling, as well as during the pulse and the chase.

Subcellular fractionation

Cells were washed twice with 5 ml of 250 mM sucrose and twice in 5 ml of 50 mM sucrose followed by harvesting of the cells in 500 μl of 50 mM sucrose including protease inhibitors (110 μg/ml PMSF/20 μg/ml aprotinin/60 μg/ml leupeptin/3.8 μg/ml calpain inhibitior I). Cells were carefully scraped off with a cell scraper and homogenized on ice with 15 strokes in a Dounce homogenizer with a tight pestle. The sucrose concentration was adjusted to 250 mM followed by an additional five strokes in the homogenizer. The cell homogenate was centrifuged at 1600 ***g*** at 4 °C for 10 min, and the supernatant was recovered. The cell pellet was washed twice in 250 μl of 250 mM sucrose and the combined supernatants were loaded on a sucrose gradient in a centrifuge tube containing a 4 ml gradient of 35–50% (w/w) sucrose on top of a 400 μl cushion of 65% sucrose. All sucrose solutions used contained 3 mM imidazole, pH 7.4. Ultracentrifugation was performed at 250000 ***g*** at 12 °C for 3 h in a Beckman vertical rotor (Vti:65.2) and the fractions were collected from the bottom of the tube. Lysis buffer [50 mM Tris/HCl (pH 7.4)/150 mM NaCl/5 mM EDTA/1% (v/v) Triton X-100], containing protease inhibitors (as above) and 5 mM NEM, was added to each fraction to a final volume of 1.5 ml. Samples were sonicated three times for 2 s (intensity 15) on an MSE Soniprep 100. Collected fractions were analysed under reducing conditions by SDS/PAGE [12% (w/v) gel] followed by Western blotting. Electrophoretic transfer to a nitrocellulose membrane was performed with 48 mM Tris/39 mM glycine/0.0375% SDS/20% (v/v) methanol (pH 8.3). An anti-calnexin antiserum (Transduction Laboratories, Lexington, KY, U.S.A.) was added to the blocked membrane and detected by enhanced chemiluminescence (Amersham, Little Chalfont, Bucks., U.K.). Fractions were also analysed for galactosyltransferase and NADPH:cytochrome *c* reductase activity [18,19].

Immunoprecipitation

Immunoprecipitation was performed as described [12] with 25 μl of each antiserum or 1 μg of *Helix pomatia* lectin followed by 10 μl of a rabbit anti-(*Helix pomatia* lectin) antiserum (Serotec) Samples were precipitated with 150 μl of 10% (v/v) Pansorbin or Immunoprecipitin (formalin-fixed *Staphylococcus aureus*; Calbiochem or Life Technologies). The immunoprecipitates were washed four times in 10 mM Tris/HCl/2 mM EDTA/0.1% (v/v) Triton X-100/0.1% SDS (pH 7.4). The bound material was released from the Pansorbin or Immunoprecipitin with sample buffer [50 mM Tris/HCl/20% (v/v) glycerol/5% (w/v) SDS (pH 6.8)] with or without 5% (v/v) 2-mercaptoethanol for 5 min at 95 °C.

SDS/PAGE, SDS/agarose gel electrophoresis and autoradiography

Samples were analysed by SDS/PAGE [20] with a 3–5% (w/v) gradient gel and a 3% (w/v) stacking gel, or analysed by SDS/agarose gels with 0.8% stacking gel of SeaKem Gold (FMC) and a separation gel of 1.3% (w/v) Ultrapure agarose (Life Technologies) and 1.3% (w/v) Sea Plaque low-gelling-temperature agarose (FMC). Electrophoresis was performed as described [12]. Gels were fixed in 30% (v/v) ethanol and 10% (v/v) acetic acid, incubated in Amplify (Amersham Pharmacia Biotech), dried and then exposed to film at −70 °C, as described [12]. Two-dimensional gel electrophoresis was performed under non-reducing conditions in the first dimension. The samples in the lane were then reduced by cutting out the lane and incubating the strip for 30 min in 0.5 M Tris/HCl/0.4% SDS (pH 6.8) containing 50 mM dithiothreitol. The strip was placed on top of another gel and was overlaid with 0.8% agarose gel containing 10 mM dithiothreitol.

Rate zonal ultracentrifugation

Sedimentation on sucrose gradients by rate zonal ultracentrifugation was performed as described previously [16].

Immunofluorescent staining

LS 174T cells were seeded on glass coverslips, fixed in 3% (w/v) paraformaldehyde and permeabilized in 0.1% (v/v) Triton X-100. The cells were stained with α-MUC2N3 or α-MUC2C2 (diluted 1:100) antiserum followed by FITC-conjugated anti-(rabbit IgG) (diluted 1:100).

RESULTS

Dimerization of human MUC5AC apomucin in colon carcinoma cell lines

The initial biosynthesis of human MUC5AC mucin was studied in the colon carcinoma cell line LS 174T. Two rabbit antisera were used: α-MUC5ACCR, derived from a consensus sequence of the repeated cysteine-rich domains, and α-MUC5ACTR,

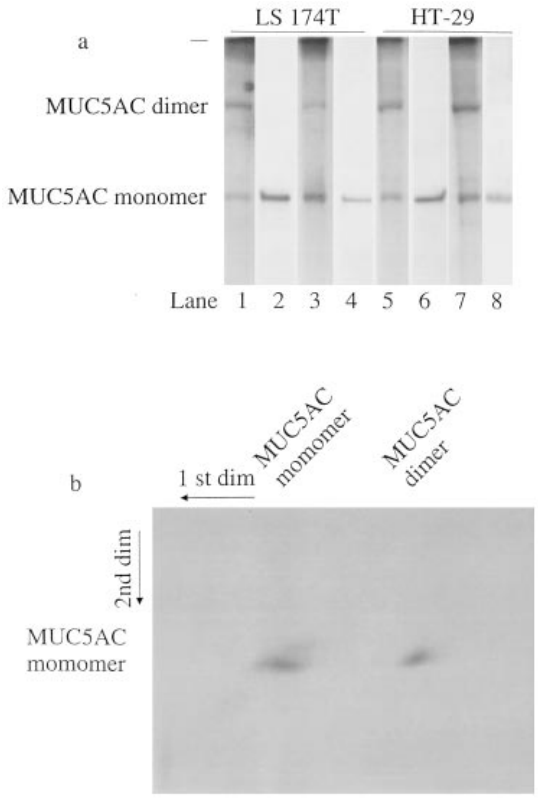

Figure 1 Dimerization of MUC5AC apomucin studied by immunoprecipitation in the colon carcinoma cell lines LS 174T and HT-29

(**a**) LS 174T (lanes 1–4) and HT-29 (lanes 5–8) cells were metabolically labelled for 2 h followed by immunoprecipitation of the cell lysate with α-MUC5ACCR (lanes 1, 2, 5 and 6) and α-MUC5ACTR (lanes 3, 4, 7 and 8). Samples in lanes with odd numbers were analysed under non-reducing conditions, and samples in lanes with even numbers were analysed under reducing conditions; in each case SDS/PAGE [3–5% (w/v) gel] was performed. The interface between the stacking and gradient gels is marked with a line at the left. (**b**) LS 174T cells labelled for 2 h were precipitated with α-MUC5ACCR and analysed by SDS/agarose gel electrophoresis under non-reducing conditions in the first dimension (right to left) and under reducing conditions in the second dimension (top to bottom).

derived from a consensus tandem repeat domain. The antisera were used in the immunoprecipitation of metabolically labelled LS 174T cells and both sera gave two bands migrating to identical positions when analysed under non-reducing conditions by SDS/PAGE (Figure 1a, lanes 1 and 3). When the samples were reduced before analysis (lanes 2 and 4), only one band, migrating as the smaller one, was observed. As a control, metabolically labelled HT-29 cells known to express MUC5AC at high levels [8,21] were immunoprecipitated with the α-MUC5ACTR and α-MUC5ACCR antisera. Also in this cell line, bands migrating to the same positions were detected under non-reducing (Figure 1, lanes 5 and 7) and reducing conditions on SDS/agarose gel electrophoresis (lanes 6 and 8). The larger band seen under non-reducing conditions was interpreted as a disulphide bond-stabilized oligomer of MUC5AC. This oligomerization was verified by two-dimensional gel electrophoretic analysis (Figure 1b) of the α-MUC5ACCR immunoprecipitates from LS 174T cells under non-reducing conditions in the first dimension and under reducing conditions in the second dimension. In the second dimension, the larger band migrated to the same position as the smaller band, showing that the larger band was an oligomer, probably a dimer, of the smaller.

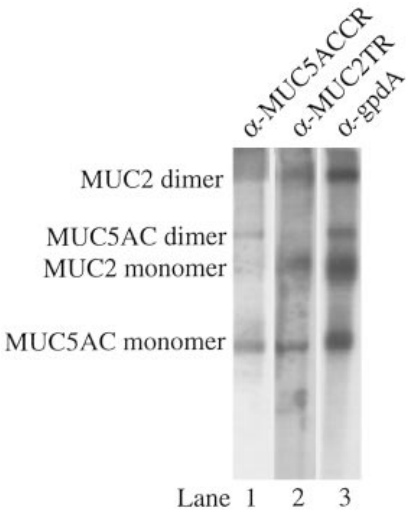

Figure 2 Expression of MUC5AC and MUC2 apomucins in LS 174T cells

LS 174T cells were metabolically labelled for 2 h followed by immunoprecipitation with α-MUC5ACCR (lane 1), α-MUC2TR (lane 2) or α-gpdA (lane 3). Samples were analysed by SDS/PAGE [3–5% (w/v) gel] under non-reducing conditions.

To verify that the larger band observed on the autoradiograms was a dimer, additional experiments were performed. The MUC5AC monomer and dimer were precipitated with α-gpdA, cross-reacting with the MUC5AC apomucin as will be described below. The samples were layered on top of a 10–40% (w/v) sucrose gradient and rate zonal ultracentrifugation was performed. The sucrose concentration of each fraction obtained was determined and the fractions were analysed by SDS/PAGE [3–5% (w/v) gel]. The sedimentation coefficients of the MUC5AC monomer and dimer were estimated as 6.9 and 8.5 respectively. The ratio between these sedimentation coefficients was thus 1.23, which is similar to the ratio between the sedimentation coefficients of the human MUC2 monomer and dimer [16]. This indicated that the human MUC5AC mucin forms a dimer.

Expression of MUC5AC and MUC2 apomucins in the LS 174T cell line

Two antisera were used previously to study the biosynthesis of the human MUC2 apomucin, α-MUC2TR against the tandem repeat of the human MUC2 mucin, and α-gpdA against the rat Muc2 mucin, cross-reacting with human MUC2 mucin [12]. To compare the expression of the MUC5AC and the MUC2 apomucins, metabolically labelled LS 174T cell lysates were precipitated with α-MUC5ACCR (Figure 2, lane 1), α-MUC2TR (lane 2) and α-gpdA (lane 3). Comparing the migrations on SDS/PAGE for the human MUC2 and MUC5AC monomers and dimers further suggests that the MUC5AC apomucin forms a dimer.

As we have shown previously [12], both α-MUC2TR and α-gpdA react with what was named the 'additional band' and its dimer, with higher mobility on SDS gels than the monomer and dimer of the MUC2 apomucin. These 'additional bands' are shown in Figure 2 (lanes 2 and 3): they migrated to the same positions as the monomer and dimer of MUC5AC apomucin (lane 1). To determine whether these 'additional bands' were indeed the human MUC5AC monomer and dimer, two series of

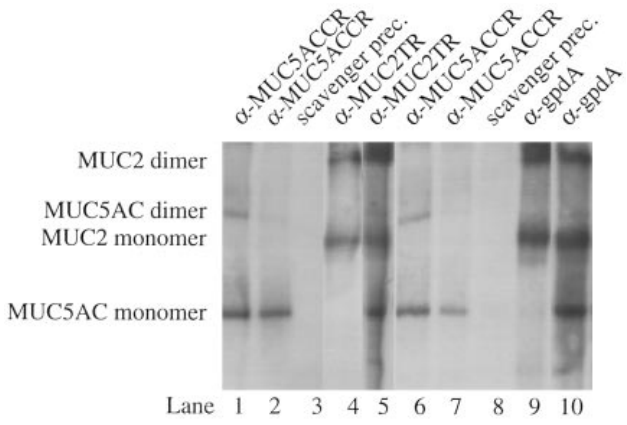

Figure 3 Cross-reactivity of α-MUC2TR and α-gpdA antisera with human MUC5AC apomucin

Two serial immunoprecipitations were performed on LS 174T cells, metabolically labelled for 2 h, with α-MUC5ACCR (lanes 1 and 6), followed by α-MUC5ACCR (lanes 2 and 7) and an additional incubation with immunoprecipitin (scavenger precipitation; lanes 3 and 8). The supernatants were then incubated with α-MUC2TR (lane 4) or α-gpdA (lane 9). As a control, LS 174T cells were immunoprecipitated directly with α-MUC2TR and α-gpdA (lanes 5 and 10 respectively). Samples were analysed by SDS/agarose gel electrophoresis under non-reducing conditions.

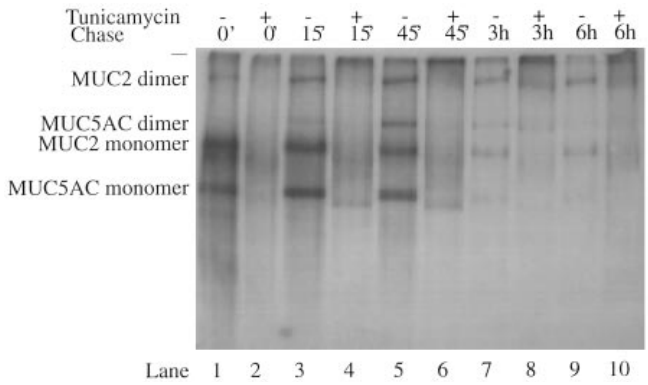

Figure 4 Comparison of dimerization of the MUC5AC and MUC2 apomucins with and without inhibition of N-glycosylation

LS 174T cells were metabolically labelled for 15 min and chased for 0 min, 15 min, 45 min, 3 h and 6 h in the presence (lanes with even numbers) or absence (lanes with odd numbers) of tunicamycin. The cell lysates were immunoprecipitated with α-gpdA and samples were analysed by SDS/PAGE [3–5% (w/v) gel] under non-reducing conditions. The interface between stacking and gradient gels is marked with a line at the left.

serial immunoprecipitations were performed on lysates from radioactively labelled LS 174T cells (Figure 3, lanes 1–4 and 6–9). In each series the cell lysate was precipitated twice with α-MUC5ACCR (Figure 3, lanes 1 and 6, and 2 and 7) to be able to precipitate most of the MUC5AC apomucin. The lysates were then incubated with immunoprecipitin (lanes 3 and 8). The supernatants were then finally precipitated with α-MUC2TR or α-gpdA (lanes 4 and 9 respectively) revealing only the two bands for MUC2. The 'additional bands' precipitated with α-MUC2TR and α-gpdA (Figure 3, lanes 5 and 10) in LS 174T cells were thus human MUC5AC apomucin. As α-gpdA is very effective in precipitating both the human MUC2 and MUC5AC apomucins this antiserum was used for studies on the biosynthesis of the two apomucins in parallel.

Pulse–chase studies on dimerization of the MUC5AC and MUC2 apomucins

The rate of dimerization of the MUC5AC and MUC2 apomucins was compared by pulse–chase labelling of LS 174T cells (Figure 4). Cells were labelled for 15 min and chased for 0–6 h, followed by immunoprecipitation of the cell lysates with α-gpdA, precipitating both the human MUC5AC and MUC2 apomucins. Samples were analysed by SDS/PAGE under non-reducing conditions. The MUC5AC and MUC2 dimers appeared within 15 min of labelling and the amounts of both the monomer and the dimer started to decrease after 3 h of chasing, with low amounts detected after 6 h of chasing. The dimerizations of the MUC5AC and MUC2 apomucins thus show similar kinetics.

Pulse–chase studies on the dimerization of the MUC5AC and MUC2 apomucins after inhibition of N-glycosylation

The dimerization of the MUC2 apomucin has been shown to be affected by the inhibition of N-glycosylation [16]. To study whether MUC5AC apomucin dimerization was similarly affected, pulse–chase labelling of tunicamycin-treated LS 174T cells was performed (Figure 4). The treated cells were labelled for 15 min and chased for 0–6 h, followed by precipitation of both the MUC5AC and MUC2 with α-gpdA. As shown, the non-N-glycosylated form of the MUC5AC and MUC2 apomucins migrated with a higher mobility on the gel than the N-glycosylated species. Dimers were formed not only by the MUC2 apomucin but also by the non-N-glycosylated MUC5AC monomers. However, the maximum quantity of dimers appeared between 45 min and 3 h of chasing compared with less than 15 min under normal conditions. The amount of non-N-glycosylated MUC2 and MUC5AC monomers and dimers did not decrease from 3 to 6 h in comparison with a decrease in cells not treated with tunicamycin. The kinetics of MUC5AC and MUC2 dimer formation with or without N-glycans were thus similar.

Subcellular fractionation of LS 174T cells on sucrose-density-gradient ultracentrifugation

To analyse whether the MUC5AC dimerization takes place in the endoplasmic reticulum, as has been shown for MUC2 apomucin, subcellular fractionation of LS 174T cells was performed by sucrose density centrifugation (Figure 5a). The sucrose gradient was adjusted to obtain a separation of the rough endoplasmic reticulum and the Golgi complex. The gradient was recovered from the bottom of the tube into 14 fractions and analysed by SDS/PAGE. The fractions were analysed by Western blotting with an anti-calnexin antiserum detecting membrane-bound calnexin, a marker for the rough endoplasmic reticulum. The fractions were also analysed for galactosyltransferase activity, a marker for the Golgi apparatus.

Metabolically labelled LS 174T cells were subcellularly fractionated as in Figure 5(a). Fractions were collected, followed by immunoprecipitation with α-gpdA; samples were then analysed by SDS/PAGE [3–5% (w/v) gel]. The autoradiographs show that most of the MUC2 and MUC5AC apomucins were precipitated from fractions co-migrating with calnexin, indicating that both apomucins formed dimers in the endoplasmic reticulum (Figure 5b).

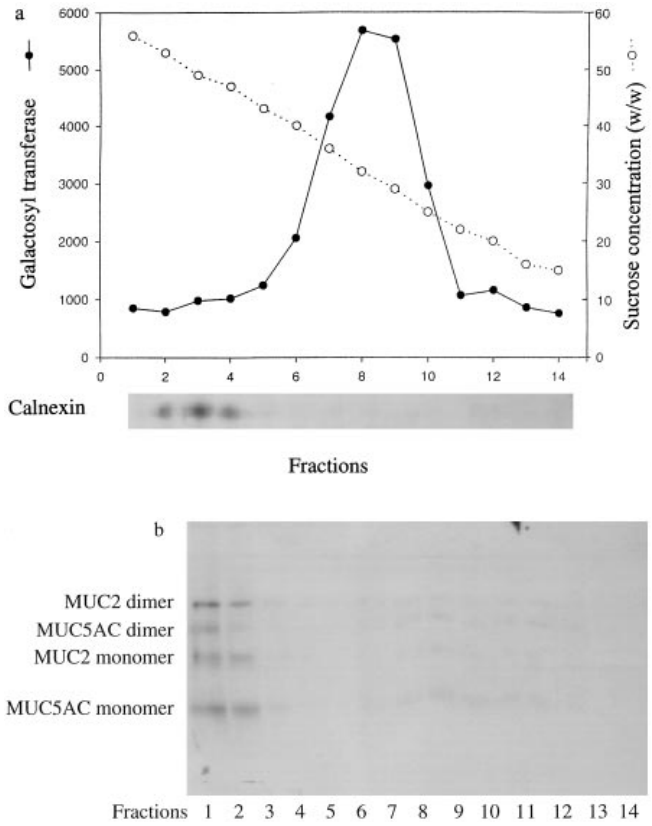

Figure 5 Subcellular fractionation of LS 174T cells by sucrose-density-gradient centrifugation

(**a**) LS 174T cells were homogenized in sucrose and subcellularly fractionated by sucrose-density-gradient centrifugation. Fractions were collected from the bottom of the tube and analysed for galactosyltransferase activity (●) and sucrose (w/w) concentration (○). The fractions were also analysed for calnexin by SDS/PAGE [12% (w/v) gel] followed by Western blot analysis with anti-calnexin antiserum (shown below the graph). (**b**) LS 174T cells were metabolically labelled for 1 h followed by subcellular fractionation as described for (**a**). Tubes were unloaded into lysis buffer containing NEM; immunoprecipitation with α-gpdA was then performed. Samples were analysed by SDS/PAGE [3–5% (w/v) gel] under non-reducing conditions.

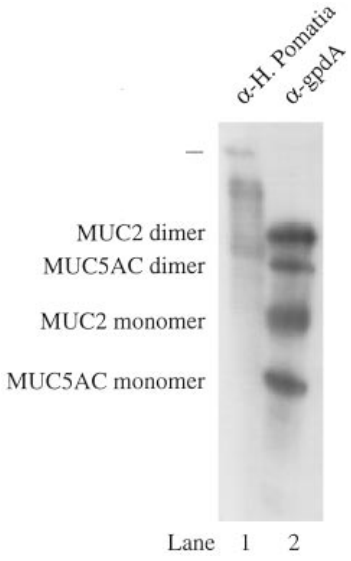

Figure 6 O-glycosylation of MUC5AC apomucin

LS 174T cells were metabolically labelled for 3 h and lysed in the presence of NEM. The cell lysate was precipitated with *Helix pomatia* lectin followed by anti-(*Helix pomatia* lectin) antiserum (lane 1). The supernatant was serially precipitated with α-gpdA (lane 2). Samples were analysed by SDS/agarose gel electrophoresis under non-reducing conditions.

The MUC5AC apomucins are not O-glycosylated

Human MUC2 mucin has been shown to form dimers before the initiation of O-glycosylation [12]. To determine whether the MUC5AC apomucin monomers and dimers were O-glycosylated, metabolically labelled LS 174T cells were precipitated with *Helix pomatia* lectin followed by anti-(*Helix pomatia* lectin) antiserum (Figure 6). The supernatant was then incubated with α-gpdA, precipitating both the MUC5AC and MUC2 apomucins. The samples were analysed by SDS/PAGE under non-reducing conditions. The α-gpdA antiserum precipitated the non-O-glycosylated MUC5AC and MUC2 monomers and dimers as shown before, whereas none of these bands were precipitated with the *H. pomatia* lectin, suggesting that the monomer and dimer bands of MUC5AC apomucin detected by α-gpdA were not O-glycosylated.

Do MUC2 and MUC5AC heterodimerize?

The high sequence similarity in the positions of the cysteines in the C-termini between the MUC5AC and the MUC2 apomucins and the similar early biosynthetic steps of the two apomucins shown here might suggest that the two mucins could form heterodimers. To study a potential heterodimerization between MUC5AC and MUC2, the expression of both or at least one of the mucins in all LS 174T cells must be shown. The expression of the MUC2 mucins was verified by incubating fixed and permeabilized cells with α-MUC2N3 or α-MUC2C2, which react with unique regions of the N-terminus and C-terminus of the MUC2 mucin respectively. As all LS 174T cells were stained, at least some cells expressed both MUC5AC and MUC2 and should thus have had the potential to form heterodimers between the two mucins. When the α-MUC5ACCR-immunoprecipitated samples from labelled LS 174T lysates were reduced, only one band corresponding to the monomeric form of the human MUC5AC was found after both one-dimensional and two-dimensional gel electrophoresis (Figure 1). No band was found in the position of the human MUC2 monomer, indicating that no heterodimers were formed between the human MUC2 and MUC5AC apomucins.

DISCUSSION

The human colon carcinoma cell line LS 174T produces human MUC5AC mucin, as revealed by Northern blot analysis [22,23]. To study the initial biosynthetic steps of MUC5AC apomucin two antisera, α-MUC5ACCR, against a repetitive cysteine-rich region of human MUC5AC, and α-MUC5ACTR, against a repetitive threonine-rich region of human MUC5AC respectively, were used in immunoprecipitations of metabolically labelled LS 174T cells. Two bands were found after SDS/agarose gel electrophoresis under non-reducing conditions. Under reducing conditions only the smaller band was observed. After two-dimensional gel electrophoresis, the smaller band was interpreted as the MUC5AC monomer and the larger one as its disulphide bond-stabilized dimer. Rate zonal ultracentrifugation on a su-

crose gradient verified the dimeric nature of the oligomer. The ratio between the calculated sedimentation coefficient of the monomer and the dimer of MUC5AC apomucin can be compared with the human MUC2 apomucin monomer and dimer [16]. For both apomucins the ratios between the sedimentation coefficients for a monomer and a dimer are 1.2. This, together with the migration on SDS/PAGE, and SDS/agarose gel electrophoresis in comparison with MUC2 apomucin, strongly suggests that human MUC5AC mucin also forms a dimer as an initial biosynthetic step.

No oligomeric forms of non-O-glycosylated MUC5AC mucin larger than a dimer were detected either by SDS/PAGE or SDS/agarose gel electrophoresis. Larger species than the monomer and dimer of MUC5AC do not enter an SDS/polyacrylamide gel. However, the trimer and tetramer should enter an SDS/agarose gel, as non-N-glycosylated putative trimers and tetramers of the MUC2 apomucin could be separated in this way [16]. Even larger species would probably be trapped and and stained in the application well or the interface between the stacking gels and separation gel. NEM was added to the lysis buffer in some experiments, to inhibit the formation of disulphide bonds *in vitro* (Figures 5 and 6). When this was done, no staining was observed in the application well or in the interface between the stacking gel and the separation gel. This indicates that no species larger than the non-O-glycosylated MUC5AC monomer and dimer were precipitated with the available antisera. In the experiments in which NEM was not included, the application well and the interface between the stacking gel and the separation gel were stained. This staining was probably due to disulphide bond-stabilized aggregates formed *in vitro*, because they were found only under non-reducing conditions and disappeared after reduction.

In biosynthetic studies of human MUC2 apomucin [12] in LS 174T cells, α-MUC2TR (against the human MUC2 tandem repeat region) and α-gpdA (against the glycopeptide of a rat small-intestinal Muc2) were used. Both sera precipitated two 'additional bands', a monomer and its putative dimer, originally interpreted as cleavage products of the human MUC2 apomucin. However, the present results show that these 'additional bands' detected in LS 174T cells were not related to MUC2 but were instead due to cross-reactivity of these antisera with human MUC5AC apomucin.

As α-MUC2TR shows some affinity for human MUC5AC, the apomucins must contain similar epitopes. The human *MUC5AC* gene does not contain repetitive threonine-rich and serine-rich regions identical to the human MUC2 apoprotein, but does contain other motifs rich in these amino acids. It is not yet known whether these similarities are sufficient for antisera cross-reactivity. However, there is no cross-reactivity of α-MUC5ACTR or α-MUC5ACCR with MUC2. The rat α-gpdA antiserum raised against the HF-deglycosylated large highly glycosylated domain (gpdA) of the rat 'insoluble' mucin, identified as part of the Muc2 mucin [14], precipitates both human MUC2 and MUC5AC. The immunization antigen for the generation of gpdA mainly contains serine, threonine and proline residues of unknown sequence, but also some cysteine-rich unique sequences [14]. It is currently difficult to predict whether the antiserum is cross-reacting with MUC5AC owing to the Ser/Thr or the Cys regions [13,14].

Subcellular fractionation of LS 174T cells was performed to identify the compartment involved in dimerization of the MUC5AC apomucin by using a sucrose gradient to separate the rough endoplasmic reticulum and the Golgi apparatus. Non-O-glycosylated MUC5AC was found mainly in the rough endoplasmic reticulum. Small amounts of monomers and dimers were found throughout the gradient, suggesting that some vesicles had been disrupted. Immunoprecipitation of MUC5AC apomucin showed that the dimerization of the mucin started within 15 min after labelling [9]. Thus both pulse–chase studies and subcellular fractionation assays suggest that the dimerization takes place in the endoplasmic reticulum.

After treatment with tunicamycin, dimerization was slower and the dimers appeared within 3 h of chasing in treated cells, compared with 15 min in untreated cells. Comparison of the results of MUC5AC dimerization with human MUC2 shown here and previously [16] indicates that the dimers of MUC2 apomucin are formed at similar rates and that dimerization is similarly dependent on N-glycosylation. By subcellular fractionation it has been possible to show that the MUC2 monomers and dimers formed during treatment with tunicamycin are not transported into the Golgi apparatus [16]. This was interpreted as being due to misfolding and trapping of MUC2 in the endoplasmic reticulum. The similar disturbances of MUC5AC processing observed here suggest that MUC5AC monomers and dimers are also retained in the endoplasmic reticulum on treatment with tunicamycin. We have shown previously that the dimerization of the human MUC2 mucin starts before O-glycosylation [16]. When the LS 174T cells were precipitated with the *Helix pomatia* lectin, no bands co-migrating with the MUC5AC monomer and dimer were detected, suggesting that dimerization precedes O-glycosylation also in the biosynthesis of the MUC5AC apomucin.

As shown and discussed here, the initial steps in the biosynthesis of the human MUC2 and MUC5AC mucins are very similar. The biosynthesis of a mucin from human stomach, now assumed to be MUC5AC, formed an oligomer in tissue explants [24]. This oligomer was interpreted as a trimer or a tetramer on the basis of estimations by SDS/PAGE. In this study, however, the oligomeric species of human MUC5AC expressed in LS 174T cells is most probably a dimer. Studies on rat gastric mucin [25] also show a different initial biosynthetic pathway from that described here for MUC5AC and MUC2 [16]. The rat gastric apomucin formed non-O-glycosylated dimers, trimers and possibly also tetramers in the endoplasmic reticulum. These discrepancies might be due to the use of different cells and tissues or to different biosynthetic pathways for different mucins.

Human MUC2 has been fully sequenced [10], as has human MUC5AC [7,8]. The similarity in the number and positions of the cysteine residues in the C-termini of both mucins is more than 90 %. There are also large sequence similarities between the positions of cysteine residues in the C-termini of these mucins with that of vWF [26], known to dimerize by disulphide bond formation between two C-terminal ends [11]. In addition, pig submaxillary mucin [27] shows sequence similarity in the positions of the cysteine residues in the C-terminal end and has also been suggested to form dimers involving the C-termini [28]. In the far C-terminal end of many growth factors there is a cysteine motif named the 'cystine knot', known to be directly involved in dimerization [29]. This 'cystine knot' motif is also found in vWF and the mucins pig submaxillary mucin, MUC5AC, MUC2, MUC5B [30,31] and MUC6 [32]. Despite the similarity in the position of the cysteine residues involved in the 'cystine knot' motif and the biosynthetic similarities of the MUC2 and MUC5AC mucins shown here, they do not heterodimerize when formed in the same cell, as shown here and also recently by van Klinken et al. [33]. This indicates that several mucin genes can be expressed in the same cell without the formation of heterodimeric mucins, suggesting that sequences outside the 'cystine knot' motif are also involved in the specificity of dimer formation.

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