The putative 'link' glycopeptide associated with mucus glycoproteins

Composition and properties of preparations from the gastrointestinal tracts of several mammals

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The existence of a discrete 'link' peptide in epithelial mucins has been debated for many years. There is evidence that at least some mucins contain a specific 'link' peptide (or glycopeptide) that enhances mucin polymerization by forming disulphide bridges to large mucin glycoprotein subunits. A major difficulty has been to know whether the reported differences in putative 'link' components represent artifacts generated by inter-laboratory differences in technical procedures used in mucin purification. The present paper outlines the results of a collaborative study involving five laboratories and 53 samples of purified gastrointestinal mucins (including salivary, gastric, small-intestinal and colonic mucins) prepared by five techniques from four different animal species. An early step in mucin purification in all cases was the addition of proteinase inhibitors. Representative mucins were analysed for their composition, electrophoretic mobility in SDS/polyacrylamide-gel electrophoresis before and after disulphide-bond reduction, and for their reactivity with monospecific antibodies developed against the 118 kDa putative 'link' glycopeptide isolated from either rat or human small-intestinal mucins. Our results indicate that, despite differences in laboratory techniques, preparative procedures, organs and species, each of the purified mucins contained a 'link' component that was released by disulphide-bond reduction and produced a band on SDS/polyacrylamide-gel electrophoresis at a position of approx. 118 kDa. After electroelution and analyses, the 118 kDa bands from the different mucins were found to have similar amino acid profiles and to contain carbohydrate. It would appear therefore that a 'link' glycopeptide of molecular mass approx. 118 kDa is common to all of the gastrointestinal mucins studied.

INTRODUCTION

Mucus glycoproteins (mucins) are the major structural elements present in the mucus layer that covers the surface of the digestive tract and protects the underlying mucosal cells (Neutra & Forstner, 1987). A major goal in mucus glycoprotein research is to relate the properties of mucins to the composition of their subunits and to the intramolecular structure, which arises from the manner in which the component subunits are joined. Two basic models have been proposed for the latter. Allen and co-workers (Pearson *et al.*, 1981; Allen *et al.*, 1984) suggested a model for pig gastric mucin in which four large glycopeptide subunits are joined by disulphide bonds to a single central peptide of molecular mass 70 kDa. An analogous peptide of 90 kDa was found in pig small-intestinal mucin (Mantle *et al.*, 1981), and in one of our

laboratories a 118 kDa 'link' glycopeptide was found to be present in mucins of rat and human small-intestinal goblet cells (Fahim *et al.*, 1983; Mantle *et al.*, 1984a,b; Fahim *et al.*, 1987a). Carlstedt & Sheehan (1984) proposed a distinctly different model of mucin structure, in which the peptide chains of large glycopeptide subunits are linked end-to-end via disulphide bonds, giving an elongated thread structure. Accumulated physical data and electron-microscopic evidence (Harding *et al.*, 1983; Slayter *et al.*, 1984; Rose *et al.*, 1984; Forstner & Forstner, 1986; Sheehan *et al.*, 1986) tend to favour this latter flexible-thread model for all mucins. However, this model has not been reconciled with the experimental observation that a putative 'link' peptide (or glycopeptide) seems to be present in many highly purified mucus glycoproteins. It has been tempting to dismiss the existence of 'link' components as being artifacts due

Abbreviations used: PBS, phosphate-buffered saline (0.15 M-NaCl/10 mM-sodium phosphate buffer, pH 7.0); GdnHCl, guanidinium chloride; PAGE, polyacrylamide-gel electrophoresis.

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to contaminants, species- or organ-specific anomalies, proteolytic fragments generated by degradation during mucin isolation, or other inter-laboratory technical differences relevant to isolation and/or characterization procedures.

We have recently had the opportunity to engage in a collaborative study involving several laboratory groups. Our goal was to determine whether gastrointestinal mucins contained a common 'link' component of approximately the same size and composition. Collectively we have purified 53 samples of mucin and have used five purification techniques, four organs and four animal species. In some cases individual mucins were purified by more than one procedure. The purified mucins were examined for their composition, for their electrophoretic mobility during SDS/PAGE before and after reduction, and for their reactivity with antibodies specific for the 118 kDa 'link' glycopeptides of rat or human small-intestinal mucins. Our data represent a synthesis of work in five laboratories, and the results suggest that, despite differences in laboratory techniques, preparative procedures, organs and species, all of the gastrointestinal mucins possess a common or 'universal' component of molecular mass approx. 118 kDa, the latter being released from native mucins by disulphide-bond reduction. In the case of human salivary mucin the 'link' component was somewhat larger and also weaker in its antigenic reactivity.

METHODS

Preparation of mucins

With the exception of methods 3 and 5, the first step of mucin purification was to subject mucosal scrapings to a brief (30 s) homogenization in PBS or 0.15 M-NaCl, pH 7.0, containing 0.02% NaN₃ and a mixture of proteinase inhibitors (2 mM-phenylmethanesulphonyl fluoride, 5 mM-EDTA and 2 mM-N-ethylmaleimide). This was followed by centrifugation at 30000 *g* at 4 °C for 30 min. [Details are provided by Fahim *et al.*, (1987a).] The supernatant was then treated by one of the following methods.

Method 1 (4B/*V*₀; 2B/*V*₀). The supernatant was concentrated by using an Amicon Diaflo membrane (exclusion limit 100 kDa), dialysed against PBS and applied to a Sepharose CL-4B chromatography column. The void-volume (*V*₀) fraction was collected, digested with nuclease and subjected to Sepharose CL-2B chromatography as described previously (Fahim *et al.*, 1983; Wesley *et al.*, 1985). The purified mucin emerged in the *V*₀ fraction with a trailing shoulder in the partly included volume. The *V*₀ fraction was pooled for subsequent study. This method was used to prepare mucins from human autopsy (less than 10 h *post mortem*) and rat small-intestinal tissues (see Table 1).

Method 2 (CsCl × 2; 2B/*V*₀). The supernatant was subjected to two sequential steps of density-gradient ultracentrifugation in CsCl (starting density 1.40 or 1.43 g/ml). The resultant carbohydrate-rich fractions (ρ 1.38–1.50 g/ml approximately) were pooled, dialysed to remove CsCl and subjected to Sepharose CL-2B chromatography. Mucin emerged in the *V*₀ fraction. [Details of the procedure are provided in Mantle & Allen (1981) and Mantle *et al.* (1984a).] This method was used

Table 1. Methods and laboratories used to prepare mucin samples

A to E refer to different laboratories. Methods 1 to 5 are given in the text. Method 2* involved two steps of CsCl-density-gradient ultracentrifugation without subsequent Sepharose CL-2B chromatography.

Source of mucin	Laboratory	No. of mucin samples (method)
Rat small intestine	A	2(1), 3(2), 1(2*), 1(3)
	B	3(2)
Human small intestine	A	6(1), 6(2)
	B	8(2)
	C	2(2)
Pig stomach (fundus)	A	1(2)
(body)	C	1(4)
(antrum)	C	1(4)
(fundus)	C	1(4)
Rabbit small intestine	B	4(2)
	E	1(2*)
Ferret small intestine	B	1(2)
Rabbit colon	B	4(2)
Human colon	A	1(2)
Human submaxillary	D	4(5)
Pig small intestine	B	2(2)

to prepare mucins from pig, ferret, human, rat and rabbit small intestine, as well as from pig gastric antrum, body and fundus and from human and rabbit colon (see Table 1). In some preparations the final step of Sepharose CL-2B chromatography was omitted. This is designated method 2* in Table 1. In a few cases (human and rat small-intestinal mucins) samples were extracted with chloroform/methanol by the method of Slomiany *et al.* (1983), with no differences noted in subsequent compositional analyses or SDS/PAGE.

Method 3 (CsCl/GdnHCl × 2; 2B/*V*₀). Scrapings of intestine were stirred for 2 h at 4 °C with the proteinase-inhibitor mixture to which was added GdnHCl (final concn. 6 M). After a brief (10 s) homogenization and centrifugation, density-gradient ultracentrifugation of the supernatant was carried out in GdnHCl (4 M)/CsCl (ρ 1.4 g/ml). A second gradient was employed in 0.2 M-GdnHCl/CsCl (ρ 1.4 g/ml) as described by Carlstedt *et al.* (1983). The carbohydrate-rich fractions were pooled, dialysed, boiled in 1% SDS for 3 min and applied to a column of Sepharose CL-2B. Mucin was eluted with 0.1 M-sodium phosphate buffer, pH 7.4, containing 1% SDS, and appeared in the *V*₀ fraction. This method was used to prepare samples of rat small-intestinal mucin (Table 1).

Method 4 (4B/*V*₀; CsCl). This method was a minor variant of methods 1 and 2, and involved application of homogenate supernatant samples to a column of Sepharose CL-4B, followed by ultracentrifugation of the *V*₀ fraction in a CsCl gradient (ρ 1.4 g/ml). The carbohydrate-rich fractions (ρ 1.40–1.50 g/ml) were pooled and designated purified mucin. This method was used to prepare four samples of pig gastric (antrum, body and fundus) mucin (Table 1).

Method 5 (GdnHCl; G-200; 2B/ V_0). Stimulated human submandibular–sublingual saliva (500 ml) was collected from one person by an appliance described by Truelove *et al.* (1967). An equal volume of 8 M-GdnHCl was added immediately after collection, the sample was concentrated 10-fold by ultrafiltration (on an Amicon YM-10 membrane with a 10 kDa cut-off) and stored at 2 °C. The sample was then applied to a column of Sephadex G-200, and the column eluted with 30 mM-Tris/HCl buffer, pH 8.0, containing 4 M-GdnHCl. The periodic acid/Schiff-positive fraction (V_0) was rechromatographed on Sepharose CL-2B. This method of purification is essentially the same as that described by Prakobphol *et al.* (1982). The purified mucin was dialysed successively against distilled water, 0.5 M-sodium acetate buffer, pH 7.2, and 30 mM- NH_4HCO_3 . After 10-fold concentration by ultrafiltration, the mucin was stored at –20 °C.

SDS/PAGE

PAGE was performed by the method of Laemmli (1970) with a 5.7% stacking gel and a 7.5% separating gel. Before application to the gels, samples were boiled for 3–5 min in 1% SDS (with or without 0.25 M-2-mercaptoethanol) in 0.1 M-Tris/HCl buffer, pH 8.6, containing 10% (v/v) glycerol and 0.05% Bromophenol Blue. The resulting gels were silver-stained by the method of Merrill *et al.* (1981).

Electroelution from gels

The band at or near 118 kDa was electroeluted with an ISCO concentrator (model 1750; ISCO, Lincoln, NE, U.S.A.), the sample was dialysed against distilled water, and SDS was removed by ion-pair extraction (Henderson *et al.*, 1979), or after ethanol (90%, v/v) precipitation of proteins.

Antibody preparation

A monospecific antibody to the rat small-intestinal 'link' glycopeptide was developed in rabbits as described previously (Fahim *et al.*, 1987a). This antibody has been shown to recognize the 'link' components (118 kDa) of both human and rat small-intestinal mucins. On Western blots of native mucin, antibody staining was confined to the region of the 5.7% stacking gel of SDS/PAGE. Reduced mucin samples, however, gave positive blots only at the 118 kDa band position. A polyclonal antibody to the human small-intestinal 'link' component of 118 kDa was also prepared in rabbits by the same procedure as used for the comparable rat 118 kDa antigen, except that affinity purification of the antibody was not required to achieve specificity for the 'link' component. Instead, the immune serum was simply enriched for IgG by precipitation with 40%-saturated $(\text{NH}_4)_2\text{SO}_4$. After thorough dialysis against PBS, the antiserum was divided into 100 μl volumes for storage at –70 °C. Pre-immune serum was subjected to the same procedure and used as a negative control in immunoassays and Western-blot experiments.

Western-blot analyses

After completion of SDS/PAGE, bands were transferred on to nitrocellulose sheets by electrophoresis, and blotting was achieved by the method of Towbin *et al.* (1979). After blotting with the antibody preparation, bands were detected by treating with a conjugate of

Protein A–horseradish peroxidase followed by the addition of the peroxidase substrate (Bio-Rad Laboratories, Richmond, CA, U.S.A.). In the case of the salivary 'link' component the Protein A complex was replaced by goat anti-(rabbit IgG) antibody and peroxidase–anti-peroxidase IgG.

E.l.i.s.a.

An indirect e.l.i.s.a. was developed to measure the reactivity of mucin samples with the antibody against the 118 kDa 'link' glycopeptide of human small-intestinal mucin. Micro-titre plate wells were coated overnight at 4 °C with 500 ng (protein) of purified human small-intestinal mucin in 50 μl of PBS. The wells were washed three times in PBS containing 0.1% Tween 20 and the remaining sites were blocked with bovine serum albumin (200 μl of a solution containing 20 mg/ml in PBS) for 1 h at room temperature. After a further washing in PBS, a 50 μl mixture containing the test mucin that had been premixed for 17 h at 37 °C with an equal volume of dilute (1:200) antibody was added to the wells and they were then incubated for 1 h at room temperature. Washing was repeated, and to the bound antibody was added 50 μl of a Protein A–horseradish peroxidase conjugate (1:1000 dilution; Zymed, San Francisco, CA, U.S.A.). After 1 h at room temperature, the plate was washed and 150 μl of peroxidase substrate was added (0.6 mg of *o*-phenylenediamine and 1 μl of H_2O_2 per ml of sodium citrate buffer, pH 5.5). After colour development (approx. 30 min), the reaction was stopped by adding 50 μl of 2.5 M- H_2SO_4 , and the wells were scanned in an e.l.i.s.a. plate reader (Bio-Rad Laboratories) with a 492 nm filter. Blank wells contained PBS instead of antigen/antibody mixtures. Suitable controls of non-immune serum were included. All assays were performed in duplicate on several dilutions of each mucin.

For rat small-intestinal mucins, the e.l.i.s.a. results were the same before and after the mucins were reduced and alkylated, indicating that the affinity of the antibody for the 118 kDa component was unchanged by disulphide-bond reduction.

Compositional analyses

During purification of mucins from columns or density gradients, protein was measured by the A_{280} value or by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard. Carbohydrate was measured by the periodic acid/Schiff reaction as described by Mantle & Allen (1978). Mucin samples were reduced and alkylated as described previously (Fahim *et al.*, 1987a). Briefly, reduction was carried out in 0.25 M-2-mercaptoethanol in 60 mM-Tris/HCl buffer, pH 6.8, containing 1% SDS for 3 min at 100 °C. Samples were alkylated with 0.5 M-iodoacetamide (for 18 h in the dark at 4 °C) and then dialysed exhaustively against distilled water. Amino acid analyses were performed, after samples had been hydrolysed for 24 h at 110 °C in 6 M-HCl, by the use of a Picotag h.p.l.c. system (Waters Associates, Mississauga, Ont., Canada).

Immunocytochemical localization

Distal ileum from male Wistar rats was immersion-fixed in 2% paraformaldehyde/1% glutaraldehyde in 0.1 M-phosphate buffer, pH 7.3, containing 2.5% polyvinylpyrrolidone for 2 h at room temperature. The tissue was trimmed, rinsed in 0.1 M-phosphate buffer,

dehydrated in increasing concentrations of ethanol and embedded in Lowicryl (Polysciences, Warrington, PA, U.S.A.). Sections were cut at 60–90 nm and mounted on Formvar-coated nickel grids.

For immunocytochemistry, the sections were blocked by floating the grids on drops of 1% bovine serum albumin in PBS for 10 min. The excess was shaken off, and the grids were floated in spot plates on 0.5 ml of the antibody to the rat 118 kDa component (diluted 1:400) overnight at 4 °C. The grids were rinsed by floating on successive PBS washes, blocked with bovine serum albumin for 10 min, and incubated on Protein A/colloidal gold with 15–20 nm gold particles (Sigma Chemical Co., St. Louis, MO, U.S.A.) for 4 h at room temperature. The grids were rinsed in successive PBS washes and air-dried. The sections were counterstained with 1% uranyl acetate and 2% lead citrate and photographed with a Philips 300 electron microscope.

RESULTS

Amino acid composition of mucins

Although mucins prepared from different species or organs normally show considerable heterogeneity with respect to carbohydrate content and oligosaccharide composition (Allen *et al.*, 1984; Neutra & Forstner, 1987), all purified mucins of the gastrointestinal tract tend to exhibit similar features in their amino acid profile. For example, protein usually constitutes less than 20% of dry weight, and the major amino acid residues (serine, threonine and proline) tend to comprise 40–50 mol% of the total. The rest of the protein is mainly accounted for by the 'minor' mucin amino acids residues: aspartic acid, glutamic acid, glycine, valine, alanine, leucine, isoleucine and cysteine. Other residues (methionine, tyrosine, phenylalanine, histidine, lysine and arginine) collectively are responsible for only about 10–15 mol% of the total. This particular pattern of amino acid distribution is quite distinctive for mucins and, together with a large molecular mass, serves as a useful index of purity. Table 2 summarizes representative amino acid data obtained in five different laboratories for mucins used in the present study. It is to be noted that protein comprised less than 20% by weight, and that between 42

and 55% of the amino acid residues were due to serine, threonine and proline combined. The bulk of the remaining protein was due to the 'minor' amino acids.

Although prepared by different methods, all mucins had a large molecular mass (eluted in the V_0 fraction from Sepharose CL-2B) and/or had a peak buoyant density between 1.43 and 1.49 g/ml (results not shown). The method of preparation did not influence the final composition of individual mucins prepared by more than one method [see Wesley *et al.* (1985) and Fahim *et al.* (1987a)]. There was also no difference in the composition of either rat or human small-intestinal mucins before and after they were each subjected to delipidation by sequential chloroform/methanol extractions (Fahim, 1985; Mantle & Forstner, 1986).

Carbohydrate analyses were performed on about half of the mucin samples (results not shown). There was negligible mannose (<1 mol%) and no uronic acid detected. As is typical of all mucins, the sugar residues were galactose, fucose, sialic acid, *N*-acetylglucosamine and *N*-acetylgalactosamine.

SDS/PAGE of mucins

When mucins were subjected to SDS/PAGE before disulphide-bond reduction, positive silver staining was seen in the 5.7% stacking gel or as a faint smear at the top (>200 kDa) of the 7.5% separating gel, but no discrete smaller-molecular-mass bands were noted (Fig. 1). Faint smears were also seen in samples D and E at molecular-mass positions ≥ 116 kDa. However, after reduction, all of the mucins revealed a major band in the region of 116–120 kDa. Comparable bands were observed for human colonic mucin and pig gastric (fundus) mucin (Fig. 2). Pig gastric body and antral mucins (results not shown) were each identical with the fundus mucin. In human submaxillary mucin (Fig. 2) the band produced by reduction was very diffuse (range 116–175 kDa), with the densest stain having a mid-position of about 150 kDa. In the case of pig and ferret small-intestinal mucins, several other less-intense bands were also observed above and below the dark band at 118 kDa (Fig. 1), suggesting that these samples were either not as highly purified or else were more heterogeneous and/or degraded than the other mucins.

Table 2. Amino acid composition of purified mucins

A to E represent different laboratories in which the mucins were prepared. Abbreviations: s.i., small intestine; N.D., not determined. Values are given for the average data for multiple isolated mucin samples, by using the same convention for methods as presented in Table 1: rat small-intestinal mucin, 1 (1), 2 (2); human small-intestinal mucin, 3 (1), 3 (2); pig small-intestinal mucin, 2 (2); rabbit small-intestinal mucin, 4 (2), 1 (2*); ferret small-intestinal mucin, 1 (2); human colonic mucin, 1 (2); rabbit colonic mucin, 4 (2); pig gastric (fundus) mucin, 1 (2); human submaxillary mucin, 4 (5)

Amino acid composition (mol%)										
Amino acids	Source of mucin ...	Rat s.i. (A, B)	Human s.i. (A, B, C)	Pig s.i. (A)	Rabbit s.i. (B, E)	Ferret s.i. (B)	Human colon (A)	Rabbit colon (B)	Pig stomach (fundus) (C, A)	Human submaxillary (D)
Ser + Pro + Thr		48.7	45.5	52.3	42.3	48.5	55.0	42.3	45.8	43.4
Asp + Glu		13.1	13.5	8.8	14.8	12.6	12.3	16.3	15.5	14.3
Gly + Val + Ala		14.7	17.6	16.6	20.9	19.3	15.9	18.8	20.5	24.1
Leu + Ile		8.3	10.5	7.4	8.2	7.9	8.2	8.4	7.6	9.6
Rest		15.2	12.9	14.9	13.7	11.7	8.6	14.2	10.6	8.6
Protein (% of dry wt.)		13.4	14.0	17.9	18.6	17.5	N.D.	17.4	N.D.	18.2

Fig. 1. SDS/PAGE of purified mucins before and after disulphide-bond reduction

Mucins containing approx. 5–8 μ g of protein were applied to 7.5 % polyacrylamide gels before or after reduction with 0.25 M 2-mercaptoethanol in 1 % SDS for 5 min at 100 °C. Detection was by silver staining. Molecular-mass markers are shown at the left. Small arrowheads show positions of 2-mercaptoethanol artifacts on reducing gels. Tract A, rat small-intestinal mucin; tract B, rabbit small-intestinal mucins; tract C, rabbit colonic mucin; tract D, human small-intestinal mucin; tract E, pig small-intestinal mucin; tract F, ferret small-intestinal mucin. Tracts A' to F' are reduced samples.

As noted in earlier studies (Mantle *et al.*, 1984a; Fahim *et al.*, 1987a), artifact (non-mucin) bands appeared on the gels (arrowheads) at positions of approx. 60 and 70 kDa whenever gels were run under reducing conditions. Similar artifacts have been discussed by Tasheva & Desseu (1983), and were seen in our experiments even in reagent-blank samples containing 2-mercaptoethanol but no mucin. Mucin bands were distinguished from artifacts because of the lower staining intensity of the latter and because intensity varied in proportion to the 2-mercaptoethanol concentration in applied samples. The chemical basis of the reduction artifacts is not understood, but they may arise from a non-specific interaction between 2-mercaptoethanol (or a minor contaminant of 2-mercaptoethanol) and acrylamide.

The pig gastric mucin was of particular interest because the band at about 118 kDa was different than the 70 kDa band noted earlier by Pearson *et al.* (1981) for reduced pig gastric (fundus plus antrum) mucin. To explore this difference, a sample of pig gastric mucin prepared in 1980 (and subsequently frozen) was obtained from Professor A. Allen (University of Newcastle upon Tyne, Newcastle upon Tyne, U.K.) and subjected to SDS/PAGE in Laboratory A (Fig. 3). Before reduction, a smear near the top of the gel was present, whereas after reduction a broad heavily stained band was observed at about 70 kDa. A fainter band was also noted at about 118 kDa. Since the amino acid composition of the 70 kDa component of this pig gastric mucin has been shown to be almost identical with the 118 kDa band of human and rat small-intestinal mucins (Fahim *et al.*, 1987a), it would seem likely that the 70 kDa and 118 kDa bands of pig gastric mucins are related to each

other and equivalent to the intestinal mucin 118 kDa component. This was examined more fully in subsequent experiments.

Composition of 118 kDa bands in mucin preparations

Some of the mucin preparations were available in sufficient quantity to permit SDS/PAGE, electroelution of the bands (range 116–120 kDa or, in the case of human submaxillary mucin, 120–175 kDa) and compositional analyses. The amino acid data are presented in Table 3 together with the published results (Pearson *et al.*, 1981) for the 70 kDa band for pig gastric mucin. Compared with the values for intact mucins (Table 2), the electroeluted bands all gave a lower proportion (approximately half) of the 'major' mucin amino acid residues serine, threonine and proline and a higher proportion (approximately double except for the salivary preparation) of the minor amino acid residues aspartic acid and glutamic acid. There was a remarkable similarity among most of the 'link' glycopeptides, including the 70 kDa component of pig gastric mucin (prepared by Pearson *et al.*, 1981) and the 118 kDa component of pig gastric (fundus) mucin prepared in Laboratory A. The 'link' glycopeptide of human colonic mucin differed somewhat from the others in having more of the 'major' and less of the 'minor' amino acid residues. In general, however, the results suggested that each mucin possesses a similar 'link' component. Each 'link' component that was assayed for carboxymethylcysteine was shown to contain this residue, in keeping with the assumed role of disulphide-bonding to large-molecular-mass mucin subunits.

The 118 kDa components of human and rat small-intestinal mucins have been shown in earlier studies

Fig. 2. SDS/PAGE of purified mucins (reduced)

After reduction in 2-mercaptoethanol, mucins were subjected to SDS/PAGE as described in the legend to Fig. 1. Tract A, human colonic mucin; tract B, pig gastric (fundus) mucin; tract C, rat small-intestinal mucin. A separate experiment (two lanes on the right) shows results for reduced human submaxillary mucin (tract D), which produced a broad band having a mid-point position at approx. 150 kDa. Molecular-mass markers are shown at the left of tract D.

(Mantle *et al.*, 1984*b*; Fahim *et al.*, 1987*a*) to contain carbohydrate. In the present study there was insufficient material for complete g.l.c. analyses, but each of the 'link' components listed in Table 3 (except pig gastric mucin of Pearson *et al.*, 1981) was subjected to a 7 h

Fig. 3. SDS/PAGE of pig gastric mucin

Pig gastric mucin was obtained from Professor A. Allen (University of Newcastle upon Tyne, Newcastle upon Tyne, U.K.) (described by Pearson *et al.*, 1981) and subjected to SDS/PAGE before (tract A) and after (tract A') reduction in 2-mercaptoethanol as described in the legend to Fig. 1. Molecular-mass markers are shown at the left.

hydrolysis in 6 M-HCl and amino sugar analyses were attempted. All samples exhibited h.p.l.c. peaks for *N*-acetylgalactosamine and *N*-acetylglucosamine, although these were too small for accurate quantification. Although limited, these findings suggest that the 'link' components probably contain oligosaccharide chains.

Western-blot analyses

Western blotting was performed on some of the mucin samples in two laboratories with the monospecific antibody to the 118 kDa component of rat small-intestinal mucin (Laboratory A, Fig. 4) and the antibody to the

Table 3. Amino acid composition of 'link' components isolated from native mucins

Abbreviations: s.i., small intestine; N.D., not determined. Analyses were done in duplicate and represent the averages for three preparations of rat small-intestinal mucin, six preparations of human small-intestinal mucin, two preparations of rabbit small-intestinal mucin, one preparation each of pig gastric mucin and human colon mucin and three preparations of human submaxillary mucin.

Amino acids	Source of mucin...	Amino acid composition (mol%)						
		Rat s.i.	Human s.i.	Rabbit s.i.	Pig stomach (fundus)	Human colon	Human submaxillary	Pig stomach (70 kDa)*
Ser + Thr + Pro		24.8	23.7	21.8	23.0	32.5	27.7	24.9
Asp + Glu		26.3	21.7	27.2	24.9	19.3	14.2	24.4
Gly + Val + Ala		19.0	25.6	20.3	24.3	21.7	31.3	30.5
Leu + Ile		11.0	8.6	8.2	8.6	9.5	13.1	11.8
Cys†		3.4	3.7	N.D.	4.7	3.8	N.D.	4.2
Rest		15.5	16.7	22.5	14.8	13.2	13.7	4.2

* Data for the 70 kDa band of pig gastric mucin were calculated from Pearson *et al.* (1981).

† As carboxymethylcysteine.

Fig. 4. Western blots of purified mucins, with an antibody to the 118 kDa 'link' glycopeptide of rat small-intestinal mucin

Purified mucins were as follows: tract A, pig gastric (fundus) mucin; tract B, human submaxillary saliva mucin; tract C, human colonic mucin; tract D, human small-intestinal mucin; tract E, rat small-intestinal mucin. After reduction the mucins were subjected to SDS/PAGE, blotted on to nitrocellulose and treated with the antibody (1:500 dilution). Antibody binding (at approx. 118 kDa) was detected by using a conjugate of horseradish peroxidase and Protein A. Arrowheads (▲) indicate the position of 2-mercaptoethanol artifacts (at approx. 60 and 70 kDa). In a separate experiment the 'link' components of rat small-intestinal mucin (tract F) and human salivary mucin (tract G) were purified and then subjected to SDS/PAGE (10% gels). Sample applications were 10 µg (for tract F) and 100 µg (for tract G).

118 kDa component of human small-intestinal mucin (Laboratory B, Fig. 5). Since the two antibodies were raised against 'link' components that are virtually identical in composition and size, it was not surprising to find that each antibody recognized the 118 kDa component of both rat and human small-intestinal mucins. Before reduction, the mucin samples gave positive staining in the upper part of the stacking gel, as reported earlier for rat mucin (Fahim *et al.*, 1987*a,b*). After reduction of each mucin, however, positive blots were obtained in a broad band having a mid-position of 118 kDa for pig fundus mucin, human colonic mucin and human and rat small-intestinal mucins (Fig. 4), and for rabbit small-intestinal and colonic mucins and ferret, pig, rat and human small-intestinal mucins (Fig. 5). In the ferret, there were mucin bands at 118 kDa and at about 70–75 kDa. As was true of silver-stained bands in Figs. 1 and 2, stained artifacts were also detected at a position of about 60–70 kDa in Western blots. The same bands were present in reagent blanks (Fig. 5) and were easily distinguished from the broader more-diffuse ferret mucin bands in the same region (tract F). Thus the smaller-molecular-mass band

Fig. 5. Western blots of purified mucins, with an antibody to the 118 kDa 'link' glycopeptide of human small-intestinal mucin

Purified mucins in tracts A to F (same identification as in the legend to Fig. 1) were reduced, subjected to SDS/PAGE, blotted on to nitrocellulose and treated with the antibody (1:1000 dilution). Antibody binding was detected with a conjugate of horseradish peroxidase and Protein A. BL refers to a blank tract (reagents only) to show the position of the 2-mercaptoethanol artifacts (▲) at about 60 and 70 kDa. The large arrowhead at the left is at a position of 118 kDa. Diffuse mucin bands in tract F (ferret small-intestinal mucin) are centred at 118, 90 and approx. 70–75 kDa.

detected in ferret mucin may represent partial degradation of the 118 kDa component. The human salivary mucin produced a very faint reaction (not clearly seen in tract B in Fig. 4). To render this more visible, SDS/PAGE of rat small-intestinal and human salivary mucins was repeated, and the 118 kDa band of rat mucin and the diffuse band (120–175 kDa) of salivary mucin were electroeluted, concentrated and re-run under reducing conditions on 10% gels with subsequent Western blotting. This time the band was slightly more visible (Fig. 4, tract G), and by comparing it with the purified rat small-intestinal 'link' component (tract F) was noted to have a molecular mass of approx. 118 kDa. In order to detect the salivary 'link' component, however, a 10-fold higher concentration of sample had to be applied (100 µg) to the gel and left to stain for a longer time period. The artifact (60–70 kDa) bands were very prominent under these conditions. The salivary 'link' component was therefore

Fig. 6. Reaction of purified mucins in an e.l.i.s.a. with the antibody to the 118 kDa 'link' glycopeptide of human small-intestinal mucin

The e.l.i.s.a. assay was carried out as described in the Methods section for several concentrations of each mucin sample: Δ , rat small-intestinal mucin; $\blacksquare$, pig small-intestinal mucin; $\circ$, human small-intestinal mucin; $\blacktriangle$, ferret small-intestinal mucin; $\square$, rabbit small-intestinal mucin; $\bullet$, rabbit colonic mucin.

much less immunoreactive than were the 'link' components of the other mucins tested, despite its similar electrophoretic mobility.

E.l.i.s.a. for 118 kDa 'link' glycopeptides

Fig. 6 presents data obtained for the e.l.i.s.a. with the antibody to the 118 kDa component of human small-intestinal mucin. Not all of the mucins were available for testing, but those that were yielded slopes that were identical with that of human small-intestinal mucin. Thus the major antigenic determinant(s) in each mucin 'link' component is (are) assumed to be similar. The fact that the ferret mucin had the same slope as the other mucins suggests that the presence of an extra immunoreactive band at 70 kDa did not alter its antigenicity. In other words, the antigenic determinants of both bands are probably the same. This is consistent with the notion that the 70 kDa band may represent a partial breakdown product of the 118 kDa component.

Immunocytochemical localization

In an earlier study (Fahim *et al.*, 1987a) it was shown by light-microscopy that the antibody to the rat 'link' glycopeptide localized to intestinal goblet cells. The same antibody was used in the present study, and localized with Protein A/colloidal gold by electron microscopy of Lowicryl thin sections of rat ileum. Staining was present over most of the granules (Fig. 7a) in the stored granule mass of nearly all goblet cells in the intestine. Labelling also appeared over the cisternae of the Golgi complex and in the condensing vacuoles arising from the Golgi complex (Fig. 7b). Virtually no labelling was found over the cisternae of the rough endoplasmic reticulum, nor on apical or basolateral membranes of goblet cells. Specific and non-specific staining of other cells was negligible. Some particles of colloidal gold appeared over secreted mucin in the lumen (Fig. 7a). These findings suggest

that the 'link' glycopeptide acquires its antigenic determinant(s) within the Golgi complex and forms an integral part of mucin molecules that are stored in granules and eventually secreted into the intestinal lumen.

DISCUSSION

Existence of a 'link' glycopeptide in gastrointestinal mucins

The goal of the present study was to provide additional evidence that a 'link' component is present in gastrointestinal mucins irrespective of different purification techniques, animal species and inter-laboratory procedures. Our results indicate that this component was present in each and every gastrointestinal mucin preparation studied, and that the 'link' components were similar in their amino acid composition, electrophoretic mobility and immunoreactivity (by e.l.i.s.a. and Western blots). Treatments of the mucins by different denaturing agents, shear forces, chromatography columns or delipidation did not eliminate the 'link' component. There was precise immunolocalization of this component to mucus-containing vesicles within rat ileal goblet cells. Therefore the 118 kDa component is unlikely to represent simply a contaminant that is co-purified with gastrointestinal mucin and remains non-covalently entangled with it.

Preservation of the 'link' component during purification probably depends upon the addition of proteinase inhibitors at the earliest step of isolation (homogenization of tissue). This assumption is based on an earlier study (Fahim, 1985; Forstner & Fahim, 1988) of rat small-intestinal mucin, in which brief exposure to trypsin (or Pronase) caused the mucin to liberate components of 200 kDa (approximately) and 118 kDa (both having the same amino acid and carbohydrate composition) and antigenically related degradation products of molecular mass 70 kDa (Fahim *et al.*, 1987b). Mucins prepared in the absence of proteinase inhibitors or prepared from secretions in which there is prolonged exposure of the mucin to proteolytic enzymes (such as gastric or tracheobronchial secretions) may be partially degraded even before the secretions are harvested. This may explain why mucin 'link' components are either not observed or else appear to range widely in size (65 to 118 kDa) (Tabachnik *et al.*, 1981; Pearson *et al.*, 1981; Fahim *et al.*, 1983; Mantle *et al.*, 1984b; Ringler *et al.*, 1987). In all likelihood, the difference in the size of the gastric mucin 'link' peptide reported by Pearson *et al.* (1981) (70 kDa) and those of gastric mucin preparations used in the present study (118 kDa) can be attributed to partial proteolytic degradation of the former mucin sample. The mucin studied by Pearson *et al.* (1981) was purified several years ago from secreted gastric mucus gel and before proteinase inhibitors were used routinely in mucin purifications.

Recent studies on mammary-gland mucin biosynthesis have failed to implicate the presence of 'link'-type glycopeptides (Burchell *et al.*, 1987; Gendler *et al.*, 1987, 1988; Hilkens & Buijs, 1988; Linsley *et al.*, 1988; Spielman *et al.*, 1988). It should be noted, however, that these mucin-like epithelial glycoproteins differ significantly from typical gel-forming gastrointestinal or salivary mucins. The former are less glycosylated (50%

Fig. 7. Localization of the 118 kDa glycopeptide in rat ileum goblet cells

(a) Localization of the 118 kDa glycopeptide in goblet cells of Lowicryl sections of rat ileum with the antibody to the 118 kDa 'link' glycopeptide of rat small-intestinal mucin. Colloidal gold is concentrated over the mucin granules (MG), with a few particles found in association with the microvilli (arrow). No particles are found over the adjacent columnar cells (C). Bar = 1 μ m (magnification $\times 21375$). (b) Localization of the 118 kDa glycopeptide in the supranuclear region of rat ileum goblet cells. Gold particles are located over the condensing vacuoles (CV) arising from the Golgi complex (G) and over the Golgi cisternae, but not over the nucleus (Nu) and very sparingly over the rough endoplasmic reticulum (RER). Bar = 0.6 μ m (magnification $\times 45885$).

compared with 80% carbohydrate), have a smaller molecular mass (500 kDa compared with 2000 kDa or more), have less serine, threonine and proline (33% combined compared with approx. 50%), have more alanine and are not decreased in size or viscosity by disulphide-bond reduction. The mammary-gland glycoproteins are membrane-bound (unlike true mucins) as well as soluble constituents. Taken altogether, these observations suggest that the mammary-gland sialomucins represent a related but distinctly different family of glycoproteins in which discrete 'link' glycopeptides are not a structural feature.

Salivary mucin 'link' component

The human salivary mucin in our study differed from the gastrointestinal mucins in producing a 'link' component with a more diffuse band on SDS/PAGE (120–175 kDa) and in showing weak reactivity with the anti-(118 kDa component) antibody. The salivary 'link' component was similar to the others, however, in that it was produced only after disulphide-bond reduction. We are aware that Timpte *et al.* (1988) have noted no cysteine in pig submaxillary mucin and no nucleotide codon for cysteine in a large cDNA fragment responsible for at least 45% of the apo-mucin. Thus pig submaxillary mucin would appear to be incapable of disulphide-dependent cross-linking of mucin subunits. This is difficult to reconcile with observations that native pig submaxillary mucin decreases dramatically in size after exposure to 2-mercaptoethanol (Shogren *et al.*, 1984). It is possible that when the rest of the pig submaxillary mucin (apo-mucin) gene is cloned in the future a 'linkage' sequence containing cysteine may be discovered. It is also possible that the apo-mucin cloned by Timpte *et al.* (1988) represents only one of two distinct mucin species normally present in saliva. Loomis *et al.* (1987), for example, have shown that in human submandibular-sublingual saliva there are two structurally distinct mucin glycoproteins. The larger mucin (MG1) (> 1000 kDa) could be split into subunits by disulphide-bond reduction, whereas the smaller mucin (MG2) (200–250 kDa) could not. Since the amino acid compositions of pig submaxillary mucin (Katzman & Eylar, 1966; De Salegui & Plonska, 1969) and human salivary mucins are very different, it would appear at the very least that salivary mucins in different species may differ considerably in their apo-mucin sequences and in the structural arrangements of subunits.

Immunocytochemical studies

In rat ileum the 'link' component of 118 kDa was localized to the Golgi complex, condensing vacuoles and mucus granules of goblet cells. The location in the Golgi complex is consistent with the presence of carbohydrate in the molecule and suggests that addition of carbohydrate may be required for antibody recognition. The lack of staining in other cell organelles and in other cell types in the small-intestinal epithelium helps to support our conclusion that the 'link' glycopeptide is an integral component of small-intestinal mucin, rather than a contaminant acquired during mucin preparation. Whether the 'link' glycopeptide indeed has a functional role in linking large subunits together, or has some other unknown role (e.g. a regulatory function relevant to storage or secretion of mucin), remains an open question and requires further study.

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