Characterization and localization of the putative 'link' component in rat small-intestinal mucin

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Rat intestinal mucin is polymerized by a putative 'link' component of M_r 118000 that can be released from the native mucin by thiol reduction [Fahim, Forstner & Forstner (1983) *Biochem. J.* **209**, 117–124]. To confirm that this component is an integral part of the mucin and independent of the mucin purification technique, rat mucin was purified in the present study by three independent techniques. In all cases, the 118000- M_r component was released after reduction. The 118 kDa band was electroeluted from SDS/polyacrylamide gels and its composition shown to resemble closely that of the link component of human intestinal mucin [Mantle, Forstner & Forstner (1984) *Biochem. J.* **224**, 345–354]. Carbohydrates were present, including significant (10 mol/100 mol) amounts of mannose, suggesting the presence of *N*-linked oligosaccharides. Monospecific antibodies prepared against the rat 118000- M_r component established its tissue localization in intestinal goblet cells. Mucins subjected to SDS/polyacrylamide-gel electrophoresis and Western blots using the same antibody, established that the link components of rat and human intestinal mucin are similar antigenically. Brief exposure (10 min) of native rat mucin to trypsin or Pronase (enzyme/mucin protein, 1:500, w/w) also released a 118000- M_r component that reacted with the monospecific antibody. Thus the 118000- M_r component is an integral part of the mucin and, although linked to large glycopeptides by disulphide bonds, this component also has proteinase-sensitive peptide bonds, presumably at terminal locations such that brief treatment with proteinases releases the molecule in a reasonably intact form. Under physiological conditions, therefore, one might expect that, after mucin is secreted into the intestinal lumen, luminal proteinases would rapidly remove the link component, thereby causing the mucin to depolymerize.

INTRODUCTION

Intestinal mucins consist of large and heterogeneous glycopeptides that are polymerized by disulphide bonds between cysteine residues of the peptide backbones (Snary *et al.*, 1970; Mantle *et al.*, 1981, 1984b; Pearson *et al.*, 1981; Fahim *et al.*, 1983; Creeth *et al.*, 1977). After thiol reduction, the viscosity and molecular masses of many mammalian mucins decrease significantly, but in addition, a variable loss of peptide occurs (Gibbons, 1968; Creeth *et al.*, 1977; Allen, 1978; Bhaskar & Reid, 1981). Considerable indirect evidence suggests that the released peptide may function as a discrete cross-linking agent for the glycopeptide subunits (Masson, 1973; Creeth *et al.*, 1977; Pearson *et al.*, 1981; Fahim *et al.*, 1983). In the case of human sputum mucin (Tabachnik *et al.*, 1981), purified pig and human gastric mucin (Pearson & Allen, 1980; Pearson *et al.*, 1981), rat, pig and human small-intestinal mucins (Fahim *et al.*, 1983; Mantle *et al.*, 1981, 1984b), discrete putative 'link' components have been demonstrated by SDS/PAGE. The isolated link component of pig gastric mucin has an M_r of 70000, represents about 25% of the total mucin protein and has a significantly higher proportion of

cysteine, aspartic acid, glutamic acid, glycine, alanine and leucine than either the parent mucin or the residual high- M_r reduced glycopeptides (Pearson *et al.*, 1981). No carbohydrates were detected. Link components of purified rat and human small-intestinal mucin have also been isolated in our laboratory (Fahim *et al.*, 1983; Mantle *et al.*, 1984b). Each contained about 30% of the total mucin protein, had an M_r of 118000 and was enriched in the same group of amino acids. Thus in some respects these link components are similar to that of pig gastric mucin. Unlike the situation with pig gastric mucin, however, carbohydrates (including mannose) were detected in the human 118 kDa component, suggesting perhaps that *N*-linked oligosaccharide chains might be a structural feature.

Since purification techniques for mucins vary from one laboratory to another, and since putative link components have not been found universally in epithelial mucins, there is some concern as to whether the presence of link components is a function of the purification procedures employed.

In the present study we show that the 118 kDa putative link component is an integral part of rat goblet-cell mucin purified by three different procedures.

Abbreviations used: PAGE, polyacrylamide-gel electrophoresis; PMSF, phenylmethanesulphonyl fluoride; NEM, *N*-ethylmaleimide; d.g.u., density-gradient ultracentrifugation; GdmCl, guanidinium chloride; PAS, periodic acid/Schiff reagent; PBS, phosphate-buffered saline (0.01 M- $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4/0.15$ M- NaCl , pH 7.4); PVP, polyvinylpyrrolidone-40T.

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We have compared its composition with previously reported values for link components from pig gastric and human intestinal mucins. By using immunological techniques we have established the tissue localization of the 118 kDa component in rat intestinal goblet cells and shown its antigenic similarity to the link component of human intestinal mucin. The link component was released intact from rat mucin by thiol reduction, confirming its normal attachment by disulphide bonds. In addition, however, the present study also demonstrates release of the link component, with minimal degradation, by brief exposure to proteinases. Thus the link component appears to comprise a distinct segment of mucin and probably has peptide bonds near each end that are highly susceptible to proteolytic cleavage.

METHODS

Isolation and purification of rat intestinal goblet-cell mucin

Rats were fasted for 16 h before being killed, the small intestine removed and washed with 40–50 ml of cold 0.15 M-saline, pH 7.0, immersed for 2 min in a mixture of proteinase inhibitors (2 mM-PMSF, 2 mM-NEM and 5 mM-EDTA), and then cut open to expose the luminal surface. The epithelium was scraped gently with a glass slide and the scrapings placed in the proteinase-inhibitor mixture in a ratio of approx. 1 g wet weight to 50 ml of solution. After homogenization for 30 s in a Waring blender and centrifugation at 30000 *g* for 30 min, the cell sap was concentrated and dialysed against 0.1 M- $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ buffer, pH 7. Mucus glycoprotein was then purified by three methods: (a) gel-filtration chromatography on Sepharose 4B; the void-volume fraction was treated with nuclease and subjected to Sepharose CL 2B chromatography as described earlier (Fahim *et al.*, 1983); (b) two sequential cycles of CsCl d.g.u., with the carbohydrate-containing fractions being harvested as described by Mantle *et al.* (1981); to ensure that lipids were removed, mucin samples were extracted by sequential chloroform/methanol as described by Slomiany *et al.* (1983); (c) CsCl d.g.u. in the presence of GdmCl by the method of Carlstedt *et al.* (1983). For the last method, tissue scrapings were collected in a solution of proteinase inhibitors and GdmCl (6 M final concn.), stirred for 2 h at 4 °C, homogenized for 10 s and centrifuged at 30000 *g* for 30 min. Distilled water was added to adjust the GdmCl concentration to 4 M and then CsCl added to give a density of 1.4 g/ml. Equilibrium d.g.u. was performed and the PAS-positive fractions, measured by the method of Mantle & Allen (1978), were subjected to a second d.g.u. in 0.2 M-GdmCl/CsCl, density 1.4 g/ml. PAS-positive fractions appeared over a density range of 1.35–1.49 g/ml and were pooled, dialysed and stored at –20 °C. One further step to ensure purity consisted of boiling the mucin for 3 min at 100 °C in 1% SDS and applying it to a Sepharose CL-2B column (0.9 cm × 55 cm). After elution with 0.1 M- $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ buffer, pH 7.4, in 1% SDS, all of the mucin appeared in the void-volume fraction. This fraction was dialysed against distilled water, concentrated and subjected to SDS/PAGE.

Human intestinal mucin was purified by method (b) above and was kindly supplied by Dr. M. Mantle

(University of Calgary Medical School, Calgary, Alberta, Canada).

Gel electrophoresis

Mucin was dissolved in 0.06 M-Tris/HCl buffer (pH 6.8)/1% SDS/2.5% β -mercaptoethanol, heated for 3 min in a boiling-water bath, cooled, alkylated with 0.5 M-iodoacetamide (16 h in the dark at 4 °C) and then exhaustively dialysed against distilled water.

Composite agarose/PAGE was performed as described previously (Fahim *et al.*, 1983) and the gel was stained with silver by the method of Switzer *et al.* (1979). SDS/PAGE was performed by the method of Laemmli (1970) and silver-stained by the method of Merrill *et al.* (1981).

The link component (M_r 118000) was electroeluted from polyacrylamide gels by using a sample concentrator apparatus (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).

Amino acid and carbohydrate analysis

Amino acid analyses were performed on either a Durrum model D-500 analyser or on a Waters h.p.l.c. amino acid analysis system after hydrolysis of the samples for 20 h in 5.5 M constant-boiling HCl at 110 °C. For the estimation of cysteine, samples were reduced and alkylated before hydrolysis and the resultant carboxymethylcysteine quantified in the analyser.

Carbohydrates were analysed either on a Varian Aerograph series 2100 or a Varian model 3700 gas chromatograph with a 3% OV-210 column by the method of Zanetta *et al.* (1972). A temperature program of 110–210 °C at 1 °C/min was used to separate the *O*-methyl glycoside derivatives. *N*-Terminal amino acids were determined by the dansyl method of Gray (1972) on polyamide sheets (Woods & Wang, 1967), with aq. 1.5% formic acid, benzene, acetic acid (9:1, v/v) and ethyl acetate/methanol/acetic acid (20:1:1, by vol.) solvents in sequence.

Preparation of antibody

Antibody to the rat 118000- M_r link component was prepared by injecting 40 μ g of protein mixed with Freund's complete adjuvant into each of three New Zealand rabbits. Before the first injection, about 50 ml of blood was collected and the serum saved from each rabbit, to serve as a control (preimmune serum). Injections of antigen were repeated with Freund's incomplete adjuvant at 2 and 4 weeks, and thereafter every 2 months. Antiserum production was monitored either by Ouchterlony (1968) double immunodiffusion or counter-immunoelectrophoresis (Kenny & Foy, 1975).

Monospecific antibody to the link protein was prepared by using affinity columns of Reacti-gel (Pierce Chemical Co., Rockford, IL, U.S.A.) to which was bound the purified link protein (200 μ g in 1 ml of gel). Antiserum was applied, the unbound material washed off the column with 25 ml of PBS, concentrated, and reapplied twice to the column. The column-bound material was eluted with 0.1 M-glycine/HCl, pH 2.8, neutralized immediately with concentrated Tris (Sigma Chemical Co.) and stored in aliquots at –20 °C.

Electroblotting (Western blot)

Electrophoretic transfer of mucins and detection with monospecific antibody (1:200 dilution) were performed by the method of Towbin *et al.* (1979).

Immunofluorescent localization

Intestinal tissue segments approx. 0.5 cm in length were fixed in 10% (v/v) neutral buffered formalin for 4 h at room temperature and left in 30% (w/v) sucrose in water overnight at 4 °C (Kopp *et al.*, 1968). The segments were embedded in O.C.T. compound (Canlab, Mississauga, Ont., Canada) and sectioned at -20 °C. Sections approx. 4 µm thick were placed on microscope slides and frozen in air-tight bags at -70 °C until use. Sections were fixed in cold acetone for at least 15 min, washed in PBS, incubated with the antibody or preimmune serum for 1 h, and then washed thoroughly in PBS. Fluorescent-conjugated second antibody (sheep anti-rabbit IgG; Miles, Elkhart, IN, U.S.A.) was centrifuged, diluted 1:20 (v/v) in PBS and incubated with the sections for 30 min. After washing in PBS, the sections were observed under a Leitz Orthoplan fluorescence microscope.

Immunoperoxidase localization

Intestinal tissue for immunoperoxidase localization was prepared by vascular perfusion of two rats under sodium pentobarbitol anaesthesia. Under anaesthesia, the abdominal and thoracic walls were opened and the vascular system was rinsed for 1.5 min. with 0.1 M-phosphate buffer containing 0.5% procaine, heparin (40000 units/l) and 2.5% PVP (Sigma, St. Louis, MO, U.S.A.). The animal was then perfusion-fixed with 2.0% paraformaldehyde with 2.5% PVP in 0.1 M-sodium phosphate buffer for 5 min. When the perfusion-fixation was started, the intestinal lumen was gently perfused with the same solution. A segment of the ileum was then excised and fixed by immersion for a total of 60 min before being cut into 4–6 mm-thick rings, rapidly dehydrated in 70% and then 95% (v/v) ethanol and embedded in glycol methacrylate (JB-4; Polysciences, Warrington, PA, U.S.A.). Semi-thin (1–2 µm) sections were pretreated with methanol for 15 min, rinsed in PBS, incubated with antibody or normal rabbit serum for 1–4 h at room temperature and then rinsed extensively in PBS. Peroxidase-conjugated second antibody (goat anti-rabbit IgG; Pel-Freez Biologicals, Rogers, AR, U.S.A.) was absorbed on freeze-dried rat liver powder, centrifuged for 15 min at 3000 g, diluted 1:50 (v/v) in PBS and incubated with the sections for 1–4 h at room temperature. The sections were then rinsed in PBS and incubated with 0.05% diaminobenzidine/0.001% H₂O₂ in 0.1 M-Tris at pH 7.4 for 20 min. The slides were then washed extensively and a portion were counterstained with Mayer's haematoxylin. The sections were observed and photographed with an Olympus Vanox research microscope.

Incubation of rat mucin with trypsin

Purified mucin (containing 2 µg of protein) was incubated with trypsin [Sigma type 1; 10000 N-α-benzoyl-L-arginine ethyl ester ('BAEE') units/mg], giving an enzyme/mucin protein ratio of 1:500 or 1:200 (w/w), for 10 min at 37 °C in 60 mM-Tris/HCl, pH 7 (final vol. 50 µl). The reaction was stopped by adding PMSF (final

concn. 2 mM), and the mixture subjected to SDS/PAGE and Western blots by using the monospecific antibody to the 118 kDa component.

RESULTS

Native rat mucin and the 118 kDa component

Goblet-cell mucin was purified from scrapings of rat intestine by three different procedures, each of which included exposure to proteinase inhibitors at an early stage, and subsequently either gel filtration or d.g.u. From two of the procedures [(a) and (b) of the Methods section] the purified mucins were subjected to compositional analyses (Table 1). Mucin purified by CsCl d.g.u. was also analysed after delipidation. No important differences were detected among the three preparations. In each case the major amino acids were serine, threonine and proline (> 55 mol%), and total protein made up only 14–16% (w/w). The only N-terminal amino acid detected was alanine. Mannose was either not detected or present in only trace amounts. Previous studies showed that mucins prepared by these techniques were free of DNA and uronic acid, and gave no reaction with antibodies to rat serum proteins or IgA (Forstner *et al.*, 1977). Mucins purified by all three methods [(a), (b)

Table 1. Amino acid and carbohydrate analysis of goblet-cell mucin

Amino acid or sugar	Content (mol/100 mol)		
	Gel-filtration Chromatography	CsCl d.g.u. (lipid extraction)	
		Before	After
Asp	4.8 (4.5–4.9)	4.6 (4.4–4.8)	4.6
Thr	30.7 (28.2–32.6)	31.0 (30.1–31.6)	31.2
Ser	13.6 (13.0–14.9)	15.7 (14.6–16.0)	16.0
Glu	6.7 (5.8–7.1)	6.2 (5.8–6.6)	6.6
Pro	10.8 (10.4–11.9)	14.7 (13.3–15.7)	13.3
Gly	7.3 (5.8–8.0)	5.9 (5.3–6.7)	6.7
Ala	3.5 (2.9–3.7)	2.4 (2.3–2.4)	2.4
Val	2.5 (2.3–2.7)	3.1 (2.5–3.5)	3.2
½-Cys	1.8 (1.8)	NM	NM
Met	0.5 (0.5)	0.8 (0.7–0.9)	0.8
Ile	3.9 (3.2–4.1)	3.2 (3.0–3.5)	3.5
Leu	5.1 (4.0–5.8)	3.1 (3.0–3.1)	3.1
Tyr	2.7 (1.8–3.2)	1.6 (1.5–1.7)	1.6
Phe	3.0 (2.1–3.8)	2.9 (2.3–3.4)	2.3
His	2.3 (2.0–2.5)	1.5 (1.4–1.7)	1.4
Lys	1.5 (1.4–1.7)	2.0 (1.8–2.2)	2.1
Arg	1.1 (1.1–1.2)	1.2 (1.0–1.3)	1.2
Fuc	14.1 (13.4–15.1)	16.5 (16.0–17.4)	16.2
Gal	27.2 (26.4–27.4)	27.0 (27.1–27.8)	27.8
GlcNAc	21.8 (20.3–22.6)	20.3 (19.3–20.9)	20.8
GalNAc	20.9 (19.2–22.4)	23.2 (22.6–24.4)	22.6
Sialic acid	16.0 (14.4–16.4)	12.6 (12.3–13.0)	12.6

and (c) of the Methods section] were subjected to electrophoresis on composite agarose/acrylamide and Laemmli (1970) SDS/polyacrylamide gels. Before thiol reduction all mucins gave only the usual high- M_r bands (Fahim *et al.*, 1983) at the top of composite gels (Fig. 1), whereas after reduction at 100 °C for 3 min in 2.5% mercaptoethanol and 1% SDS, all mucin samples showed a decrease of the polymer on the top of the gel and the appearance of a broad band at the M_r -118000 position. This value of M_r was assigned by comparing the R_F value of the midpoint of the band with a series of molecular-mass markers ranging from 14.3 to 200 kDa (Fahim *et al.*, 1983). Treatment with SDS and/or boiling alone did not cause these changes. No other lower- M_r

Fig. 1. Electrophoresis of rat intestinal mucin on 0.5% agarose/2% polyacrylamide composite slab gels, with detection by silver stain

Samples containing approx. 2 μ g of mucin protein were applied after heating at 100 °C for 5 min in 1% SDS, pH 8.6, without (left track) and with (right track) 2.5% 2-mercaptoethanol. Electrophoresis was performed for 4 h at 25 mA. 'O' represents the origin.

bands were seen. Essentially the same pattern was observed on SDS/PAGE (Fig. 5 below, tracks 2 and 3). Thus-purified mucins obtained from the three different techniques liberated a component of M_r 118000 after reduction, but no bands of lower M_r .

The 118 kDa component was electroeluted from preparative SDS/polyacrylamide gels and checked for homogeneity by re-electrophoresis on 7.5% (w/v) polyacrylamide gels or a gradient gel (5.5–12.5%). Fig. 2 shows that a single (although somewhat diffuse) band at M_r 118000 was the only component. Compositional analyses of the 118 kDa component are presented in Table 2 and compared with a similar 118 kDa band found previously in our laboratory from human small-intestinal mucin (Mantle *et al.*, 1984b) and an analogous component of 70000 M_r isolated after reduction of pig gastric mucin (values calculated from those of Pearson & Allen, 1980). Initially the rat 118 kDa component was thought to be devoid of carbohydrate (Fahim *et al.*, 1983), but in the present study sufficient material was available to permit g.l.c. analysis of 5-fold more material (50 μ g) and carbohydrates were detected. As with the human 118 kDa component, the usual mucin sugars were present, with differences possibly accounted for by ABH- and Lewis-blood-group specificities. In addition, however, mannose was also present in significant amounts. Within the 118 kDa component, carbohydrate comprised

Fig. 2. SDS/PAGE of the purified 118 kDa component

After preparative gel electrophoresis of rat intestinal mucin, the band at the M_r -118000 position was electroeluted and then applied to a gradient (5.5–12.5%) polyacrylamide gel. Only one band appeared at the M_r -118000 position (arrowhead). High- M_r standards are as shown on the left.

Table 2. Composition of putative link components of intestinal mucins

Amino acid values for the rat link component are the means of three analyses (with total range in parentheses). Carbohydrates were analysed once. Abbreviation used: ND, not detected.

Amino acid or sugar	Content (mol%)		
	Intestinal mucin		Pig gastric mucin†
	Rat	Human*	
Asp	11.5 (9.9–12.0)	10.2	10.0
Thr	8.5 (7.0–9.0)	7.6	7.7
Ser	8.8 (8.5–10.0)	9.1	10.1
Glu	13.5 (12.5–14.6)	11.5	14.4
Pro	6.5 (6.1–8.0)	7.0	7.1
Gly	9.3 (8.2–10.8)	12.5	11.5
Ala	6.3 (6.0–6.5)	8.3	9.2
Val	4.2 (3.2–5.2)	4.8	9.8
$\frac{1}{2}$ -lys	4.1	3.7	4.2
Met	0.9 (0.8–1.0)	1.2	0.8
Ile	4.4 (4.2–4.9)	2.8	3.8
Leu	8.5 (6.9–12.9)	5.8	8.0
Tyr	2.6 (2.3–2.9)	2.7	4.4
Phe	4.8 (4.4–5.5)	2.6	4.2
His	3.0 (2.5–3.6)	2.1	1.8
Lys	3.8 (3.5–4.0)	3.7	3.7
Arg	3.4 (3.1–3.6)	3.3	3.9
Fuc	10.1	24.0	ND
Gal	21.6	24.1	ND
GlcNAc	30.2	20.8	ND
GalNAc	22.1	14.2	ND
Sialic acid	6.0	8.0	ND
Man	10.0	8.9	ND

* Values are the means for two patients (T.B. and C.A.M.) reported by Mantle *et al.* (1984b).

† Values calculated from those given by Pearson *et al.* (1981).

about 50% (w/w). Although substantial, this amount comprised less than 5% of the total carbohydrate of native rat mucin and probably accounts for the failure to detect mannose in the native mucin (Table 1).

The protein content of the putative link component represents 25–30% of the native mucin protein in pig gastric, rat and human intestinal mucins (Pearson *et al.*, 1981; Fahim *et al.*, 1983; Mantle *et al.*, 1984b). The amino acid profiles (Table 2) were similar, but all were distinct from those of the respective parent (native) mucin, with enrichment of cysteine, aspartic acid, glutamic acid, alanine, leucine and glycine, and significantly lower serine, threonine and proline. No *N*-terminal amino acid could be detected in the rat 118 kDa component, which may suggest that the *N*-terminus is blocked. The same failure to detect an *N*-terminal residue has been reported for the two other isolated link components.

Antibody to the 118 kDa component of rat mucin

The 118 kDa component of rat mucin was electroeluted from SDS/PAGE and injected into rabbits to prepare antiserum. Further purification of antiserum was achieved by affinity chromatography as described in the Methods section. The resultant monospecific antibody

was localized by immunofluorescence to goblet cells of rat jejunum and ileum (Figs. 3a and 3b) and in some areas to secreted mucin adherent to the mucosal surface. To gain more precise definition and localization, immunoperoxidase staining was also performed using much thinner sections (1–2 μ m) of rat ileum.

All goblet cells of the crypt and villus reacted with the antibody. Within the goblet cells (Fig. 4a) staining was seen in mature mucus granules undergoing discharge and in the supranuclear region of the cell, which contains the Golgi and developing mucus granules. In some cells the material was clearly localized in the subnuclear region (Fig. 4b), which is the domain of the rough endoplasmic reticulum in goblet cells. Interestingly, the mucus granules of most goblet cells were not uniformly stained with the antibody, but rather exhibited a somewhat patchy distribution. This may indicate that individual granules differ in their content of the 118 kDa component. Mucosal membranes and columnar cells did not react with the antibody, thus excluding the possibility that the 118 kDa component represents a contaminant in mucus arising from membrane glycoprotein from goblet- or adjacent columnar-cell glycoproteins. Immunoperoxidase localizations using non-immune serum in place of the specific antibody resulted in virtually no non-specific peroxidase activity in the epithelium (result not shown).

Specificity of the antibody was further examined using silver staining and Western blots of mucin samples after SDS/PAGE (Fig. 5). Before reduction the mucin did not enter the gels, whereas after reduction reactivity was confined to the 118 kDa band. Both the silver-stained band and Western blots of the purified 118 kDa component had the same mobility.

Polyclonal antibodies developed previously in our laboratory against native rat and human intestinal mucins did not show species cross-reactivity in Western blots (results not shown). However, since the present study indicated that the 118 kDa component from human mucin had a size and composition similar to that of the rat mucin, it was of interest to compare their antigenic reactivity with the antibody to the 118 kDa component. As shown in Western blots (Fig. 6), both mucins after reduction gave immunoreactivity at the M_r -118000 position. In the case of the human mucin, faint bands corresponding to lower M_r values were also seen, suggesting perhaps that some degree of degradation had occurred.

Reduced pig gastric mucin (kindly supplied by Dr. A. Allen, Department of Physiological Sciences, University Medical School, Newcastle upon Tyne, U.K.) gave no reaction with the rat 118 kDa antibody. To date, therefore, it appears that link components of rat and human intestinal mucin, but not of pig gastric mucin, are antigenically as well as compositionally similar. Other purified mucins of different organs and species have not been tested.

Generation of rat 118 kDa component by proteolysis

Previous work established that the 70 kDa putative link component of pig gastric mucin (Pearson *et al.*, 1981) and the 118 kDa putative link component of human (Mantle *et al.*, 1984b) and rat (Fahim *et al.*, 1983; Fahim, 1985) intestinal mucins are generated by thiol reduction and are also susceptible to proteolytic degradation. With human intestinal mucin, for example, the 118 kDa band disappeared from gels after 8 h of

Fig. 3. Immunofluorescent localization of antibody to the 118 kDa component of rat intestinal mucin (*a* and *b*); the control (preimmune) serum is shown in (*c*)

Fluorescein-labelled sheep anti-rabbit IgG was used for staining. Specific fluorescence was confined to goblet cells and secreted mucus. Note the specific staining of the rough endoplasmic reticulum around the nucleus (arrow). Magnification $\times 400$.

Fig. 4. Immunoperoxidase localization of the antibody to the 118 kDa component of rat intestinal mucin

(a) Illustrates the somewhat patchy staining of the entire apical-granule mass and the supranuclear Golgi region of the cell (arrow). To show the epithelium better, the section was counterstained with Mater's haematoxylin, resulting in the staining of the nuclei. Magnification $\times 1100$. (b) Illustrates the same preparation without counterstaining, but photographed with phase optics. Staining by peroxidase is strictly limited to intracellular and secreted mucin of goblet-cell origin. Note the staining of the nascent granules in the Golgi of the cell (arrow). Magnification $\times 800$. Peroxidase-labelled goat anti-rabbit IgG was used for staining. Control sections stained either with non-immune rabbit serum or second antibody alone showed no staining of the epithelium (result not shown).

Pronase digestion (Mantle *et al.*, 1984a). In the present study we took advantage of the specificity of the 118 kDa antibody to determine whether very brief exposure of rat mucin to minute amounts of either trypsin or Pronase might release some of the 118 kDa component. After incubation for only 10 min at 37 °C, trypsin [enzyme/mucin protein 1:200 and 1:500 (w/w)] caused the appearance of a prominent immunoreactive band at the M_r -118000 position (Fig. 7). The same result was seen after brief exposure to similar concentrations of Pronase (result not shown). Subsequent reduction of the mucin released the remaining 118 kDa component from mucin polymers at the top of the gel, but did not change the proteolytically released 118 kDa band, although a few faint bands appeared in the general region of M_r 60000–70000. Our results suggest that, within the mucin, there may be a limited number of peptide bonds that are exquisitely sensitive to proteinases, and that these bonds are located near to the ends of the 118 kDa link component. Since reduction after brief trypsin (or Pronase) digestion did not cause a major change in mobility of the 118 kDa band, the uniquely sensitive peptide bonds are likely located close to the S–S bridges that link the 118 kDa component to the larger glycopeptides in the native mucin.

In summary, the 118 kDa putative link component is a distinct glycoprotein region which appears to be an integral part of small-intestinal mucin; it has been localized to rat intestinal goblet cells; it shares compositional features with the only other two link proteins which have been isolated from intestinal mucins; it is antigenically related to the analogous human 118 kDa component; and it can be released rapidly from mucin polymers either by thiol reduction or by brief exposure to proteolytic enzymes.

DISCUSSION

The possibility that there are discrete cross-linking peptides responsible for S–S polymerization of mucin glycopeptides has been discussed and debated for at least 20 years. In many mucins such as bronchial (Creeth *et al.*, 1977), gastric (Snary *et al.*, 1970), cervical (Gibbons, 1968) and ovarian-cyst (Dunstone & Morgan, 1965), thiol reduction causes mucins to become more soluble, to decrease in M_r and to exhibit a selective loss of aspartic acid, glutamic acid and cysteine residues, with preservation in the remaining glycopeptides of threonine, serine and proline. Masson (1973) presented the first hypotheti-

Fig. 5. SDS/PAGE of rat mucin and purified 118 kDa link component before and after thiol reduction

Track 1, M_r standards; tracks 2 and 3, native rat mucin before and after reduction respectively (silver stained); tracks 4 and 5, the same as 2 and 3, but detection was by Western blot, the antibody to the 118 kDa component being used; tracks 6 and 7, purified 118 kDa component detected by silver stain (track 6) and by Western blot (track 7). Separation of stack (4%) and running gel (7.5%) is marked by arrows (top), and arrowheads indicate reduction artefacts.

cal models of mucin structure that featured a discrete cross-linking peptide connected by S-S bonds to high- M_r glycopeptides. In 1977, Creeth *et al.* analysed bronchial mucin before and after reduction and calculated that the peptide released by reduction had an M_r of about 100 000 and a buoyant density of 1.31 g/ml. Its function, they proposed, was to cross-link glycoprotein into aggregates by S-S bridges. Similar conclusions were reached by Bhaskar & Reid (1981) after a study of mucin from sputa of asthmatic patients. The first report of the isolation of a putative link protein from mucin came from Pearson *et al.* (1981) and Mantle *et al.* (1981), and, with the exception of the two intestinal mucins studied in our laboratory and one reported for human sputum mucin (Tabachnik *et al.*, 1981), no other discrete link or cross-linking proteins have been analysed. At the present time it should be noted that the linkage function of these proteins is a sensible hypothesis, but not a proven fact.

In the present study we have demonstrated that the 118 kDa link component of rat intestinal mucin was present independent of the method of purification, although in each method precautions were taken to employ proteinase inhibitors. The 118 kDa component was not generated simply by exposing mucins to denaturants or chaotropic agents such as CsCl, SDS or GdmCl, which tends to rule out the possibility that it simply co-purified with the mucin. A specific antibody was used to confirm the localization of the 118 kDa component to intestinal goblet cells. Thus the 118 kDa component appears to be an integral, but antigenically

Fig. 6. Western blot of human (left track) and rat (right track) intestinal mucin on SDS/PAGE (7.5% gel) using affinity-purified antibody to the 118 kDa component of rat mucin

Samples (approx. 3 μ g of protein) of each mucin were reduced by boiling in 1% SDS and 2.5% mercaptoethanol before electrophoresis.

distinct, region of the native mucin. As shown previously (Fahim *et al.*, 1983) the 118 kDa component plays a role in disulphide-bond polymerization of mucin glycopeptides, but not all of the heterogeneous glycopeptide 'subunits' of purified mucin are necessarily attached to it. The native mucin is pictured therefore as a complex mixture, consisting of polymerized glycopeptides (linked by the 118 kDa component) and non-polymerized, but large and heterogeneous, glycopeptides spanning a wide range of M_r values.

The rat 118 kDa component was shown to resemble that isolated from human intestinal mucin. For example, both had the same M_r on gels, both reacted with an

Fig. 7. SDS/PAGE and Western blot of rat mucin after trypsin treatment

Samples were incubated without (tracks 1 and 6) or with trypsin [trypsin/mucin protein, 1:500 (w/w) (tracks 2 and 4) and trypsin/mucin protein, 1:200 (w/w) (tracks 3 and 5)]. Reactions were stopped by adding PMSF (final concn. 2 mM) and heating for 3 min at 100 °C in the presence of 1% SDS, pH 8.6, without (tracks 1, 2, and 3) or with (tracks 4, 5 and 6) 2.5% 2-mercaptoethanol. SDS/PAGE was performed on 7.5%-polyacrylamide separating gels, and Western blots were allowed to react with the affinity-purified antibody to the 118 kDa component.

antibody developed against the rat 118 kDa glycopeptide, both had similar, although not identical, amino acid profiles, both contained carbohydrate and both resembled in amino acid composition the 70 kDa putative link component of pig gastric mucin. These findings suggest that the peptide portion of gastrointestinal link components may be similar in mucins of different species.

The relatively high concentration (~10 mol%) of mannose in the purified rat (and human) link component is unusual, and was not detected in the intact mucin before fractionation. Normally, mannose is a quantitatively insignificant component of native mammalian mucins. Some exceptions have been noted, especially in isolated salivary mucins or selected subfractions of them (Roukema *et al.*, 1976; Levine *et al.*, 1978; Malinowski & Herp, 1981; Dutta & Rao, 1982; Prakobphol *et al.*, 1982). In the case of goat submaxillary-gland mucin, Dutta & Rao (1982) isolated two specific and unusual glycopeptide fractions that each contained > 10 mol% mannose and glucose. Mannose was located in a unique position at the non-reducing ends of some *O*-linked oligosaccharides. In mannose-containing salivary mucins of Chinese swallows (genus *Collocalia*) (Hanisch & Uhlenbruch, 1984) and mouse submandibular glands (Nieuw Amerongen *et al.*, 1983), both *O*- and *N*-glycosidically linked oligosaccharides were found. The mannose was assumed to be in *N*-linked chains. For the present we assume that this is also the case in the 118 kDa component of human and rat intestinal mucin. The well-accepted generalization that epithelial mucins are devoid of *N*-linked oligosaccharide chains may not,

therefore, be entirely accurate, although for rat intestinal mucin these chains may be confined to the link component.

Brief (10 min) exposure of rat mucin to trypsin [enzyme/mucin protein ratio 1:500 and 1:200 (w/w)] was shown by Western blots to liberate a component of M_r about 118000. Because of its size and reactivity with the 118 kDa antibody, we assume that the 118 kDa component was released in an almost intact form. Subsequent reduction removed the remaining 118 kDa component from mucin polymers, but caused very little change in the molecular size of the proteolytically released component. Thus peptide bonds near each end of the 118 kDa component appear to be very susceptible to proteinases. These bonds may be located within the 118 kDa component itself or at the 'naked' ends of the larger glycopeptides to which the 118 kDa component is disulphide-bonded.

Because of this sensitivity to proteinases, we might expect that mucins *in vivo* would lose the 118 kDa link component and become depolymerized rapidly once they are secreted and exposed to luminal proteinases. This may represent an early physiological step of mucin degradation and may partly explain why many investigators fail to observe a link component in mucins which are purified from proteinase-containing epithelial secretions.

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