Mucus glycoproteins from pig gastric mucosa: different mucins are produced by the surface epithelium and the glands

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An antibody (PGM2B) recognizing a pig gastric-mucin apoprotein reacts with the surface epithelium of pig gastric mucosa. Virtually no reactivity was observed over the mucin-producing cells in the glands, which were recognized by the GlcNAc-selective *Griffonia simplicifolia* II (GSA-II) lectin. Mucins from the glandular tissue of the cardiac region, corpus and antrum were purified using isopycnic density-gradient centrifugation in CsCl/guanidinium chloride. In the cardiac region, two major mucin populations at 1.5 and 1.4 g/ml were identified. The high-density population reacted preferentially with the PGM2B antibody and resembled mucins from the surface epithelium of this region, whereas the low-density population reacted strongly with the GSA-II lectin and appeared to originate from the glands. In the glandular tissue of corpus, a component with strong GSA-II lectin reactivity, which was distinctly different from the surface mucins from this region, was found at 1.4 g/ml, thus resembling the gland component from the cardiac region. Mucins from antrum glandular tissue contained at least two GSA-II lectin-reactive populations banding at 1.5 and 1.4 g/ml, respectively. Gland mucins from all regions were large oligomeric glycoproteins and heterogeneous with respect to charge properties, as shown by using rate-zonal centrifugation and ion-exchange HPLC, respectively. Gel chromatography of mucin glycopeptides showed that gland mucins from antrum and corpus contained significantly longer glycosylated domains than those from the surface mucosa. Thus, mucins from pig gastric glandular tissue comprise a number of large and oligomeric glycoproteins that differ from those from the surface epithelium in buoyant density, apoprotein structure and carbohydrate substitution.

INTRODUCTION

The gastric surface is covered by a mucus layer that protects the mucosa against HCl and pepsin [1]. The polymer matrix of mucus is formed by high-molecular-mass oligomeric glycoproteins, the so-called mucus glycoproteins or mucins. Gastric mucins are produced by cells in the surface epithelium, as well as in the glands, and secretions from these two sources may form laminated structures in the mucus layer [2]. In some species, 'surface' and 'gland' mucins can be distinguished by using lectins or carbohydrate-specific monoclonal antibodies. For example, the *Griffonia simplicifolia* II (GSA-II) lectin reacts preferentially with the mucous neck cells, as well as with the glands in rat gastric mucosa [3,4], and in human gastric mucosa, type 1 backbone structures (Le^a and Le^b) are present in the surface epithelium, whereas type 2 backbone structures (Le^x and Le^y) are found in the glands [5,6]. In addition, differences in the expression of mucin apoproteins between the surface epithelium and the glands have been demonstrated in several species. An antibody (anti-PGM-HF) against deglycosylated pig gastric mucins reacts only with the surface epithelial cells and has been proposed to recognize the pig MUC5AC homologue [7]. Putative MUC5AC homologues have also been identified in the surface gastric mucosa of mouse [8] and rat [9]. The M1 antigen, which is associated with the mucin apoprotein, is confined to the gastric-surface epithelium of pig and man [10,11]. In the human gastric antrum, studies using both *in situ* hybridization and immunohistochemistry have shown that the surface epithelial cells secrete the MUC5AC mucin, whereas the glands produce MUC6 [12–14], following the expression of the Le^a/Le^b and the Le^x/Le^y structures, respectively.

Previously, we have shown that the large oligomeric mucins from the surface epithelium of pig gastric mucosa comprise several distinct components that vary in 'solubility', 'acidity', oligosaccharide substitution and cellular origin [15,16]. Here, we have isolated the major high-molecular-mass oligomeric mucins from the glands and compared them with those from the surface epithelium. A number of distinct populations of gland mucins were identified, which differed from those of the surface epithelium in buoyant density, apoprotein identity, size of the highly glycosylated domains and carbohydrate substitution.

MATERIALS AND METHODS

Materials

Trypsin [EC 3.4.21.4; type XIII, L-(1-tosylamido-2-phenyl) ethyl chloromethyl ketone-treated], pepsin (type A, EC 3.4.23.1), iodoacetamide, 3-aminopropyl-triethoxy-silane, 3,3′-diaminobenzidine (DAB) and guanidinium chloride were obtained from Sigma. Stock solutions (approx. 8 M) of guanidinium chloride were treated with charcoal and filtered through an Amicon PM-10 ultrafiltration membrane (nominal cut-off 10 kDa) before use. BSA (fraction V, pH 7.0) was from Serva (Heidelberg, Germany), CHAPS was from Boehringer, di-isopropyl phosphorofluoridate (DFP) was from Fluka and 1,4-dithiothreitol (DTT) was from Merck. Sephacryl S-500 and a Mono Q column were purchased from Pharmacia.

Abbreviations used: DAB, 3,3′-diaminobenzidine; DIG, digoxigenin; DFP, di-isopropyl phosphorofluoridate; DTT, 1,4-dithiothreitol; GSA-II, *Griffonia simplicifolia* II; Le, Lewis structure; TBS, Tris-buffered saline; HD, high density; LD, low density.

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Antibodies and lectins

Biotinylated GSA-II lectin was purchased from EY Laboratories, San Francisco, CA, U.S.A. or Vector, Burlingame, CA, U.S.A. Alkaline phosphatase-conjugated streptavidin and anti-digoxigenin antibodies (anti-DIG) were from Boehringer, alkaline phosphatase-conjugated swine anti-rabbit immunoglobulins were from Dako (Denmark), and alkaline phosphatase-conjugated goat anti-mouse IgM and monoclonal anti-gastric mucin antibody (45M1) were from Sigma. The monoclonal anti-Le^b antibody (96 FR 2.10) was obtained from Biotest, Dreieich, Germany and clone 2-25LE was a kind gift from Dr. J. Bara, U-55 INSERM, Paris, France. The polyclonal antibody PGM2B was raised in rabbits against a synthetic peptide with the sequence RNQDQGGKFRIC from a putative pig gastric MUC5AC homologue [7]. 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 4 weeks and the antiserum was collected 2 weeks thereafter.

Histochemical methods

For lectin staining and immunohistochemistry, sections (4 μm) of formalin-fixed paraffin-embedded porcine gastric tissue were prepared on slides coated with 3-aminopropyl-triethoxy-silane. Sections were dewaxed, rehydrated and treated with 10 mM sodium citrate buffer, pH 6, at 100 °C for 5 min in a microwave oven. All subsequent steps were conducted at room temperature in immunostaining chambers (Coverplate, Shandon, U.K.). Endogenous peroxidase activity was quenched by treating sections with 3 % (v/v) hydrogen peroxide in water for 30 min. Sections were washed with Tris-buffered saline (TBS; 0.15 M NaCl/0.1 M Tris/HCl buffer, pH 7.4) prior to blocking of non-specific binding with normal goat serum diluted 1:5 in TBS (for immunohistochemistry) or 1 % BSA in TBS/Tween [TBS containing 0.05 % (v/v) Tween 20; for lectin staining] for 1 h. For immunohistochemical localization, sections were then incubated with the PGM2B antibody (diluted 1:2000 in TBS), the 45M1 antibody (diluted 1:10000 in TBS) or the Le^b antibodies (diluted 1:100 in TBS) for 1 h followed by the StreptABComplex/HRP Duet kit (Dako). For lectin histochemistry, a modified version of the StreptABComplex/HRP method was used [17]. Sections were probed with biotinylated GSA-II lectin (0.25 μg/ml in TBS/Tween containing 0.1 mM $CaCl_2$) for 1 h and then incubated with the streptavidin/biotinylated horseradish peroxidase part of the StreptABComplex/HRP kit for 30 min. Binding of the lectin and the antibodies was visualized using DAB (0.6 mg/ml) in TBS containing 0.03 % (v/v) hydrogen peroxide for 15 min. Control sections were incubated with the pre-immune serum from the PGM2B rabbit (diluted 1:2000 in TBS), with TBS alone instead of the PGM2B antibody, or with GSA-II lectin, and subsequent steps were carried out as outlined above. After visualization of binding with DAB antibody/GSA-II lectin sections were counterstained with Mayer's haematoxylin. Microscopy was performed using a Nikon Optifot microscope.

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 aliquots (100 μl) coated on to microtitre plates (polyvinyl chloride, Falcon 3912) overnight in a humidified chamber at room temperature. After washing in 0.15 M NaCl/5 mM sodium phosphate buffer, pH 7.4, containing 0.05 % (v/v) Tween 20 (washing solution), unbound sites were blocked for 1 h with 200 μl of 1 % (w/v) BSA in washing solution (blocking solution). The wells were then incubated with primary antibody for 1 h (anti-Le^b, clone FR 96 2.10, diluted 1:250 or PGM2B antiserum diluted 1:1000 in blocking solution). After washing and incubation with the secondary antibody [alkaline phosphatase-conjugated goat anti-mouse IgM (diluted 1:40000) or alkaline phosphatase-conjugated swine anti-rabbit immunoglobulins (diluted 1:2000)] for 1 h, plates were washed, and bound secondary antibodies were detected with *p*-nitrophenyl phosphate (2 mg/ml) in 0.5 mM $MgCl_2$/1 M diethanolamine buffer, pH 9.8, as a substrate. Absorbance at 405 nm was measured after 1 h.

Lectin-binding assays were performed essentially as the ELISA procedure. After blocking unbound sites with 1 % (w/v) BSA in 0.15 M NaCl/0.05 M Tris/HCl buffer, pH 7.6, containing 0.05 % (v/v) Tween 20 (blocking solution for lectin-ELISA), the wells were incubated for 1 h with biotinylated lectin (2 μg/ml), diluted in blocking solution for lectin-ELISA containing 0.1 mM $CaCl_2$. After washing, bound lectin was detected with alkaline phosphatase-conjugated streptavidin as described above.

Analytical methods

Density measurements were performed using a Carlsberg pipette as a pycnometer. Sialic acid was assayed by the method of Jourdian et al. [18] with an automated procedure [19], as modified by Davies et al. [20]. Hexose/fucose was measured with the anthrone method using a manual procedure [21] for density-gradient fractions and an automated procedure [22] for fractions from the chromatographic runs. Carbohydrate was also measured as periodate-oxidizable structures in a microtitre-plate assay [23]. In this assay, samples (100 μl) were coated on to microtitre plates overnight. After washing in 0.15 M NaCl/5 mM sodium phosphate buffer, pH 7.4, containing 0.05 % (v/v) Tween 20, oxidation was performed with 100 μl of 25 mM metaperiodate in 0.1 M sodium acetate buffer, pH 5.5, for 20 min. The wells were washed and incubated with 100 μl of DIG-succinyl-ϵ-caproic acid hydrazide (0.6 μg/ml) for 1 h, followed by washing and detection of bound DIG with 100 μl of alkaline phosphatase-conjugated anti-DIG antibody (diluted 1:5000). Bound secondary antibody was detected with *p*-nitrophenyl phosphate (2 mg/ml) in 0.5 mM $MgCl_2$/1 M diethanolamine buffer, pH 9.8, as a substrate. Absorbance at 405 nm was read after 1 h. All incubations were performed in a humidified chamber at room temperature.

Isolation of mucins and mucin subunits

The surface mucosa of the cardiac region, corpus and antrum of pig stomachs was removed by gently scraping the tissue with a glass microscope slide. The glandular mucosa was then dissected from the underlying muscle layer, quickly frozen in liquid nitrogen and stored at −70 °C. The glandular tissue was immersed in liquid nitrogen and pulverized in a Retsch tissue pulverizer (1 min) before extraction. Frozen material (2g) was thawed in the presence of DFP (1 ml of a 0.1 M solution in propanol) for 5 min before the addition of 50 ml of ice-cold 6 M guanidinium chloride/5 mM Na_2EDTA /5 mM *N*-ethylmaleimide/10 mM sodium phosphate buffer, pH 6.5 (extraction buffer). After dispersion with a Dounce homogenizer (four strokes; loose pestle), the tissue was subjected to slow stirring overnight at 4 °C. The 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

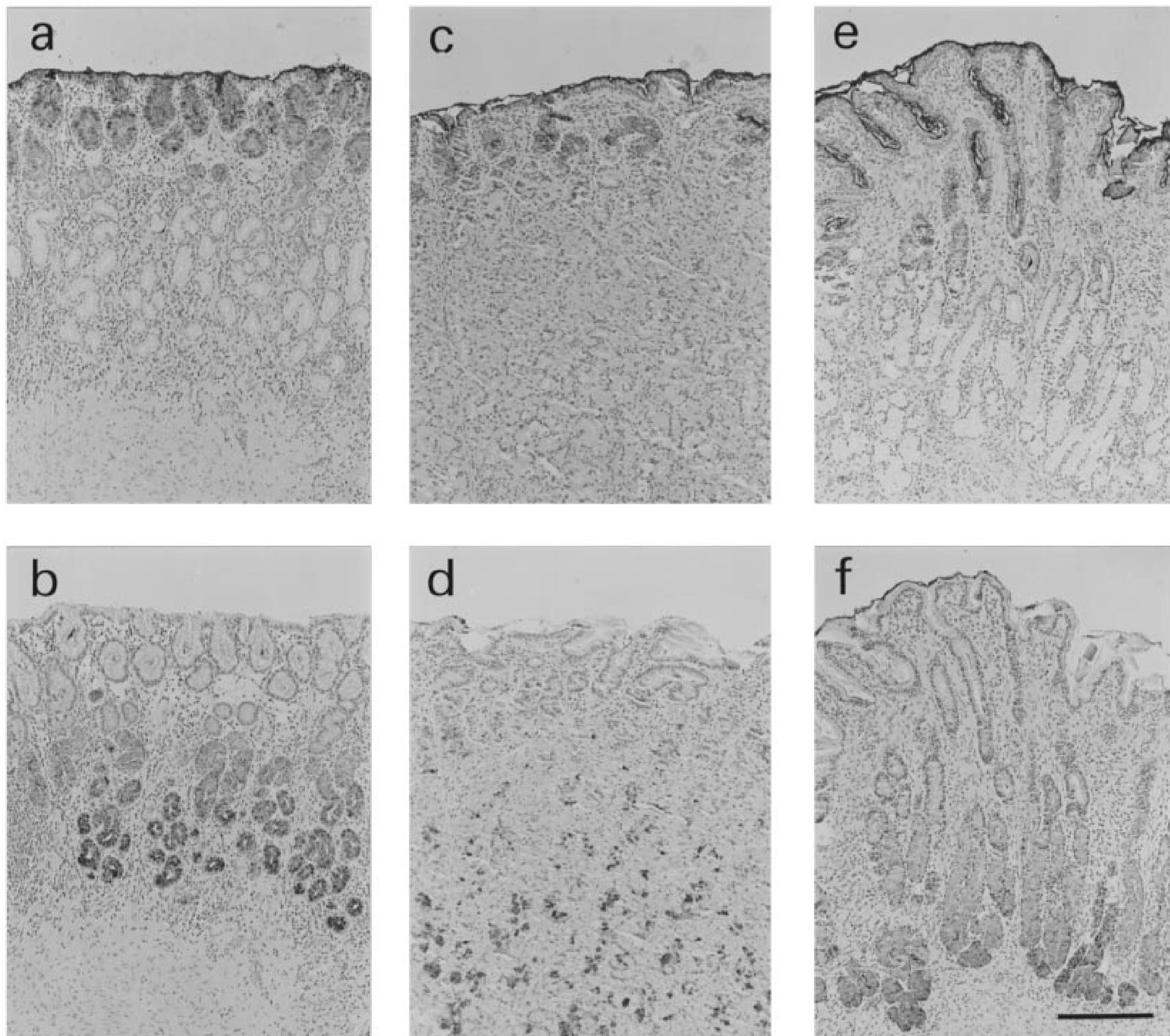

Figure 1 Histochemical localization of mucins in pig gastric mucosa

Tissue sections (4 μm) from the cardiac region (**a**,**b**), corpus (**c**,**d**) and antrum (**e**,**f**) were stained with the PGM2B antibody (**a**,**c**,**e**) or the GSA-II lectin (**b**,**d**,**f**), as described in the text, and counterstained with Mayer's haematoxylin. The bar indicates 200 μm.

buffer. Mucins were purified from lipids and non-mucin proteins by using isopycnic density-gradient centrifugation in CsCl/4 M guanidinium chloride and from DNA in a second density-gradient run in CsCl/0.5 M guanidinium chloride as described previously [15].

Reduced mucin subunits were obtained by reduction of the cognate mucins with 10 mM DTT in 6 M guanidinium chloride/5 mM EDTA/0.1 M Tris/HCl buffer, pH 8, for 5 h at 37 °C, followed by alkylation with 25 mM iodoacetamide overnight at room temperature. Mucins from the surface epithelium of the cardiac region, corpus and antrum were isolated as described previously [15].

Rate-zonal centrifugation

Solutions of whole mucins and reduced subunits in 5 M guanidinium chloride (100 μl) were layered on to a 6–8 M guanidinium chloride gradient [24]. The tubes were spun (40000 rev./min, 2 h 45 min, 20 °C; Beckman SW-41 rotor) and fractions (400 μl) taken from the top of the gradients and analysed for carbohydrate and lectin reactivity.

Ion-exchange HPLC of reduced subunits

Reduced mucin subunits were dialysed against starting buffer [6 M urea/10 mM piperazine/perchlorate buffer, pH 5, containing 0.1 % (w/v) CHAPS] and applied to a Mono Q HR5/5 column as described [15]. The column was eluted (0.5 ml/min) with starting buffer for 10 min followed by a linear gradient (60 min) up to 0.4 M $LiClO_4$ in starting buffer. Fractions (0.5 ml) were diluted with 4 M guanidinium chloride/10 mM sodium phosphate buffer, pH 7, and analysed for carbohydrate and reactivity with the GSA-II lectin.

Gel chromatography of high-molecular-mass glycopeptides

Tryptic fragments were obtained after digestion of the cognate reduced subunits in 0.1 M NH_4HCO_3, pH 8, for 5 h at 37 °C

using approx. 20 μg of trypsin/mg of subunits. Pepsin digestion of reduced subunits was carried out in 50 mM glycine/HCl, pH 2.2, for 12 h at 37 °C using approx. 10 μg of pepsin/mg of subunits. Reduced subunits and proteolytic fragments were subjected to gel chromatography on a Sephacryl S-500 column (1.6 × 50 cm) eluted with 0.1 M ammonium acetate at a flow rate of 0.15 ml/min. Fractions (1 ml) were collected and analysed with the anthrone method.

RESULTS

Histology

In all regions, the PGM2B antibody showed a strong and uniform reactivity over the surface mucosa (Figures 1a, 1c and 1e). The staining extended deep into the bottom of the gastric pits and, in antrum, possibly even into the uppermost part of the gland region, making it difficult to decide whether the staining was associated with the foveolar cells or the glands (Figure 1e). In corpus, a weak diffuse staining was also present over the glands (Figure 1c). A staining pattern similar to that of the PGM2B antibody was also observed with a monoclonal antibody (45M1) against the M1 antigen [25], except that the latter antibody did not react with the corpus glands (results not shown). The reactivity with the (96 FR 2.10) Le^b antibody was relatively weak and patchy but followed largely the distribution of the PGM2B and 45M1 antibodies. In contrast, the (2-25LE) Le^b antibody did not react at all (results not shown). In all regions, the GlcNAc-selective GSA-II lectin reacted mainly with the gland cells, although material in the gastric-gland lumen and on the luminal surface was also stained (Figures 1b, 1d and 1f).

Isolation of mucins

Material reacting with both the anthrone and sialic acid assays was present in CsCl/4 M guanidinium chloride gradients of extracts from the glandular tissue of all regions. In the cardiac region, a major sialic acid-reactive peak at a density of 1.47 g/ml coincided with a component absorbing strongly at 280 nm, which probably corresponded with DNA ([26]; Figure 2a). This material was separate from a small peak of sialic acid-containing molecules at 1.4 g/ml and low-density material at the top of the gradient. Reactivity with the (96 FR 2.10) Le^b and PGM2B antibodies was mainly associated with the population at 1.47 g/ml, whereas the lower-density species reacted with the GSA-II lectin, in particular with the population banding at 1.4 g/ml (Figure 2b). In corpus, a mucin population banding at 1.37 g/ml was separated from a major A_{280}-absorbing peak at 1.47 g/ml (Figure 2c). The latter population was associated with some reactivity with the (96 FR 2.10) Le^b and PGM2B antibodies, whereas strong reactivity with the GSA-II lectin was observed over the low-density population at 1.37 g/ml (Figure 2d). In antrum, the predominant sialic acid-containing molecules coincided with DNA at 1.47 g/ml (Figure 2e). Antibody and lectin reactivity revealed the presence of two partially separated populations (Figure 2f) with the (96 FR 2.10) Le^b and PGM2B antibody reactivities shifted towards the high-density part of the

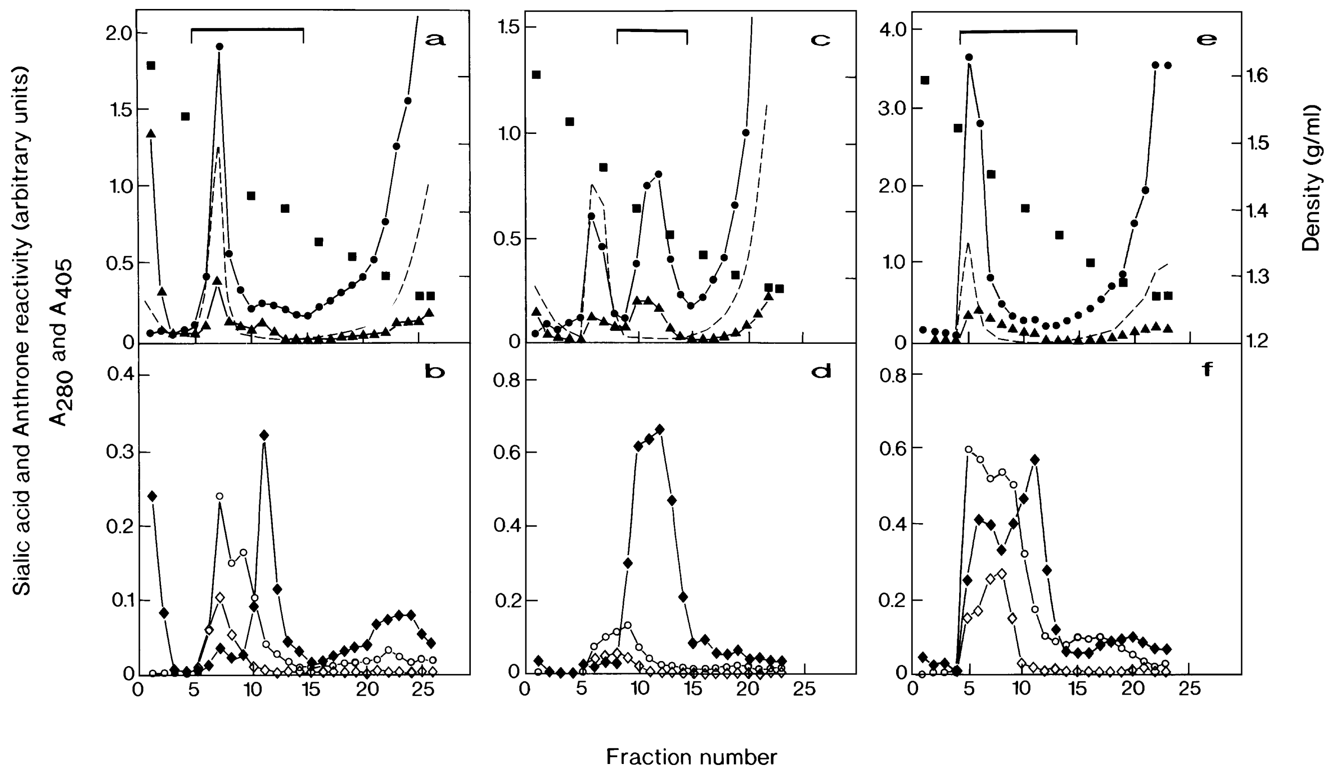

Figure 2 Isopycnic density-gradient centrifugation, in CsCl/4 M guanidinium chloride, of mucins from the glands of the cardiac region (a,b), corpus (c,d) and antrum (e,f)

Extracts from the glandular tissue of the cardiac region, corpus and antrum were subjected to centrifugation (36000 revs/min, 90 h, 15 °C) with a starting density of 1.39 g/ml. Fractions were collected from the bottom of the tubes and analysed for density (■), A_{280} (– – –), sialic acid (●), hexose/fucose (▲), reactivity against the (96 FR 2.10) Le^b (◇) and PGM2B antibodies (○) and the GSA-II lectin (◆). Fractions were pooled according to the bars.

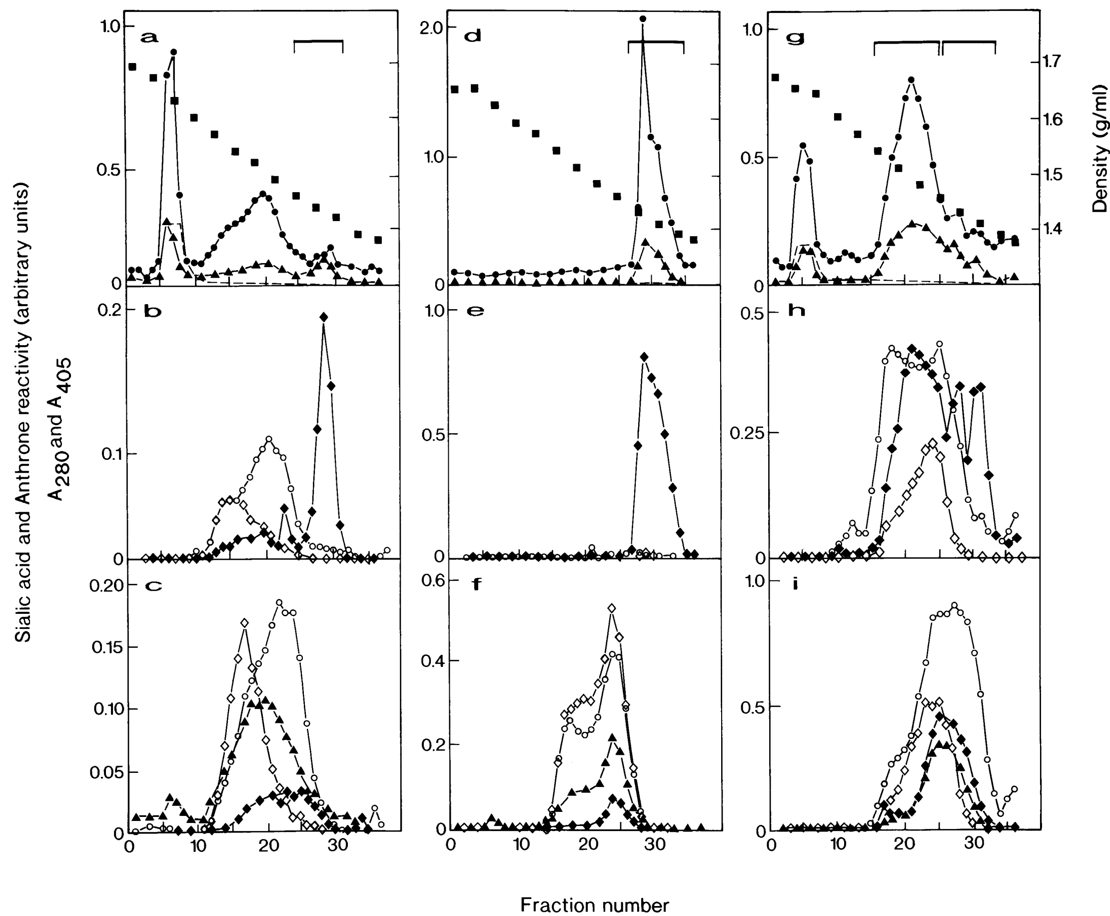

Figure 3 Isopycnic density-gradient centrifugation in CsCl/0.5 M guanidinium chloride

Mucins purified from the glands (**a**,**b**) and surface epithelium (**c**) of the cardiac region, the glands (**d**,**e**) and surface epithelium (**f**) of corpus and the glands (**g**,**h**) and surface epithelium (**i**) of antrum were subjected to centrifugation in CsCl/0.5 M guanidinium chloride (36000 revs/min, 90 h, 15 °C) with a starting density of 1.5 g/ml. Fractions were collected from the bottom of the tubes and analysed for density (■), A_{280} (– – –), sialic acid (●), hexose/fucose (▲), reactivity against the (96 FR 2.10) Le^b (◇) and PGM2B antibodies (○) and the GSA-II lectin (◆). Fractions were pooled according to the bars.

distribution, whereas the GSA-II lectin showed a stronger relative reactivity on the low-density side. Mucin-containing fractions were pooled as indicated and subjected to a second density-gradient-centrifugation step in CsCl/0.5 M guanidinium chloride.

In CsCl/0.5 M guanidinium chloride, mucins from the cardiac glands were well separated from DNA with absorbance at 280 nm and unspecific reactivity with the assay for sialic acid at 1.62 g/ml (Figure 3a). The major mucin population was broad and heterogeneous, spanning the density range 1.60–1.45 g/ml, with a less-pronounced population occurring at about 1.43 g/ml. The broad high-density population reacted with the (96 FR 2.10) Le^b and PGM2B antibodies but only weakly with the GSA-II lectin, whereas the material banding at 1.43 g/ml gave rise to a sharp peak with the lectin and virtually no reactivity with the two antibodies (Figure 3b). Mucins from the surface epithelium were recognized by both antibodies and occurred as a broad peak between 1.55 and 1.47 g/ml, with the Le^b and PGM2B reactivities shifted towards the high-density and low-density sides of the distribution, respectively (Figure 3c). These mucins are thus similar to the high-density mucins from the glandular tissue and it is, therefore, likely that the latter molecules originated from the surface epithelium whereas the low-density population with strong GSA-II-lectin reactivity represented a component from the glands.

Mucins from the glandular tissue of corpus appeared as a sharp peak at 1.4 g/ml (Figure 3d), which showed strong reactivity with the GSA-II lectin and was not recognized by the (96 FR 2.10) Le^b and PGM2B antibodies (Figure 3e). In contrast, mucins from the surface epithelium, banding, as expected, as two partially separated components {corpus-HD (high-density) and corpus-LD (low-density); [15]} at 1.5 and 1.45 g/ml respectively (Figure 3f), reacted strongly with both antibodies. Although corpus-LD reacted slightly with the GSA-II lectin, mucins from the glandular tissue were entirely different from those from the surface epithelium, but resembled the GSA-II-lectin-reactive component from the cardiac region.

Mucins from the glandular tissue of antrum separated into a major high-density mucin at 1.5 g/ml and a heterogeneous mixture of low-density material (Figure 3g). The high-density population reacted with the (96 FR 2.10) Le^b and PGM2B antibodies as well as with the GSA-II lectin, whereas the low-density components showed a stronger reactivity with the GSA-II lectin, relative to the two antibodies (Figure 3h). Mucins from the surface epithelium of antrum banded as a broad distribution around 1.45 g/ml (antrum-LD) with a high-density shoulder

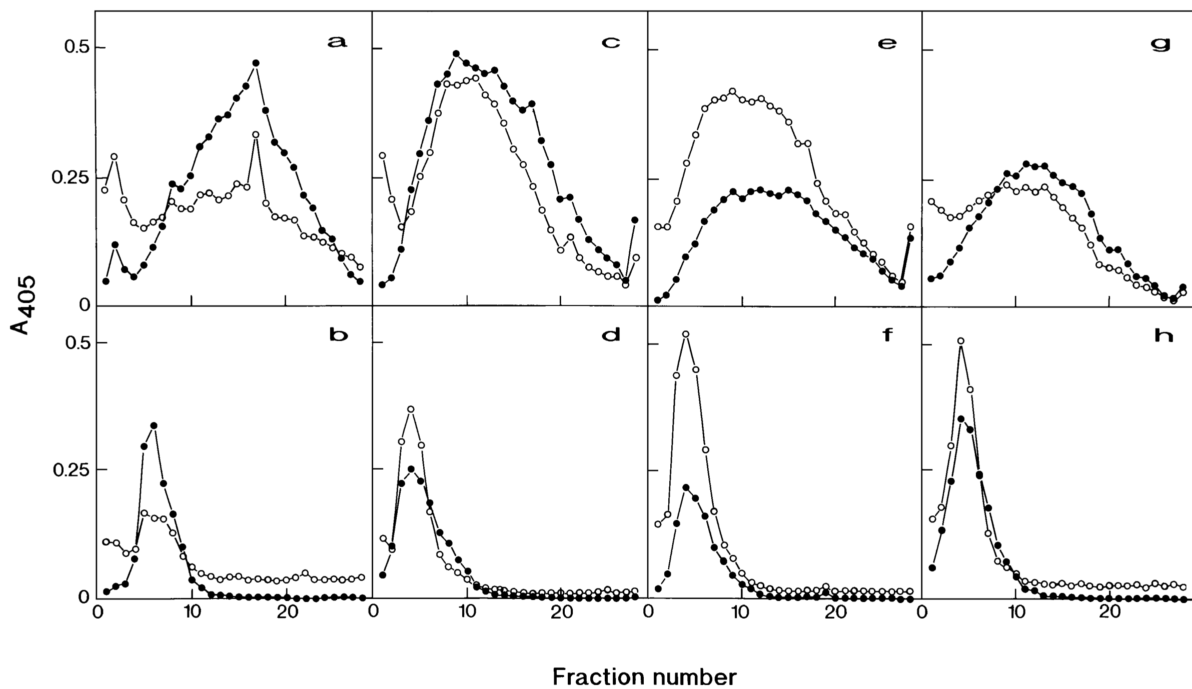

Figure 4 Rate-zonal centrifugation of pig gastric gland whole mucins and the cognate reduced subunits from the cardiac region (a,b), corpus (c,d) and the high-density (e,f) and low-density (g,h) populations from antrum

Samples (100 μl) in 5 M guanidinium chloride were layered on top of gradients (6–8 M) of guanidinium chloride. Whole mucins (**a**,**c**,**e**,**g**) or reduced subunits (**b**,**d**,**f**,**h**) were centrifuged (2 h 45 min, 20 °C) and fractions recovered from the top of the tubes. Fractions were analysed for carbohydrate (○) using a microtitre-plate carbohydrate assay and for reactivity with the GSA-II lectin (●).

(antrum-HD) at 1.5 g/ml (Figure 3i). Both populations were recognized by the (96 FR 2.10) Le^b and PGM2B antibodies, whereas the GSA-II lectin reacted preferentially with the population banding at 1.45 g/ml. However, density-gradient centrifugation alone did not provide a clear distinction between mucins from the surface epithelium and those from the glandular tissue in this region.

Size and subunit composition of glandular mucins

Whole mucins and reduced subunits from the glandular tissue of the cardiac region, corpus and antrum were subjected to rate-zonal centrifugation to study their size distribution and oligomeric nature. Whole mucins from all regions showed a broad distribution in the gradient (Figures 4a, 4c, 4e and 4g), with those from the cardiac region (Figure 4a) possibly being somewhat larger than those from the other regions (Figure 4). In all cases, reduction gave rise to fragments that were considerably smaller than the cognate whole mucins (Figures 4b, 4d, 4f and 4h), showing that gland mucins from all regions are large, oligomeric glycoproteins composed of subunits, as shown previously for mucins derived from the surface epithelium [15].

Protease digestion of reduced subunits

To compare the subunit structures of the gland mucins from corpus and antrum with those from the surface epithelium, reduced subunits were subjected to trypsin or pepsin digestion and then to gel chromatography on Sephacryl S-500 (Figure 5). Subunits from all mucin populations were included on the column. In addition, a peak of low-molecular-mass material was present close to the total volume, suggesting that some small

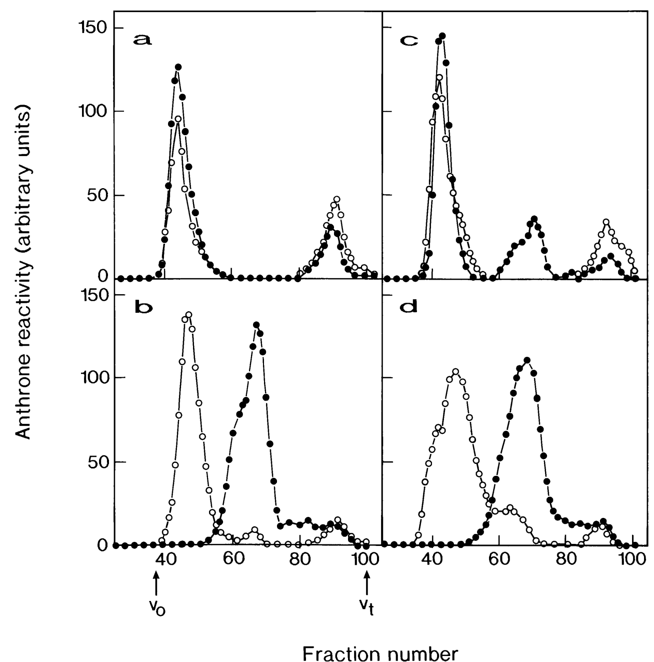

Figure 5 Gel chromatography on Sephacryl S-500 of reduced subunits and tryptic fragments thereof from the glands (a) and surface epithelium (b) of corpus and the glands (c) and the surface epithelium (d) of antrum

Reduced subunits (○) and high-molecular-mass glycopeptides obtained after trypsin digestion (●) were chromatographed on a Sephacryl S-500 column eluted with 0.1 M ammonium acetate at a flow rate of 0.15 ml/min. Fractions (1 ml) were collected and analysed for hexose/fucose. V_0, void volume; V_t, total volume.

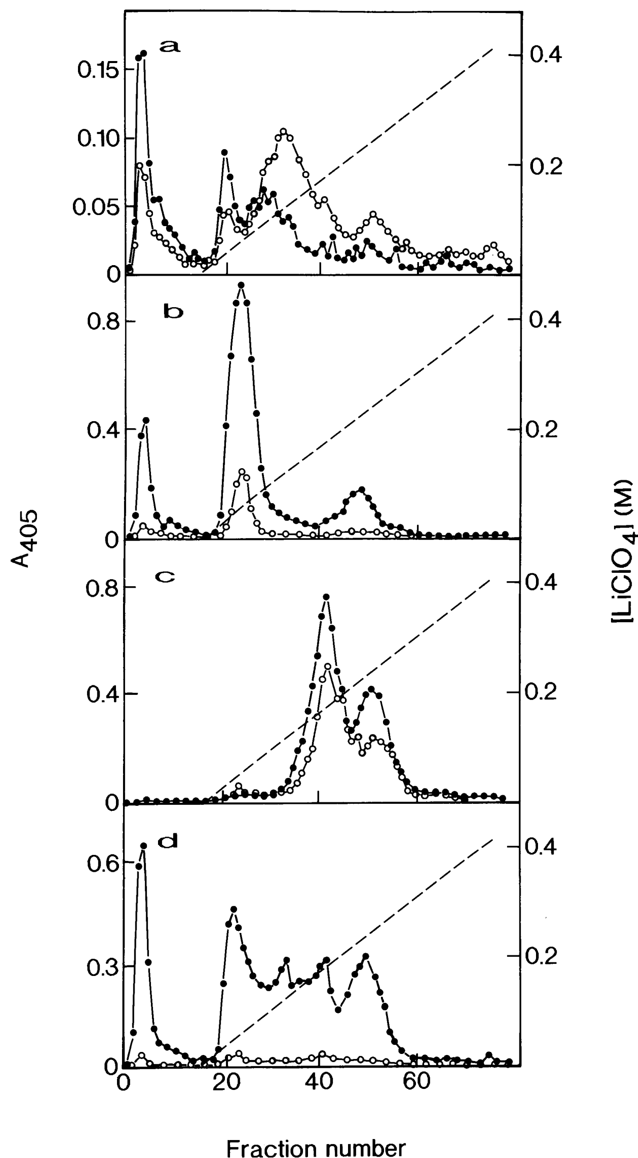

Figure 6 Ion-exchange HPLC on a Mono Q column of reduced subunits from the gland mucins from the cardiac region (a), corpus (b) and the high-density (c) and the low-density (d) populations from antrum

Reduced subunits were chromatographed on a Mono Q column eluted with a linear gradient. Buffer A, 6 M urea/0.1 % (w/v) CHAPS/10 mM piperazine/$HClO_4$ buffer, pH 5; buffer B, 0.4 M $LiClO_4$ added to buffer A. Samples were eluted with buffer A for 10 min followed by a linear gradient of 0–0.4 M $LiClO_4$ over 60 min. Fractions were analysed for carbohydrate (○) using a microtitre-plate carbohydrate assay and for reactivity with the GSA-II lectin (●).

glycosylated fragments were released from the mucins upon reduction. Trypsin digestion of subunits from the glands of corpus and antrum did not change the elution position, although in antrum, a peak of smaller fragments appeared in fractions 60–70 (Figures 5a and 5c). In contrast, mucin subunits from the surface epithelium were extensively degraded (Figures 5b and 5d), and the major fragments occurred at the same position (fractions 60–70) as the smaller fragments produced by digestion of the glandular material. Similar results were seen when mucins were digested with pepsin (results not shown). The data show that the glycosylated domains of the gland mucins from corpus and antrum have considerably larger hydrodynamic volumes and, thus, that gland mucins are structurally different from those of the surface epithelium. The presence of some gland glycopeptides eluting at the same position as the major glycopeptide population from the surface mucins in antrum suggests that there was some admixture of surface and gland mucins. The gland mucins from antrum thus comprised a distinct population, although no separation was achieved in the density-gradient.

Ion-exchange HPLC of reduced subunits

The charge density of the gland mucins from the different regions was investigated by subjecting reduced subunits to ion-exchange HPLC. Analysis for carbohydrate showed mucins from the cardiac region to elute as a number of populations, one of which was not retained on the column (Figure 6a). All populations reacted with the GSA-II lectin. In corpus, the major population, as shown by both the carbohydrate assay and GSA-II reactivity, eluted as a sharp peak early in the gradient (Figure 6b). The high-density species from antrum occurred as two lectin-reactive populations, which eluted relatively late in the gradient (Figure 6c). Most of the mucins from the low-density population from antrum eluted as several GSA-II-reactive species that were retained by the column (Figure 6d). The low-density population from the antrum surface mucins (antrum-LD) was not retained by the column or eluted at the start of the gradient [15], showing that this population was different from the gland mucins. However, some overlap in elution behaviour was noticed for antrum-HD and the gland mucins (results not shown).

DISCUSSION

Mucin-producing cells in the surface epithelium of pig gastric mucosa reacted avidly with the polyclonal antibody PGM2B raised against a peptide sequence in a pig gastric-mucin apoprotein, identified by Turner and collaborators [7]. The same tissue distribution was obtained with a monoclonal antibody against the human M1 antigen present in the human stomach (45-M1) [25], and Bara et al. have also shown that this antibody stains mucin-producing cells in the surface mucosa of pig stomach [27]. Apart from corpus, where some diffuse staining with the PGM2B antibody, but not with 45-M1 antibody, was observed over the glands, no reactivity was noticed with these antibodies in the glands, suggesting that gland and surface mucins have different apoproteins. Similar results were obtained by Turner and collaborators [7] with the anti-PGM-HF antibody raised against deglycosylated pig gastric mucins, and the findings are paralleled in human gastric mucosa, where the MUC5AC mucin is confined to the surface epithelium, whereas MUC6 mucin originates from the glands [12–14]. In contrast, the GSA-II lectin virtually only reacted with the glands, in agreement with observations made in other species, for example the rat, where the GSA-II lectin reacts preferentially with the glands and the mucous neck cells [3,4]. In man, type 1 (e.g. Le^a and Le^b) and type 2 backbone structures (e.g. Le^x and Le^y) are confined to the surface epithelium and glands, respectively [5,6]. To our knowledge, the Le^b antigen has not been described in the pig; however, a patchy but relatively weak reactivity with a Le^b antibody (clone 96 FR 2.10) was observed over the surface epithelium. This reactivity is likely to reflect the lack of absolute specificity of Le^a and Le^b antibodies. Accordingly, the monoclonal antibody 2-25LE, which apparently only reacts with the Le^a and Le^b structures [28], showed no reactivity over the surface epithelium (results not shown). Despite the lack of specificity for the Le^b structure, the 96 FR 2.10 antibody could be used to distinguish between surface and gland mucins during the biochemical

purification procedures (see below) and, again, no reactivity was observed with the 2-25LE clone. The 96 FR 2.10 antibody possibly reacts with the H1 structure that may be present in pig gastric mucins [16], and with which the antibody is known to react [28].

By gentle mechanical treatment, the surface epithelium was largely removed, thus enriching the glandular tissue. Density-gradient centrifugation, first in CsCl/4 M guanidinium chloride and then in CsCl/0.5 M guanidinium chloride, revealed the presence of mucin species that reacted with the (96 FR 2.10) Le^b and PGM2B antibodies, and thus resembled the mucins from the cognate surface epithelium, as well as those that reacted strongly with the GSA-II lectin. In the cardiac region and corpus, the latter population occurred as a distinctive peak at approx. 1.4 g/ml in the density-gradient, well separated from the surface mucins. In antrum, however, the situation was more complex, since surface and gland mucins were not well separated during the isolation procedure. High- and low-density populations were identified in the glandular tissue where reactivity with the GSA-II lectin was stronger, relative to PGM2B, in the low-density population. The lectin-reactive species with the lowest buoyant density were likely to originate from the gastric glands, but since the density range of the bulk of the mucins from the glandular tissue overlapped significantly with that of the mucins from the surface epithelium, it was not possible to distinguish between the gland and surface mucins at this level. However, proteolytic digestion showed that gland mucins contain much larger glycosylated domains than those from the surface epithelium (see below) and thus appear to constitute a structurally different population.

The size distribution of the various gland mucins showed that they are all large and composed of subunits linked by disulphide bonds and, therefore, like those from the surface epithelium, belong to the family of large oligomeric mucins of which, in man, MUC5AC [29], MUC5B [30] and MUC2 [14] are members. Treating the reduced subunits from the GSA-II-lectin-reactive glandular mucins from corpus and antrum with trypsin or pepsin revealed the presence of much larger protease-resistant domains than those present in the surface-epithelial mucins from the same regions. Since the hydrodynamic volume of mucins, and thus their elution position after gel chromatography, does not change significantly until the link GalNAc residue is removed [31], our data suggest that the glycosylated regions within the gland mucins are significantly longer than those in the surface-epithelial mucins. Finally, ion-exchange HPLC showed that the gland mucins from all regions could be fractionated into a number of populations. These differences in 'acidity' could well be explained by the existence of 'glycoforms' of the same mucin apoprotein, although this cannot be confirmed until the nature of the protein core has been identified. In this study it was not possible to decide whether the observed charge heterogeneity resulted from differences in sialic acid and/or sulphate content. However, in tissue sections, staining with the sulphate-selective high-iron diamine procedure was observed only over a sub-population of cells within the antrum glands (results not shown), suggesting that the differences were partially due to sulphate content.

In summary, in all regions, mucins specific to the glandular tissue were identified which differed from surface mucins in apoprotein content, carbohydrate substitution and the length of the highly glycosylated domains. In the cardiac region and corpus, the buoyant densities of surface and gland were also significantly different. Possibly, the surface and gland mucins identified here may represent the porcine equivalents of the human MUC5AC and MUC6 mucins, respectively, although the oligomeric nature of the human MUC6 mucin remains to be established. The gland mucins appear to be less sensitive to proteolytic degradation than those from the surface, and may play a particular role in protecting the cells lining the acid-producing gland. The functional significance of having mucins from two cellular sources with different structures is not known. However, mucins in the stomach have been shown to form laminated structures, which are likely to contain different mucins, overlaying the surface mucosa [2]. Differential secretion of surface and gland mucins possibly represents a mechanism by which this architecture of the mucus gel is maintained and modulated.

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