Gastric MUC5AC and MUC6 are large oligomeric mucins that differ in size, glycosylation and tissue distribution

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Gastric MUC5AC and MUC6 mucins were studied using polyclonal antibodies. Immunohistochemistry showed MUC5AC to originate from the surface epithelium, whereas MUC6 was produced by the glands. Mucins from the surface epithelium or glands of corpus and antrum were purified using CsCl/4M guanidinium chloride density-gradient centrifugation. MUC5AC appeared as two distinct populations at 1.4 and 1.3 g/ml, whereas MUC6, which was enriched in the gland tissue, appeared at 1.45 g/ml. Reactivity with antibodies against the Le^b structure (where Le represents the Lewis antigen) followed the MUC5AC distribution, whereas antibodies against the Le^y structure and reactivity with the GlcNAc-selective *Solanum tuberosum* lectin coincided with MUC6, suggesting that the two mucins are glycosylated differently. Rate-zonal centrifugation of whole mucins and reduced subunits showed that both gastric MUC5AC and MUC6 are oligomeric glycoproteins composed of disulphide-bond linked subunits and that oligomeric MUC5AC was apparently smaller than MUC6. A heterogeneous population of 'low-density' MUC5AC mucins, which were smaller than the 'high-density' ones both before and after reduction, reacted with an antibody against a variable number tandem repeat sequence within MUC5AC, suggesting that they represent precursor forms of this mucin. Following ion-exchange HPLC, both MUC5AC and MUC6 appeared as several distinct populations, probably corresponding to 'glycoforms' of the mucins, the most highly charged of which were found in the gland tissue.

Key words: carbohydrate, mucosal gland, stomach, surface epithelium.

INTRODUCTION

The gastric mucosa is protected against the action of luminal acid and proteolytic enzymes by a firmly adherent mucus gel [1]. High-molecular mass glycoproteins (mucins) provide the matrix of the gel and immunohistological studies have shown that the mucus layer is formed by secretions originating from both the surface epithelium and the glands [2]. Mucins from these two sources may form laminated structures in mucus, the function of which is unknown [3]. Immunohistological studies [4] and *in situ* hybridization [5] have shown that, in normal gastric mucosa, MUC5AC is produced in the surface epithelium, whereas *MUC6* is expressed in the glands. In addition, MUC2 has been detected in areas of intestinal metaplasia and in gastric cancer [6,7]. Differences in the reactivity with carbohydrate-specific antibodies between the surface epithelium and glands of gastric mucosa have also been noted with the surface epithelium expressing type 1 backbone structures (Le^a and Le^b, where Le represents the Lewis antigen), whereas type 2 structures (Le^x and Le^y) are found in the glands [8–10].

Many investigators have demonstrated that mucus-forming mucins secreted on to mucosal surfaces are oligomeric macromolecules linked by disulphide bonds (for example, see [11–14]). The full organization of MUC5AC cDNA has recently been described, and sequence similarities in the N- and C-terminal regions of this mucin, as well as in the C-terminal domain of MUC6, to the D domains involved in the polymerization of the von Willebrand factor indicate that both these mucins may form oligomeric structures [15,16]. Metabolic labelling experiments in LS174T cells suggest that the mucins encoded by genes residing in the gene cluster on the human chromosome 11p15.5 (*MUC2*, *MUC5B*, *MUC5AC*, *MUC6*) at least form dimers early during synthesis [17,18]. However, the extent to which gastric MUC5AC and MUC6 are oligomerized further has not been demonstrated.

The aim of the present study was to investigate the properties and tissue localization of gastric MUC5AC and MUC6 mucins. Polyclonal antibodies raised against sequences within non-glycosylated domains of MUC5AC and MUC6 were used to identify these mucins in tissue sections as well as in extracts of gastric mucosa. The two mucins were partially separated using isopycnic density-gradient centrifugation and shown to differ in reactivity with the *Solanum tuberosum* (STA) lectin and monoclonal antibodies against the Le^b and Le^y structures. MUC5AC was found in two forms, one of which represents a putative precursor. Both MUC5AC and MUC6 could be separated into several distinct populations depending on charge-properties, suggesting the presence of mucin glycoforms.

EXPERIMENTAL

Materials

Iodoacetamide and guanidinium chloride were obtained from ICN Biochemicals. Stock solutions of guanidinium chloride (8 M) were treated with charcoal and filtered through an Amicon PM-10 ultrafiltration membrane before use. CHAPS was purchased from Roche Molecular Biochemicals, di-isopropyl phos-

Abbreviations used: AP, alkaline phosphatase; DFP, di-isopropyl phosphorofluoridate; DTT, dithiothreitol; HRP, horseradish peroxidase; Le, Lewis antigen; PBST, PBS containing 0.05% (v/v) Tween 20; STA, *Solanum tuberosum*; TBS, Tris-buffered saline; TBST, TBS containing 0.05% (v/v) Tween 20; VNTR, variable number tandem repeat.

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phorofluoridate (DFP) was from Fluka, biotin hydrazide was from Vector Laboratories Burlingame, CA, U.S.A. and dithiothreitol (DTT) was from Merck. BSA (fraction V, pH 7.0) was obtained from Serva, Heidelberg, Germany. Sephacryl S-500 HR and a Mono Q column were purchased from Amersham Biosciences.

Antibodies and lectins

Polyclonal antisera recognizing MUC2 (LUM2-3), MUC5B (LUM5B-2) and MUC5AC (LUM5-1) have been described previously [19–21]. An antiserum against MUC6 (LUM6-3) was raised in rabbits using a peptide with the sequence RPLHSYEQQLELPC (where the single-letter amino-acid notation has been used) from the MUC6 apoprotein [16]. The peptide was conjugated to keyhole-limpet haemocyanin and rabbits were injected with approx. 100 μg of conjugate in Freund's complete adjuvant. A booster dose using the same amount of antigen in Freund's incomplete adjuvant was given after approx. 4 weeks, and the antiserum was collected 2 weeks thereafter. A monoclonal anti-(human gastric mucin) antibody (clone 45M1) was purchased from Sigma and a monoclonal antibody (clone CLH2) against the variable number tandem repeat (VNTR) region of the MUC5AC apoprotein [22] was kindly given by Dr L. David, Institute of Molecular Pathology and Immunology, University of Porto, Portugal. Biotinylated STA lectin was obtained from Vector Laboratories. A monoclonal anti-Le^b antibody (clone 2-25 LE) was kindly given by Dr J. Bara, U-55 INSERM, Paris, France. The monoclonal anti-Le^y antibody (clone 77/180) was used as described previously [4]. Alkaline phosphatase (AP)-conjugated streptavidin was from Roche Molecular Biochemicals, AP-conjugated swine anti-rabbit immunoglobulins were from Dako (Denmark) and AP-conjugated swine anti-mouse IgM and anti-mouse IgG were from Sigma.

Histochemical methods

Sections (4 μm) of formalin-fixed paraffin-embedded gastric tissue, prepared on Superfrost Plus slides (Mensel Gläser, Braunschweig, Germany), were dewaxed, rehydrated and treated with 10 mM sodium citrate buffer (pH 6) at 100 °C for 5 min in a microwave oven. Sections stained with the LUM2-3 or LUM6-3 antisera were reduced with 10 mM DTT in 0.1 M Tris/HCl (pH 8.0) at 37 °C for 30 min and then alkylated with 25 mM iodoacetamide in the same buffer at 20 °C for 30 min. Endogenous peroxidase activity was quenched using 3 % (v/v) H_2O_2 in water for 30 min, and the sections were washed with Tris-buffered saline [TBS; 0.15 M NaCl in 0.05 M Tris/HCl (pH 7.4)]. Non-specific binding was blocked using normal goat serum diluted 1:5 in TBS (for immunohistochemistry) or 0.5 % (w/v) BSA in TBST [TBS containing 0.05 % (v/v) Tween 20; for lectin staining] (TBS blocking solution) for 1 h, and endogenous biotin was blocked by treatment with the Dako biotin blocking kit, according to the manufacturer's instructions. For immunohistochemical localization, sections were incubated with the LUM5-1 (1:1000), LUM2-3 (1:1000) or LUM6-3 (1:500) antisera or the 45M1 (1:4000) or CLH2 (1:50) antibodies diluted in TBS for 1 h, followed by the StreptABComplex/HRP Duet kit (Dako). Some sections showed a background staining with the LUM5-1 and LUM6-3 antisera which could be removed by preadsorption of the antisera with keyhole-limpet haemocyanin. Lectin histochemistry was performed using a modified version of the StreptABComplex/horseradish peroxidase (HRP) method [23]. Sections were incubated with the biotinylated STA lectin (0.25 μg/ml) diluted in TBST containing 0.1 mM $CaCl_2$ for 1 h, followed by the streptavidin/biotinylated HRP component of the StreptABComplex/HRP kit for 30 min. Antibody and lectin binding were visualized using 3,3′-diaminobenzidine (0.6 mg/ml) in TBS containing 0.03 % (v/v) hydrogen peroxide for 15 min, and sections were counterstained with Mayer's haematoxylin.

Analytical methods

Density measurements were performed using a Carlsberg pipette as a pycnometer. Sialic acid was determined by using an automated procedure [24] of the original method described by Jourdain et al. [25]. Carbohydrate was also determined as periodate-oxidizable structures with a microtitre-plate assay (glycan detection) as described by Devine [26]. In this assay, samples (100 μl) were coated on to multi-well assay plates (3912, Falcon) overnight in a humidified chamber at 20 °C. After washing in PBST [0.15 M NaCl in 5 mM sodium phosphate buffer (pH 7.4) containing 0.05 % (v/v) Tween 20], oxidation was performed with 25 mM sodium metaperiodate in 0.1 M sodium acetate buffer (pH 5.5) for 20 min at 20 °C. The wells were washed and incubated for 1 h with 100 μl of biotin hydrazide (2.5 μM) in 0.1 M sodium acetate buffer (pH 5.5), followed by washing and detection of bound biotin with 100 μl of AP-conjugated streptavidin diluted 1:10000 in PBS blocking solution [PBST containing 0.5 % (w/v) BSA]. Bound AP-conjugated streptavidin was detected with *p*-nitrophenyl phosphate (2 mg/ml) in 1 M diethanolamine/HCl buffer (pH 9.8) containing 0.5 mM $MgCl_2$ as a substrate. Absorbance at 405 nm was read after 1 h.

ELISA

Fractions from CsCl density-gradients, gel chromatography, rate-zonal centrifugations and ion-exchange HPLC were diluted in 4 M guanidinium chloride/10 mM sodium phosphate buffer (pH 7), and samples (100 μl) were coated on to multi-well assay plates (3912, Falcon) overnight at 20 °C. For the LUM2-3, LUM5B-2 and LUM6-3 antisera, samples were reduced and alkylated before coating. Briefly, samples were diluted in 6 M guanidinium chloride/5 mM sodium EDTA/10 mM Tris/HCl (pH 8) before the addition of DTT to a final concentration of 2 mM. After reduction at 37 °C for 1 h, samples were alkylated with 5 mM iodoacetamide in 6 M guanidinium chloride/5 mM sodium EDTA/10 mM Tris/HCl (pH 8) for 1 h at 20 °C. All subsequent incubations were performed in a humidified chamber at 20 °C for 1 h. After washing, unbound sites were blocked with PBS blocking solution. The wells were then incubated with 100 μl of the LUM5-1 (1:2000), LUM5B-2 (1:1000), LUM6-3, (1:1000) or LUM2-3 (1:1000) antisera or the 45M1 (1:4000), CLH2 (1:100), anti-Le^b (1:100) or anti-Le^y (1:5) antibodies diluted in PBS blocking solution. After washing and incubation with 100 μl of the secondary antibody (AP-conjugated swine anti-rabbit immunoglobulins or goat anti-mouse IgM or IgG), plates were washed and bound secondary antibody was detected with 100 μl of *p*-nitrophenyl phosphate as described above. Absorbance at 405 nm was measured after 1 h.

Lectin-binding assays were performed essentially as for the ELISA. After blocking of unbound sites with TBS blocking solution, the wells were incubated with biotinylated lectin (2 μg/ml) diluted in TBS blocking solution containing 0.1 mM $CaCl_2$. After washing, bound lectin was detected with AP-conjugated streptavidin.

In the sandwich ELISA, monoclonal anti-(human gastric mucin) antibodies [45M1, 10 μg/ml in 0.1 M sodium bicarbonate buffer (pH 9.5)] were coated on to multi-well assay plates for 1 h at 20 °C. As a negative control, an unrelated monoclonal

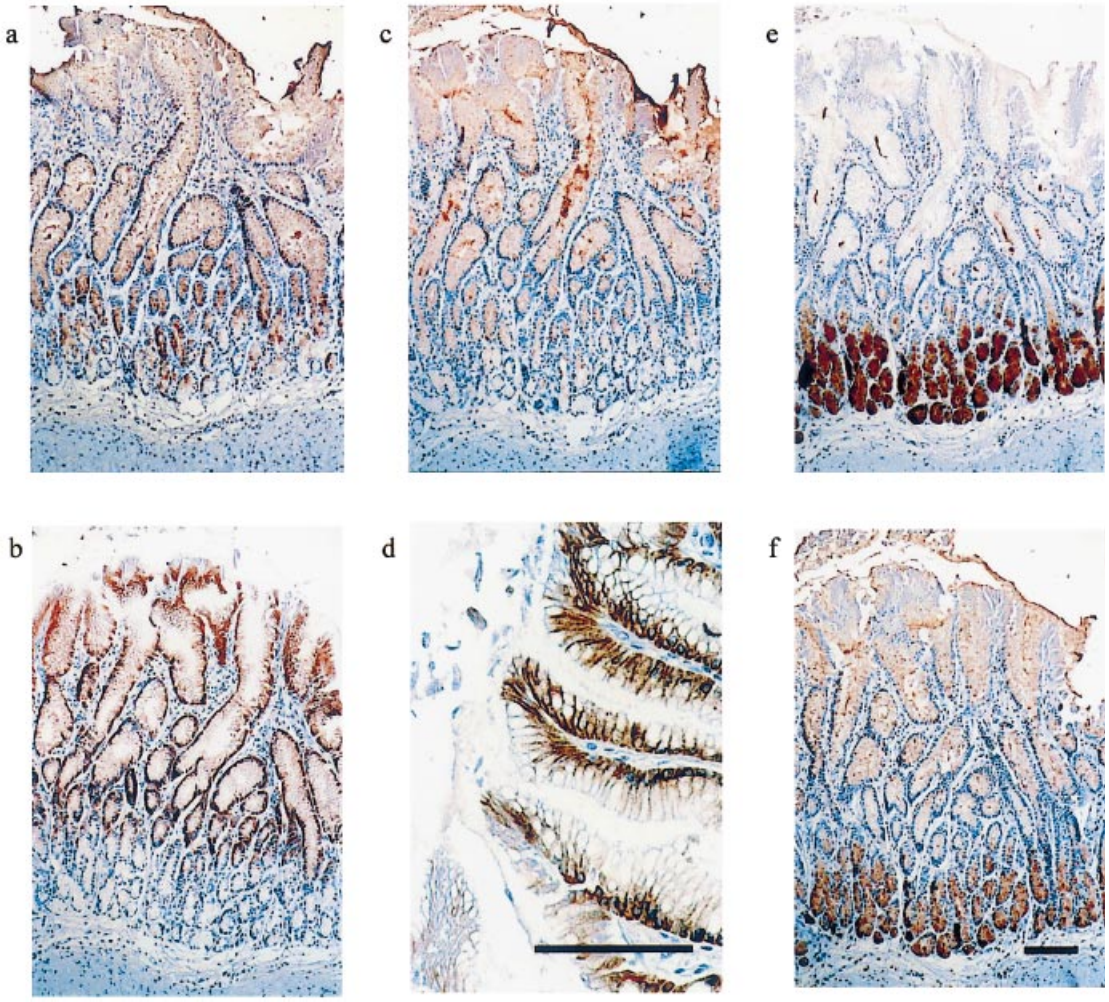

Figure 1 Immunohistological localization of human gastric mucins in antrum

Sections (4 μm) of formalin-fixed tissue from antrum were probed with the LUM5-1 (**a**), CLH2 (**b**, **d**), 45M1 (**c**), LUM6-3 (**e**) antibodies/antisera or STA lectin (**f**). Sections were counterstained with Mayer's haematoxylin. The bars indicate 100 μm. The bar in (**f**) also applies to (**a**–**c**) and (**e**).

antibody [anti-(α_1-microglobulin)] was used at the same concentration. The plates were washed and non-specific binding was blocked with PBS blocking solution. Fractions from density-gradient centrifugations were diluted (1:100) in 'catch' buffer [PBST containing 0.25% (w/v) BSA] and 100 μl was added to the antibody-coated plates. The 'catch' was performed for 2 h with orbital shaking (400 rev./min) in a Labsystems iEMS microtitre-plate incubator/shaker at 37 °C. After washing, the LUM5-1 antiserum was used to detect bound MUC5AC mucin. Bound antibodies were detected with AP-conjugated anti-rabbit immunoglobulins, as for the direct ELISA-procedure.

Purification of mucins

Specimens (approx. 3 cm × 3 cm) of both corpus and antrum (from two individuals) or antrum alone (from two individuals) were obtained distant from the tumour from patients undergoing resection for gastric cancer and stored at −20 °C until use. The frozen tissue pieces were covered with PBS containing 2 mM DFP and then thawed. The surface epithelium was gently scraped off with a microscope slide and immersed in liquid nitrogen. The residual gland mucosa was dissected from the underlying muscle layer, washed with ice-cold PBS and frozen in liquid nitrogen before pulverisation in a Retsch tissue pulveriser (1 min). Frozen material from surface epithelium and pulverised gland mucosa was thawed in the presence of DFP (1 ml of a 0.1 M solution in propanol) and immersed in 50 ml of ice-cold extraction buffer [6 M guanidinium chloride/10 mM sodium phosphate buffer (pH 6.5) containing 5 mM Na_2EDTA and 5 mM *N*-ethylmaleimide]. Samples were dispersed with a Dounce homogenizer (four strokes; loose pestle) and subjected to slow stirring overnight at 4 °C. Tissue extracts were centrifuged (17000 rev./min, 50 min, 4 °C; Beckman JA-20 rotor) and insoluble material was re-extracted twice with 25 ml of extraction buffer. Material which remained insoluble after three rounds of extraction was brought into solution by reduction in 6 M guanidinium chloride/10 mM sodium phosphate buffer (pH 6.5) containing 5 mM Na_2EDTA and 10 mM DTT at 37 °C for 5 h, and then alkylated by incubating in iodoacetamide (25 mM) overnight in the dark at 4 °C. Mucosal extracts were subjected to isopycnic density-gradient centrifugation in CsCl/4 M guanidinium chloride at an initial density of 1.39 g/ml (36000 rev./min, 85 h, 15 °C; Beck-

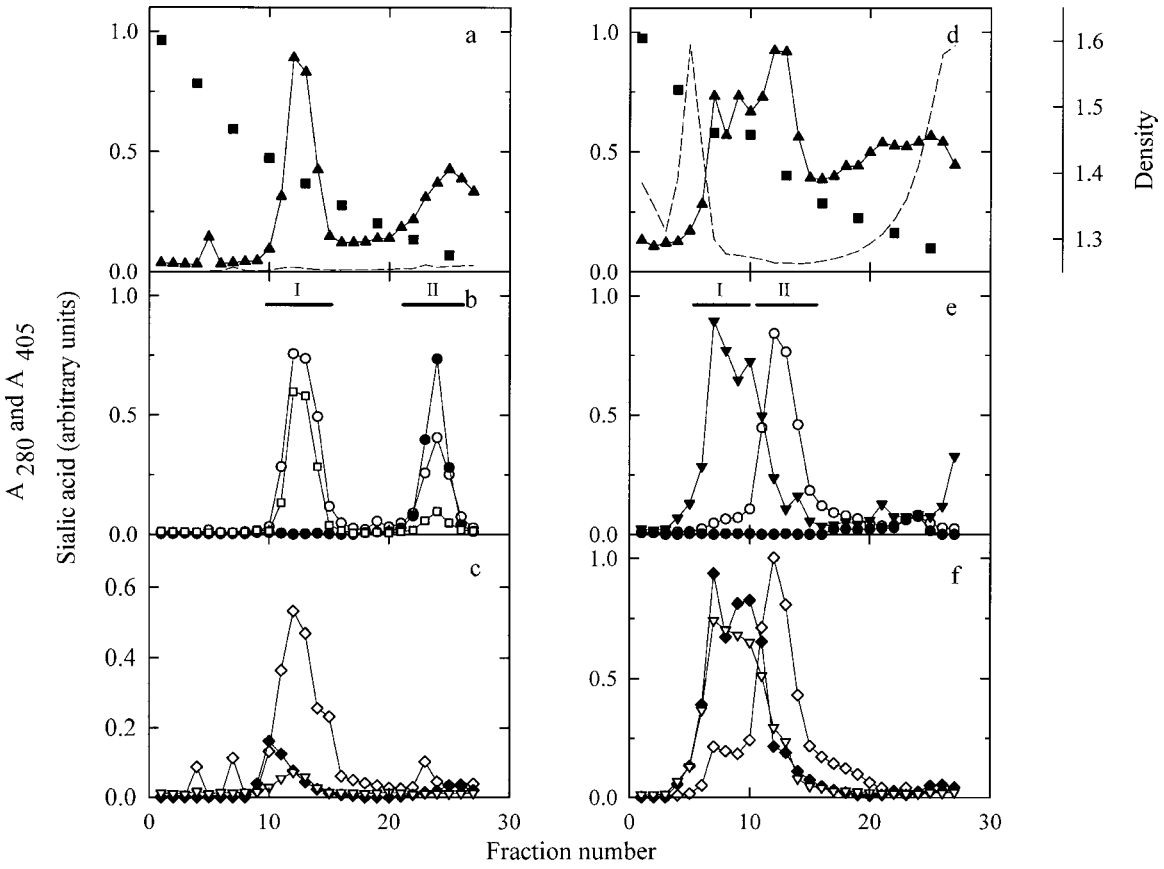

Figure 2 Isopycnic density-gradient centrifugation, in CsCl/4 M guanidinium chloride, of human gastric mucins from the surface epithelium (a, b, c) and the glands (d, e, f) of corpus

Corpus tissue, solubilized in 6 M guanidinium chloride, was subjected to density-gradient centrifugation (36000 rev./min, 85 h, 15 °C; 50.2 Ti rotor). Fractions were retrieved from the bottom of the tubes and analysed for density (■), A_{280} (—), carbohydrate (▲), and reactivity with the LUM5-1 (○), LUM6-3 (▼), CLH2 (●), anti-Le^b (◇) or anti-Le^y (◆) antibodies/antisera and STA lectin (▽). For sandwich ELISA, fractions, diluted in PBS blocking solution, were added to plates coated with anti-(human gastric mucin) antibodies (45M1). After the 'catch' was performed, bound MUC5AC was detected with the LUM5-1 antiserum (□). Fractions were pooled as shown by the bars in (**b**) and (**e**).

man 50.1 Ti rotor). Fractions were collected from the bottom of the tubes and analysed for density, A_{280}, carbohydrate, sialic acid and for reactivity with the LUM5-1, LUM2-3, LUM5B-2 and LUM6-3 antisera, CLH2, anti-Le^b and anti-Le^y antibodies and the STA lectin.

Rate-zonal centrifugation

Samples (100 μl) of whole mucins and reduced subunits in 5 M guanidinium chloride were layered on to a gradient of 6–8 M guanidinium chloride in 10 mM sodium phosphate buffer (pH 7) [27]. Samples were spun in a Beckman SW-41 rotor at 40000 rev./min at 20 °C for either 2 h 45 min or 10 h. Fractions (300 μl) were taken from the top of the tubes and analysed for reactivity with the LUM5-1 and LUM6-3 antisera.

Gel chromatography and ion-exchange HPLC

Gel chromatography was performed on a Sephacryl S-500 HR column (1.6 cm × 50 cm), and fractions were eluted with 4 M guanidinium chloride/10 mM sodium phosphate buffer (pH 7) at a flow rate of 0.15 ml/min. Fractions (1 ml) were collected and analysed for carbohydrate and antibody reactivity. For ion-exchange HPLC, reduced subunits obtained after reduction and alkylation of whole mucins were dialysed against buffer A [6 M urea/10 mM piperazine/perchlorate (pH 5) containing 0.2% (w/v) CHAPS] and applied to a Mono Q HR5/5 column connected to a 2150 LKB pump, 2152 LKB controller, 2040-203 LKB mixing valve and Pharmacia V-7 injector. Samples were eluted with buffer A for 10 min, followed by a linear gradient of 0–0.4 M $LiClO_4$ in buffer A over 60 min. Fractions (0.5 ml) were diluted with 4 M guanidinium chloride/10 mM sodium phosphate buffer (pH 7) and analysed for carbohydrate and antibody reactivity.

RESULTS

Immunohistochemistry

In tissue sections from antrum, staining for MUC5AC with the LUM5-1 antiserum (Figure 1a), the CLH2 antibody (Figure 1b) and the 45M1 antibody (Figure 1c) were observed in the surface epithelium, whereas reactivity with the LUM6-3 antiserum, raised against a peptide sequence in MUC6, was found both over the glands and the contents of the gland lumen (Figure 1e). Although some overlap was seen in the staining patterns for MUC5AC and MUC6, these data suggest that the LUM6-3 antiserum recognizes the MUC6 mucin and does not cross react with MUC5AC. Staining with the GlcNAc-selective STA lectin (Figure 1f)

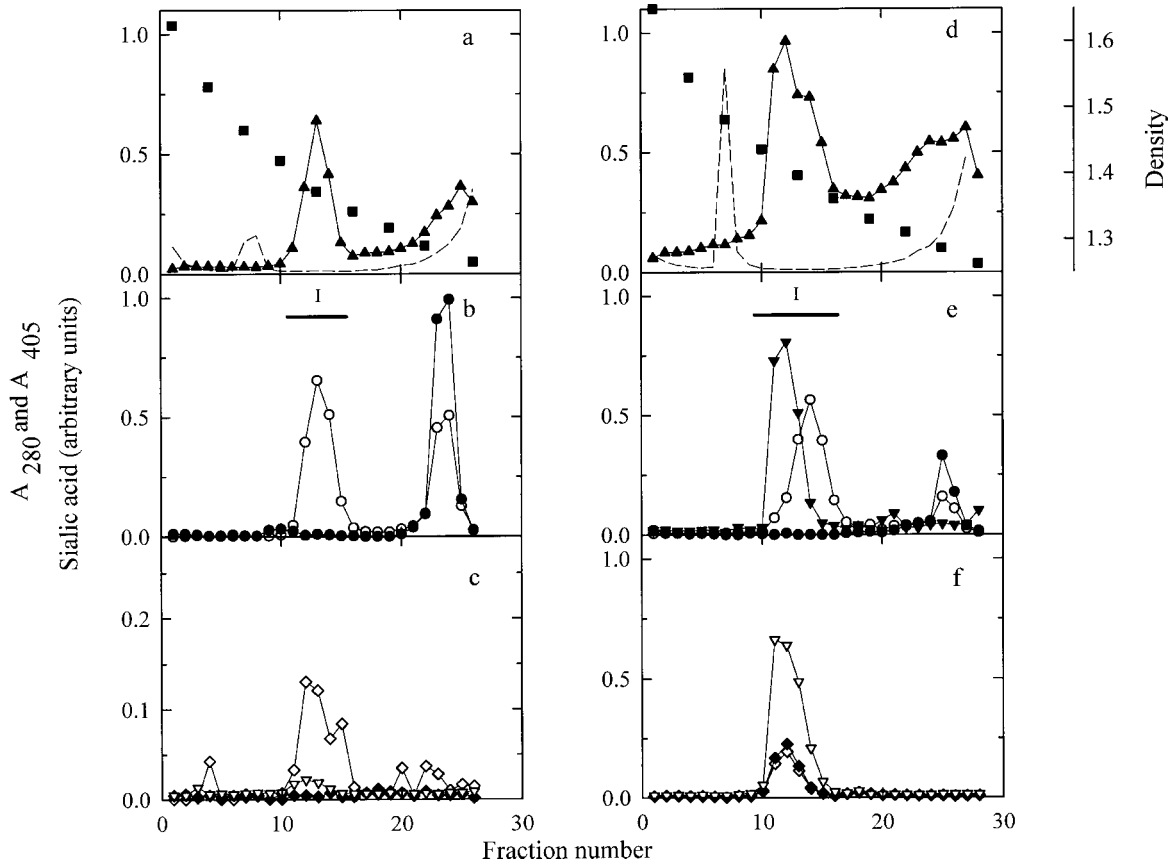

Figure 3 Isopycnic density-gradient centrifugation, in CsCl/4M guanidinium chloride, of human gastric mucins from the surface epithelium (a, b, c) and the glands (d, e, f) of antrum

Antrum tissue, solubilized in 6 M guanidinium chloride, was subjected to density-gradient centrifugation (36000 revs/min, 85 h, 15 °C; Beckman 50.2 Ti rotor). Fractions were retrieved from the bottom of the tubes and analysed for density (■), A_{280} (—), carbohydrate (▲), and reactivity with the LUM5-1 (○), LUM6-3 (▼), CLH2 (●), anti-Le^b (◇) or anti-Le^y (◆) antibodies/antiseara and STA lectin (▽). Fractions were pooled as shown by the bars in (**b**) and (**e**).

followed that with the LUM6-3 antiserum, whereas reactivity with the 45M1 and CLH2 antibodies showed a similar pattern to that of the LUM5-1 antiserum. However, although the whole theca region of the epithelial cells was stained with the 45M1 and LUM5-1 antibodies, reactivity with the VNTR antibody (CHL2) was confined to the supranuclear region and around the theca of the cells (Figure 1d). No staining was obtained with the LUM2-3 antiserum (results not shown).

Surface epithelial mucins from corpus and antrum

In the surface epithelial extracts of corpus tissue, the major carbohydrate-containing material, as detected using the glycan detection assay, was found as a peak at 1.4 g/ml (Figure 2a). Some carbohydrate was also present at a lower density at the top of the gradient. Reactivity with the assay for sialic acid was weak, but the distribution broadly followed that for carbohydrate (results not shown). The main mucin peak was well separated from DNA, identified as a peak with high absorbance (A_{280}) at 1.47 g/ml and low-density UV-absorbing material at the top of the gradient. Reactivity with the LUM5-1 antiserum coincided with the major carbohydrate peak at 1.4 g/ml (Figure 2b). In addition, a second population at 1.3 g/ml appeared close to the top of the gradient and thus MUC5AC appears as 'high-density' and 'low-density' populations. The CLH2 antibody, recognizing the VNTR region of MUC5AC, reacted with the 'low-density' population, but not with the 'high-density' one. The monoclonal M1 antibody (45M1) has been demonstrated to react with MUC5AC mucins [28]. To confirm that the 45M1 antibody and LUM5-1 antiserum recognized the same molecules, fractions were subjected to a sandwich ELISA, where the 45M1 antibody was used as the 'catch' antibody and bound mucins were detected with the LUM5-1 antiserum. The results in Figure 2(b) show that the 45M1 antibody was able to 'catch' both the 'high-density' and 'low-density' MUC5AC mucin species. No reactivity with the LUM6-3 antiserum was observed (results not shown). Strong reactivity with the anti-Le^b antibody coincided with the 'high-density' MUC5AC mucins, whereas very little reactivity was seen with either the anti-Le^y antibody or the STA lectin (Figure 2c).

Surface epithelial mucins from antrum, as identified by the glycan detection assay, banded at a similar density (1.4 g/ml) to those from corpus (Figure 3a). Again, the reactivity with the sialic acid assay relative to that of the glycan detection assay was very low (results not shown). The LUM5-1 antiserum showed that MUC5AC was present as two populations at 1.4 and 1.3 g/ml (Figure 3b), whereas MUC6 was not detected (results not shown). The anti-Le^b antibody reacted with the 'high-density' MUC5AC mucins, but little, or no, STA lectin or anti-Le^y antibody reactivity was seen (Figure 3c).

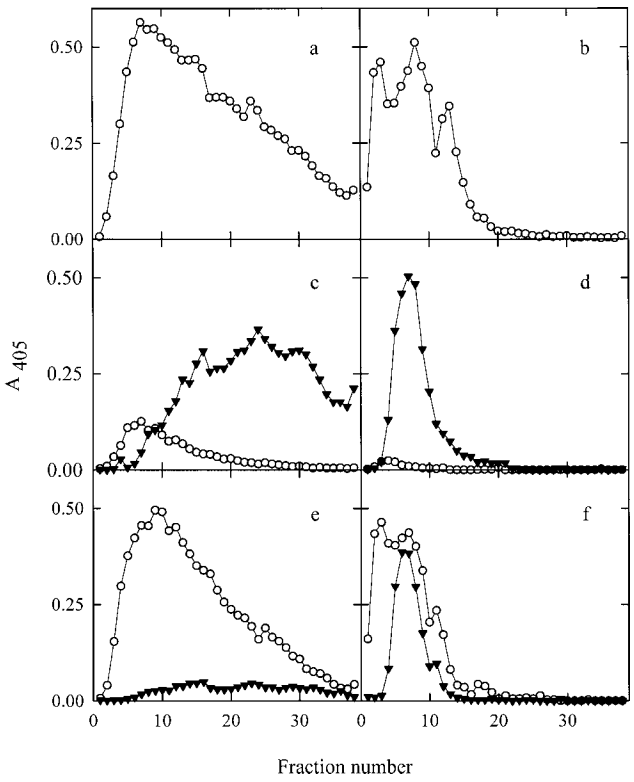

Figure 4 Rate-zonal centrifugation of whole gastric mucins (a, c, e) and the cognate reduced subunits from corpus surface epithelium and glands (b, d, f)

Mucin samples (100 μl) in 5 M guanidinium chloride were layered on top of a gradient (6–8 M guanidinium chloride). 'High-density' mucins from the surface epithelium (**a**, **b**) and 'high-density' (**c**, **d**) and 'low-density' (**e**, **f**) populations from the glands were centrifuged (2 h 45 min, 20 °C) and fractions were recovered from the top of the tubes. Fractions were analysed for reactivity with the LUM5-1 (○) or LUM6-3 (▼) antiserum.

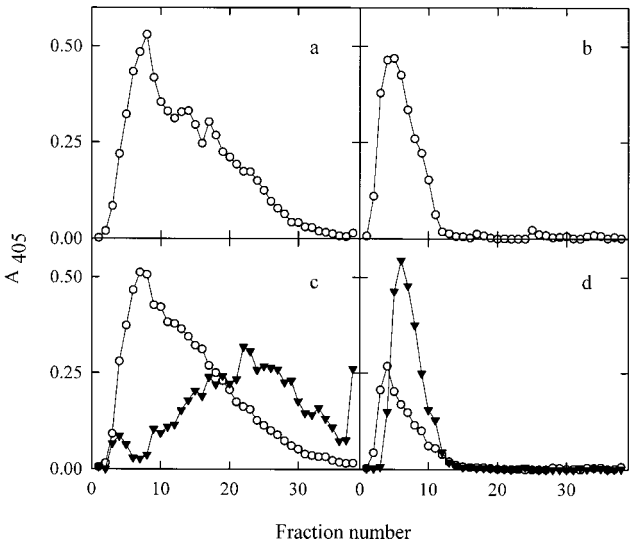

Figure 5 Rate-zonal centrifugation of whole gastric mucins (a, c) and the cognate reduced subunits from antrum surface epithelium and glands (b, d)

Mucin samples (100 μl) in 5 M guanidinium chloride were layered on top of a gradient (6–8 M guanidinium chloride). 'High-density' mucins from the surface epithelium (**a**, **b**) and the antrum gland mucin population (**c**, **d**) were centrifuged (2 h 45 min, 20 °C) and fractions were recovered from the top of the tubes. Fractions were analysed for reactivity with the LUM5-1 (○) or LUM6-3 (▼) antiserum.

Gland mucins from corpus and antrum

Mucins in the glandular extract from corpus were distributed between 1.45 and 1.38 g/ml as shown by the assay for carbohydrate (Figure 2d). In addition, carbohydrate-rich molecules and material reacting with the assay for sialic acid (results not shown) were present at the top of the gradient. Absorbance at 280 nm, at 1.47 g/ml and at the top of the gradient indicated the presence of DNA and proteins respectively. A population of MUC5AC mucins at the same density as the 'high-density' ones from the surface epithelium was revealed by LUM5-1 reactivity (Figure 2e). In addition, a major component banding between 1.42 and 1.45 g/ml reacted with the LUM6-3 antiserum. As in the surface epithelium, the antibody to the Le^b structure reacted with material corresponding to the MUC5AC population. In contrast, reactivity with the anti-Le^y antibody and the STA lectin coincided with the MUC6 mucins (Figure 2f).

In the extract from the antrum glands, the carbohydrate assay revealed a peak of mucins between 1.45 and 1.38 g/ml (Figure 3d). The LUM6-3- and LUM5-1-reactive populations were again partially separated, but MUC6 from antrum was found at a slightly lower density than that from the glands of corpus (Figure 3e). In addition, a small peak of reactivity with the LUM5-1 antiserum and CLH2 antibody was present at a similar low density to that seen in the extract from the surface epithelium of antrum. Small amounts of reactivity with the anti-Le^b antibody, as well as the anti-Le^y antibody and the STA lectin, occurred at a density similar to that with the LUM6-3 antiserum (Figure 3f). No reactivity with the LUM2-3 or LUM5B-2 antisera was observed in the surface epithelial or glandular extracts from corpus and antrum (results not shown).

Non-extractable mucins

The insoluble residues remaining after guanidinium chloride extraction of the surface epithelium and the glandular tissue were each subjected to reduction and alkylation followed by density-gradient centrifugation, as described for the soluble material. The insoluble residues from the surface epithelium of both corpus and antrum were dominated by a mucin population at 1.4 g/ml, which reacted with the LUM5-1 antiserum and corresponded to the 'high-density' MUC5AC population seen in the soluble material (results not shown). The 'low-density' MUC5AC population was absent. In the residue from the glandular tissue, MUC5AC was again the dominant mucin with only small amounts of LUM6-3-reactive material present, suggesting that a larger fraction of MUC5AC was insoluble compared with MUC6. In addition, investigation with the LUM2-3 antiserum indicated that MUC2 mucins were absent from this fraction. The total amount of material in the non-extractable fractions, estimated as the area under the curve for the carbohydrate analysis of the peak occurring at 1.4 g/ml, was 20–30 % for both corpus and antrum.

Size and subunit composition of gastric MUC5AC and MUC6 mucins

Mucins from corpus and antrum were pooled as shown in Figures 2 and 3 and subjected to rate-zonal centrifugation before and after reduction of disulphide bonds. In corpus, the major

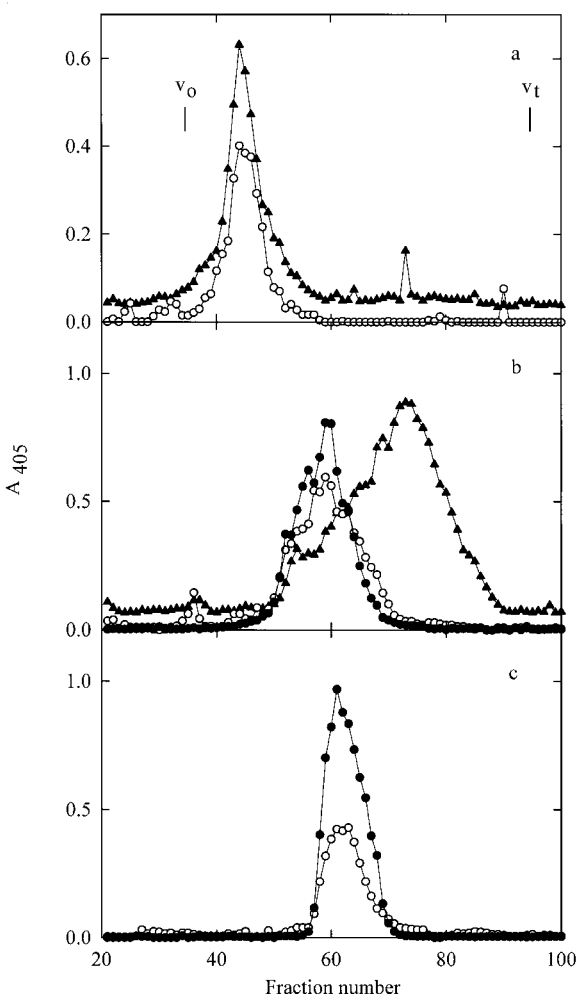

Figure 6 Gel chromatography on Sephacryl S-500 HR of mucins from corpus surface epithelium

Reduced subunits from the 'high-density' population (**a**), native mucins from the 'low-density' population (**b**) and reduced subunits from the 'low-density' population (**c**) were chromatographed on a Sephacryl S-500 HR column eluted with 4 M guanidinium chloride/10 mM sodium phosphate buffer (pH 7) at a flow rate of 0.15 ml/min. Fractions (1 ml) were analysed for carbohydrate (▲) and for reactivity with the LUM5-1 (○) antiserum and the CLH2 antibody (●). V_o, void volume; V_t, total volume.

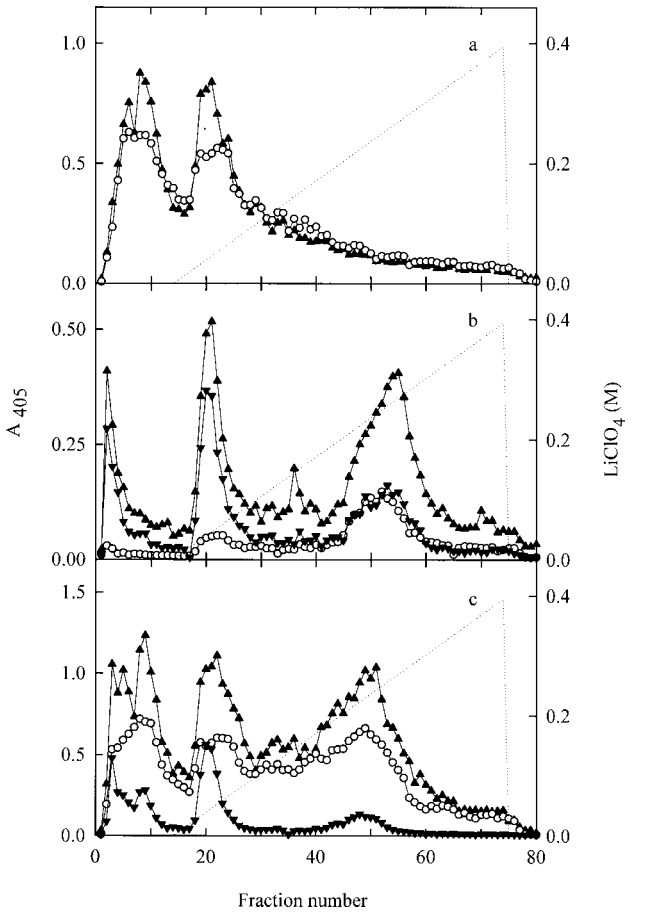

Figure 7 Ion-exchange HPLC of reduced subunits from mucins from corpus

Reduced subunits from the 'high-density' population from the surface epithelium (**a**) and 'high-density' (**b**) and 'low-density' (**c**) populations from the glands were chromatographed on a Mono Q column. Samples were eluted with buffer A for 10 min, followed by a linear gradient of 0–0.4 M $LiClO_4$ in buffer A over 60 min. The nominal gradient is shown by the dotted line. Fractions were analysed for carbohydrate (▲) and for reactivity with the LUM5-1 (○) or LUM6-3 (▼) antiserum.

MUC5AC population from the surface epithelium (Figure 2b, pool I), as well as MUC5AC from the glands (Figure 2e, pool II), were distributed mainly over the first half of the gradient (Figures 4a and 4e). In both cases, MUC5AC mucins became smaller upon reduction and the subunits separated into different populations (Figures 4b and 4f). MUC6 mucins from the glands of corpus (Figure 2e, pool I) distributed further down the gradient than MUC5AC, suggesting that they are larger (Figure 4c). Again, reduction resulted in a smaller molecular size, demonstrating that the MUC6 mucins are oligomeric structures (Figure 4d). The 'high-density' population from the surface epithelium of antrum (Figure 3b, pool I) showed a similar pattern to that of the same mucins from corpus, although there was no clear separation of reduced subunits into distinct populations (Figures 5a and 5b). Due to the poor separation between MUC5AC and MUC6 in the density gradient of material from the glands of antrum, the two mucins were pooled (Figure 3e, pool I) and subjected to rate-zonal centrifugation together (Figure 5c). The apparently larger MUC6 mucins were partially separated from MUC5AC. After reduction, subunits from MUC6 appeared to be somewhat larger than those from MUC5AC, although the two distributions overlapped (Figure 5d). This was confirmed by subjecting one sample to a longer centrifugation run under the same conditions (results not shown). In these experiments, the two species were separated further, although a large subpopulation of MUC5AC subunits overlapped with subunits of MUC6. In agreement with the density-gradients, the MUC5AC subunits reacted with the anti-Le^b antibody, whereas MUC6 subunits showed reactivity with the anti-Le^y antibody and the STA lectin.

Following gel chromatography on a Sephacryl S-500 HR column, subunits from the higher-density MUC5AC population from corpus surface epithelium (Figure 2b, pool I) were eluted as a single unimodal peak in the included volume (Figure 6a). The native 'low-density' MUC5AC molecules (Figure 2b, pool II) were more retarded on the gel than the subunits from the 'high-density' ones, suggesting that they are smaller (Figure 6b). In addition, the former molecules were partially separated from

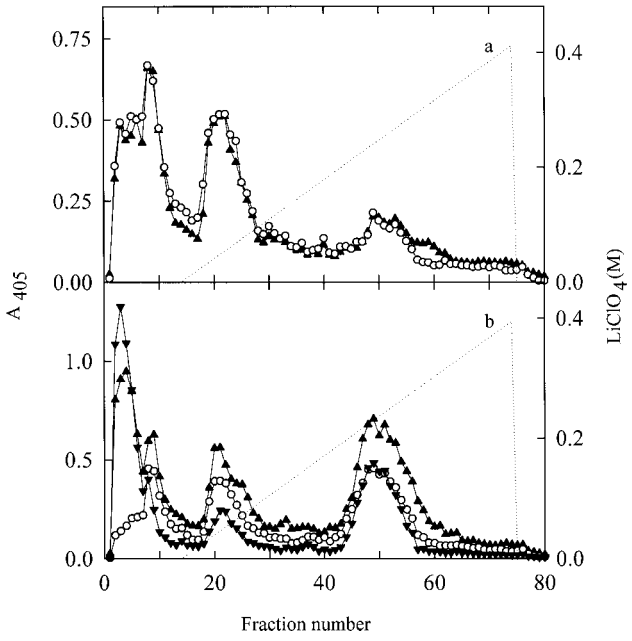

Figure 8 Ion-exchange HPLC of reduced subunits from mucins from antrum

Reduced subunits from the 'high-density' population from the surface epithelium (**a**) and gland mucins (**b**) were chromatographed on a Mono Q column. Samples were eluted with buffer A for 10 min followed by a linear gradient of 0–0.4 M $LiClO_4$ in buffer A over 60 min. The nominal gradient is shown by the dotted line. Fractions were analysed for carbohydrate (▲) and for reactivity with the LUM5-1 (○) and LUM6-3 (▼) antiserum.

carbohydrate-containing components, which were further included on the column. After reduction, the 'low-density' MUC5AC mucins were slightly more retarded, suggesting that at least a subpopulation became smaller upon reduction (Figure 6c).

Charge density of MUC5AC and MUC6 mucins

Reduced mucin subunits from the 'high-density' and 'low-density' populations from corpus surface epithelium and glands were subjected to ion-exchange HPLC (Figure 7). The 'high-density' MUC5AC mucins from the surface epithelium of corpus (Figure 2b, pool I) were separated into two major populations, one of which was not retained on the column, whereas the other one was eluted early in the gradient (Figure 7a). In the 'high-density' mucins from the glands (Figure 2e, pool I) at least three populations were evident (Figure 7b). One LUM6-3-reactive species was not retained by the column, whereas a second was eluted early and a third population late in the gradient. The two populations retained on the column showed reactivity with both the LUM5-1 and LUM6-3 antisera. The 'low-density' population from the glands (Figure 2e, pool II) contained two MUC5AC peaks, corresponding with those identified in the surface epithelium as well as the later-eluting species coinciding with that found in the 'high-density' population from the glands (Figure 7c). At least three peaks with reactivity with the LUM6-3 antiserum were also present at elution positions similar to those in the 'high-density' population.

Reduced subunits from antrum mucins showed elution patterns similar to those of corresponding species from corpus (Figure 8). The surface epithelial MUC5AC mucins (Figure 3, pool I) were separated into one population that was not retained by the column, one that was eluted early and one that was eluted late in the gradient (Figure 8a). Both MUC5AC and MUC6 (Figure 3, pool I) from the glands were eluted as several peaks (Figure 8b). The most retained population from the glands reacted with high-iron diamine reagent, suggesting that the higher-charge density displayed by these mucins, at least in part, is due to the presence of sulphate groups (results not shown).

DISCUSSION

Normal gastric mucosa has been shown to express both the *MUC5AC* and *MUC6* genes. Immunohistochemistry using antibodies directed against the tandem repeat regions of these mucins has identified MUC5AC as originating from the surface epithelium, whereas MUC6 is produced in the glands [4,5]. In the present study, staining with the LUM5-1 and LUM6-3 antisera confirmed this distribution, with MUC5AC predominantly localized to the surface epithelium and MUC6 in the glands. The 45M1 monoclonal antibody stained the same cells as the LUM5-1 antiserum as well as being able to 'catch' molecules recognized by the LUM5-1 antiserum from tissue extracts. Our present study thus lends weight to the data indicating that the M1 epitope recognized by the 45M1 antibody is present within the MUC5AC molecule [28].

Since the MUC5AC and MUC6 mucins had a differential distribution, the gastric surface mucosa was scraped from the glandular tissue prior to further biochemical analysis to enrich mucins from the surface epithelium and gland cells respectively. Isopycnic density-gradient centrifugation was used to separate the different mucins in each of the extracts and the apoprotein identity of the dominating species in each fraction was investigated using the LUM5-1 and LUM6-3 antisera. In keeping with the immunochemical data, MUC6 mucins were found predominantly in the gland preparations. MUC5AC however, was found in both the gland and surface epithelial fractions. Immunohistochemical staining showed that, in addition to being present in the surface epithelium, MUC5AC is produced in cells deep within the gastric pits. In a previous study using porcine gastric tissue [14], we have shown that it is difficult to harvest the cells deep in the gastric pits by scraping the mucosa and that this is likely to explain the presence of MUC5AC in the extracts from the gland tissue.

Mucins residing in the gene cluster at 11p15.5 (*MUC2*, *MUC5B*, *MUC5AC* and *MUC6*) have been shown to share sequence similarities in the C-terminal and, for *MUC2*, *MUC5AC* and *MUC5B*, in the N-terminal regions, which has led to the suggestion that they all encode large oligomeric mucins [18]. This has been demonstrated for human respiratory MUC5AC [29], salivary, respiratory and cervical MUC5B [21,30,31] and intestinal MUC2 [20], and all these mucins have been shown to dimerize during synthesis [17,18]. However, the degree of oligomerization of gastric MUC5AC and MUC6 has not been established fully. In the present study, rate-zonal centrifugation indicated that both MUC5AC and MUC6 represent large oligomeric mucins composed of subunits linked by disulphide bonds. Native MUC6 mucins and reduced MUC6 subunits, corresponding to mucin monomers, appeared to be of larger molecular size than MUC5AC. Thus, in this respect, human gastric mucins are similar to porcine gastric mucins, where those originating from the glands were larger than those from the surface epithelium [14,32].

Although in normal gastric mucosa the predominantly expressed mucin genes are *MUC5AC* and *MUC6*, both intestinal metaplasia and gastric cancer are characterized by the appearance of MUC2 expression [5,7]. Staining for MUC2 was absent from

the sections prepared from tissue used in the present study, suggesting that neither intestinal metaplasia nor gastric cancer was present in the tissue. The presence of MUC2 mucins was also investigated in the tissue extracts. Since MUC2 in the intestine appears as a complex, which is insoluble in guanidinium chloride, the presence of MUC2 mucins was also studied in the insoluble residues from the tissue pieces which were brought into solution with reduction [20,33]. No MUC2 was detected confirming that the resected tissue used for isolation of mucins was not significantly affected by either intestinal metaplasia or cancer.

In addition to the 'high-density' MUC5AC and MUC6 mucins, a 'low-density' population of MUC5AC, as shown by reactivity with the LUM5-1 antisera, was also present in the tissue extracts from the surface epithelium. The reactivity of this population with the CLH2 antibody, raised against a sequence within the tandem repeat region of MUC5AC [22], indicates either that the apoprotein is unglycosylated or glycosylated sparsely so that the epitope is still accessible. In addition, the 'low-density' species were eluted later from a Sephacryl S-500 HR gel-filtration column than reduced subunits from the 'high-density' MUC5AC mucins, suggesting a smaller hydrodynamic volume. Since substitution with the link GalNAc is largely responsible for endowing mucin apoproteins with their extended conformation, and thus their large hydrodynamic volume [34], it is likely that the 'low-density' population represents a non-glycosylated precursor form of the large 'high-density' MUC5AC mucins. Reduction of disulphide bonds led to a slight shift in elution position and, since the glycosylation should not be affected by this treatment, this is likely to reflect a decrease in size. Possibly, the 'low-density' peak represents a mixed population of monomeric and disulphide-bond-linked non-glycosylated MUC5AC precursors. Such precursors have been identified previously by other workers [17,18], where MUC2, MUC5AC, MUC5B and MUC6 apoproteins were all shown to form dimers prior to the initiation of glycosylation.

Analysis of gastric MUC5AC and MUC6 using carbohydrate-specific antibodies and lectins revealed differences in glycosylation between the gene products, with Le^b (type 1) structures found mainly on MUC5AC and Le^y (type 2) structures and terminal GlcNAc, as detected with the STA lectin, found on MUC6. These data are in agreement with a previous study showing the expression of FUT2 fucosyltransferase and MUC5AC in the gastric surface epithelium and FUT1 and MUC6 in the glands [10]. The 'mature' MUC5AC mucins from the surface epithelium were separated into two populations on the basis of charge density, suggesting the presence of additional differences in glycosylation. The populations of MUC6 and 'high-density' MUC5AC mucins from the gastric glands were each separated into three main populations, with one neutral and one moderately charged species as well as one highly charged population of each mucin. Since the mucins from the glands reacted poorly with the analysis for sialic acid, these variations in charge density could not be attributed to differences in sialic acid content, but may, in part, be due to differences in the level of sulphation. The reactivity of the most highly charged mucin population with high-iron diamine lends weight to this suggestion. Since the mucins were isolated from tissue, it is not currently known whether the differently glycosylated forms of MUC5AC and MUC6 correspond to partially glycosylated biosynthetic intermediates or whether heterogeneity amongst the mucin-producing cells, with respect to glycosyltransferases and/or the existence of different glycosylation programmes, give rise to true glycoforms. However, studies in porcine gastric mucosa have shown that the mucous neck cells produce a more highly charged mucin than the surface epithelial and submucosal cells [12]. Although the significance of different mucin glycoforms is not well understood, highly charged mucin species from porcine stomach have been shown to interact more strongly with the gastric pathogen *Helicobacter pylori* at low pH than those that are less charged [35].

In summary, both MUC5AC and MUC6 of gastric origin were shown to be large oligomeric mucins and native MUC6 mucins to be larger than MUC5AC. Distinct glycosylation differences were noted between MUC5AC and MUC6 with regard to Lewis determinants, and both mucins appeared as several glycoforms. MUC5AC was found in two forms, one representing the apparently mature fully glycosylated MUC5AC and the other a low-glycosylated oligomerized form, likely to be a MUC5AC 'precursor'. Gastric mucus is thus composed of two mucins that differ in size, glycosylation and tissue distribution, and differential secretion of mucins from the surface epithelium and glands may provide a mechanism for modulation of the composition of the protective mucus layer related to acid secretion or the presence of bacteria and noxious agents in the lumen.

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REFERENCES

1 Allen, A., Flemström, G., Garner, A. and Kivilaakso, E. (1993) Gastroduodenal mucosal protection. Physiol. Rev. **73**, 823–857
2 Ota, H., Katsuyama, T., Ishii, K., Nakayama, J., Shiozawa, T. and Tsukahara, Y. (1991) A dual staining method for identifying mucins of different gastric epithelial mucous cells. Histochem. J. **23**, 22–28
3 Ota, H. and Katsuyama, T. (1992) Alternating laminated array of two types of mucin in the human gastric surface mucous layer. Histochem. J. **24**, 86–92
4 de Bolós, C., Garrido, M. and Real, F. X. (1995) MUC6 apomucin shows a distinct normal tissue distribution that correlates with Lewis antigen expression in the human stomach. Gastroenterology **109**, 723–734
5 Ho, S. B., Roberton, A. M., Shekels, L. L., Lyftogt, C. T., Niehans, G. A. and Toribara, N. W. (1995) Expression cloning of gastric mucin complementary DNA and localization of mucin gene expression. Gastroenterology **109**, 735–747
6 Ho, S. B., Shekels, L. L., Toribara, N. W., Kim, Y. S., Lyftogt, C., Cherwitz, D. L. and Niehans, G. A. (1995) Mucin gene expression in normal, preneoplastic, and neoplastic human gastric epithelium. Cancer Res. **55**, 2681–2690
7 Reis, C. A., David, L., Correa, P., Carneiro, F., de Bolós, C., Garcia, E., Mandel, U., Clausen, H. and Sobrinho-Simoes, M. (1999) Intestinal metaplasia of human stomach displays distinct patterns of mucin (MUC1, MUC2, MUC5AC, and MUC6) expression. Cancer Res. **59**, 1003–1007
8 Mollicone, R., Bara, J., Le Pendu, J. and Oriol, R. (1985) Immunohistologic pattern of type 1 (Lea, Leb) and type 2 (X, Y, H) blood group-related antigens in the human pyloric and duodenal mucosae. Lab. Invest. **53**, 219–227
9 Sakamoto, J., Furukawa, K., Cordon-Cardo, C., Yin, B. W. T., Rettig, W. J., Oettgen, H. F., Old, L. J. and Lloyd, K. O. (1986) Expression of Lewis a, Lewis b, X, and Y blood group antigens in human colonic tumors and normal tissue and in human tumor-derived cell lines. Cancer Res. **46**, 1553–1561
10 Lopéz-Ferrer, A., de Bolós, C., Barranco, C., Garrido, M., Isern, J., Carlstedt, I., Reis, C. A., Torrado, J. and Real, F. X. (2000) Role of fucosyltransferases in the association between apomucin and Lewis antigen expression in normal and malignant gastric epithelium. Gut **47**, 349–356
11 Snary, D., Allen, A. and Pain, R. H. (1970) Structural studies on gastric mucoproteins: lowering of molecular weight after reduction with 2-mercaptoethanol. Biochem. Biophys. Res. Commun. **40**, 844–851
12 Carlstedt, I., Lindgen, H. and Sheehan, J. K. (1983) The macromolecular structure of human cervical-mucus glycoproteins. Studies on fragments obtained after reduction of disulphide bridges and after subsequent trypsin digestion. Biochem. J. **213**, 427–435
13 Sheehan, J. K., Oates, K. and Carlstedt, I. (1986) Electron microscopy of cervical, gastric and bronchial mucus glycoproteins. Biochem. J. **239**, 147–153
14 Nordman, H., Davies, J. R., Herrmann, A., Karlsson, N., Hansson, G. C. and Carlstedt, I. (1997) Mucus glycoproteins from pig gastric mucosa: identification of different mucin populations from the surface epithelium. Biochem. J. **326**, 903–910

15 Escande, F., Aubert, J. P., Porchet, N. and Buisine, M. P. (2001) Human mucin gene MUC5AC: organization of its 5′-region and central repetitive region. Biochem. J. **358**, 763–772
16 Toribara, N. W., Ho, S. B., Gum, E., Gum, J. R., Lau, P. and Kim, Y. S. (1997) The carboxyl-terminal sequence of the human secretory mucin, MUC6. Analysis of the primary amino acid sequence. J. Biol. Chem. **272**, 16398–16403
17 Asker, N., Baeckström, D., Axelsson, M. A. B., Carlstedt, I. and Hansson, G. C. (1995) The human MUC2 mucin apoprotein appears to dimerize before O-glycosylation and shares epitopes with the 'insoluble' mucin of rat small intestine. Biochem. J. **308**, 873–880
18 Van Klinken, B. J. W., Einerhand, A. W. C., Buller, H. A. and Decker, J. (1998) The oligomerization of a family of four genetically clustered human gastrointestinal mucins. Glycobiology **8**, 67–75
19 Hovenberg, H. W., Davies, J. R., Herrmann, A., Lindén, C.-J. and Carlstedt, I. (1996) MUC5AC, but not MUC2, is a prominent mucin in respiratory secretions. Glycoconjugate J. **13**, 839–847
20 Herrmann, A., Davies, J. R., Lindell, G., Mårtensson, S., Packer, N., Swallow, D. M. and Carlstedt, I. (1999) Studies on the 'insoluble' glycoprotein complex from human colon. Identification of reduction-insensitive MUC2 oligomers and C-terminal cleavage. J. Biol. Chem. **274**, 15828–15836
21 Wickström, C., Davies, J. R., Eriksen, G. V., Veerman, E. C. I. and Carlstedt, I. (1998) MUC5B is a major gel-forming, oligomeric mucin from human salivary gland, respiratory tract and endocervix: identification of glycoforms and C-terminal cleavage. Biochem. J. **334**, 685–693
22 Reis, C. A., David, L., Nielsen, P. A., Clausen, H., Mirgorodskaya, K., Roepstorff, P. and Sobrinho-Simoes, M. (1997) Immunohistochemical study of MUC5AC expression in human gastric carcinomas using a novel monoclonal antibody. Int. J. Cancer **74**, 112–121
23 Hsu, S. M. and Raine, L. (1982) Versatility of biotin-labeled lectins and avidin-biotin-peroxidase complex for localization of carbohydrate in tissue sections. J. Histochem. Cytochem. **30**, 157–161
24 Davies, J. R., Hovenberg, H. W., Lindén, C.-J., Howard, R., Richardson, P. S., Sheehan, J. K. and Carlstedt, I. (1996) Mucins in airway secretions from healthy and chronic bronchitic subjects. Biochem. J. **313**, 431–439
25 Jourdian, G. W., Dean, L. and Roseman, S. (1971) The sialic acids. A periodate-resorcinol method for the quantitative estimation of free sialic acids and their glycosides. J. Biol. Chem. **246**, 430–435
26 Devine, P. L. (1992) A sensitive microplate assay for glycoproteins that utilizes an immunological digoxigenin-based detection system. BioTechniques **12**, 160–162
27 Sheehan, J. K. and Carlstedt, I. (1987) Size heterogeneity of human cervical mucus glycoproteins. Studies performed with rate-zonal centrifugation and laser light-scattering. Biochem. J. **245**, 757–762
28 Bara, J., Chastre, E., Mahiou, J., Singh, R. L., Forgue-Lafitte, M. E., Hollande, E. and Godeau, F. (1998) Gastric M1 mucin, an early oncofetal marker of colon carcinogenesis, is encoded by the MUC5AC gene. Int. J. Cancer **75**, 767–773
29 Hovenberg, H. W., Davies, J. R. and Carlstedt, I. (1996) Different mucins are produced by the surface epithelium and the submucosa in human trachea: identification of MUC5AC as a major mucin from the goblet cells. Biochem. J. **318**, 319–324
30 Thornton, D. J., Khan, N., Mehrotra, R., Howard, M., Veerman, E., Packer, N. H. and Sheehan, J. K. (1999) Salivary mucin MG1 is comprised almost entirely of different glycosylated forms of the MUC5B gene product. Glycobiology **9**, 293–302
31 Thornton, D. J., Howard, M., Khan, N. and Sheehan, J. K. (1997) Identification of two glycoforms of the MUC5B mucin in human respiratory mucus. Evidence for a cysteine-rich sequence repeated within the molecule. J. Biol. Chem. **272**, 9561–9566
32 Nordman, H., Davies, J. R. and Carlstedt, I. (1998) Mucus glycoproteins from pig gastric mucosa: different mucins are produced by the surface epithelium and the glands. Biochem. J. **331**, 687–694
33 Carlstedt, I., Herrmann, A., Hovenberg, H., Lindell, G., Nordman, H., Wickström, C. and Davies, J. R. (1995) 'Soluble' and 'insoluble' mucins – identification of distinct populations. Biochem. Soc. Trans. **23**, 845–851
34 Gerken, T. A., Butenhof, K. J. and Shogren, R. (1989) Effects of glycosylation on the conformation and dynamics of O-linked glycoproteins: carbon-13 NMR studies of ovine submaxillary mucin. Biochemistry **28**, 5536–5543
35 Nordman, H., Borén, T., Davies, J. R., Engstrand, L. and Carlstedt, I. (1999) pH-dependent binding of *Helicobacter pylori* to pig gastric mucins. FEMS Immunol. Med. Microbiol. **24**, 175–181

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