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Effects of hydrogen peroxide, mild trypsin digestion and partial reduction on rat intestinal mucin and its disulphide-bound 118 kDa glycoprotein

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The role of the disulphide-bound 118 kDa glycoprotein of rat intestinal mucin is unknown, although it has been proposed to serve as a 'link' component for the mucin monomers. The present studies investigated release or destruction of the 118 kDa glycoprotein (monitored by gel electrophoresis and Western-blot analysis) during progressive breakdown of the mucin polymer (assessed by Sepharose 2B chromatography). H_2O_2 gradually destroyed the 118 kDa glycoprotein and dissociated the mucin polymer into components of similar size to the monomers. After 3 h, mucin samples contained almost no 118 kDa glycoprotein or its breakdown products, but 50% of the mucin was still eluted in the void volume of a Sepharose 2B column. Although mild trypsinolysis had little effect on the Sepharose 2B elution profile of the mucin, the 118 kDa glycoprotein was completely cleaved into 54–56 kDa and 60–66 kDa fragments which remained disulphide-bound to the high-molecular-mass mucin. Increasing levels of thiol reduction resulted in progressive loss of disulphide bonds, release of the 118 kDa glycoprotein and depolymerization of the mucin. Although approx. 40% of the mucin in partially reduced samples was recovered in the Sepharose 2B void volume, this material contained no 118 kDa glycoprotein and apparently consisted of disulphide-bound mucin monomers. Thus the 118 kDa glycoprotein may be destroyed by H_2O_2, extensively cleaved by trypsin or released by reduction without completely dissociating the mucin into monomers. Therefore the 118 kDa glycoprotein may not function as a 'link' component for all of the mucin monomers in the native polymer.

INTRODUCTION

Mucins, the gel-forming components of the mucous secretions, are large polydisperse macromolecules (1000 kDa) comprising a number of glycoprotein monomers (100–10000 kDa) held together by disulphide bonds (Neutra & Forstner, 1987). Over recent years, a smaller discrete (glyco)protein of 65–160 kDa has been identified in many purified mucin preparations, including pig and human gastric mucin (Pearson *et al.*, 1981), pig, human, rat, rabbit and ferret small-intestinal mucin (Fahim *et al.*, 1983, 1987*a*,*b*; Mantle & Thakore, 1988; Mantle *et al.*, 1981, 1984*a*,*b*; Roberton *et al.*, 1989), human and rabbit colonic mucin (Mantle & Thakore, 1988; Roberton *et al.*, 1989), human submaxillary mucin (Roberton *et al.*, 1989) and human and dog tracheobronchial mucin (Ringler *et al.*, 1987, 1988; Gupta & Jentoft, 1989). In all cases, the 65–150 kDa (glyco)proteins were attached to their respective mucin polymers by disulphide bonds and were only released following exhaustive thiol reduction, suggesting that they were an integral component of the native mucin macromolecule. Remarkably, the amino acid profiles of all these disulphide-bound (glyco)proteins were very similar, containing a high proportion of acidic and aliphatic residues compared with that present in the mucin monomers. Although the smaller 70 kDa and 65 kDa components of gastric and tracheobronchial mucins were not glycosylated (Pearson *et al.*, 1981; Ringler *et al.*, 1987, 1988), the larger (118 kDa and 150 kDa) components of intestinal and submaxillary mucins did contain carbohydrate (approx. 50% by weight), with a significant amount of mannose (approx. 9 mol% of the total sugars), suggesting the presence of *N*-linked oligosaccharide chains (Fahim *et al.*, 1983, 1987*a*; Mantle *et al.*, 1984*b*; Roberton *et al.*, 1989).

Although the function of the small disulphide-bound (glyco)proteins has yet to be determined, it has been proposed that they may serve as 'link' components, holding together the mucin monomers in the native polymer (Fahim *et al.*, 1983, 1987*a*,*b*; Mantle *et al.*, 1984*a*,*b*). This hypothesis is based on the observation that exhaustive thiol reduction or extensive proteolytic digestion dissociates the mucin polymer into glycoprotein monomers with the concomitant release or destruction of the disulphide-bound 'link' (glyco)protein (Fahim *et al.*, 1983, 1987*a*,*b*; Mantle & Thakore, 1988; Mantle *et al.*, 1981, 1984*a*,*b*; Pearson *et al.*, 1981; Ringler *et al.*, 1987, 1988; Roberton *et al.*, 1989). However, various pieces of evidence cast doubt on the linkage role of these disulphide-bound components. Recent studies on highly purified intestinal mucins from normal subjects and patients with cystic fibrosis demonstrated that the 118 kDa glycoprotein content of mucin preparations from separate individuals could vary approx. 10-fold (Mantle & Stewart, 1989). In some polymeric mucins, the 118 kDa glycoprotein represented as little as 4% of their protein content (or 1% by weight of the total mucin). Such a variable and sometimes very low amount of 118 kDa glycoprotein is not consistent with this moiety serving as a 'link' component in all native mucin molecules. In addition, Fahim *et al.* (1987*a*) showed that very brief trypsin or Pronase digestion of rat intestinal mucin resulted in the rapid release of 118 kDa glycoprotein despite the fact that, under the experimental conditions used, extensive degradation of the mucin polymer would not be expected to occur. Finally, examination of secreted rat intestinal mucin demonstrated the presence of dimeric 118 kDa glycoprotein not covalently attached to mucin polymers, in contrast with intracellular mucin, which only contained 118 kDa glycoprotein disulphide-bonded to polymeric material (Fahim *et al.*, 1987*b*). In view of the above findings, mucin

Abbreviation used: PAS, periodic acid/Schiff.

depolymerization under mild degradative conditions was examined in more detail in the present studies to establish whether selective release or destruction of the 118 kDa glycoprotein could be achieved without dissociating the native mucin macromolecule. Results indicate that the 118 kDa glycoprotein can be cleaved from rat intestinal mucin without complete depolymerization, strongly suggesting that this disulphide-bound component may not function as a cross-linking agent for all of the mucin monomers.

METHODS

Isolation and purification of mucin

After an overnight fast, male Sprague–Dawley rats (300–400 g) were anaesthetized with urethane, and the entire small intestine from the ligament of Trietz to the ileocaecal junction was removed. Luminal contents were rapidly removed by flushing with cold phosphate-buffered saline (0.1 M-NaCl/0.1 M-Na_2HPO_4/NaH_2PO_4, pH 7.4) containing 0.02% NaN_3 and proteolytic inhibitors (5 mM-Na_2EDTA, 1 mM-phenylmethanesulphonyl fluoride and 10 mM-*N*-ethylmaleimide) and the intestine was then everted. Mucosal scrapings were collected into the same buffer and homogenized. Mucin was purified from mucosal homogenates by equilibrium density-gradient centrifugation in CsCl (twice) followed by gel filtration on Sepharose 2B (Pharmacia Fine Chemicals, Uppsala, Sweden), as described previously (Mantle *et al.*, 1984*b*). High-molecular-mass polymeric mucin was harvested from the void-volume fractions of the column. The purity of mucin preparations was confirmed by SDS/PAGE (showing the absence of non-mucin protein bands), by DNA analysis (< 2 μg of DNA/mg of mucin, as assessed by the method of Hinegardner, 1971) and by g.l.c. [by the method of Chaplin (1982), showing the absence of glucose and uronic acids and only a low content (< 1 mol%) of mannose].

Small-intestinal mucins were also isolated from New Zealand White rabbits (Mantle & Thakore, 1988) and from a normal subject (H35) with no history of gastrointestinal disease (Mantle & Stewart, 1989) as described above for rat intestinal mucin and their purity was similarly confirmed.

H_2O_2 digestion

Purified mucin was dissolved in 0.1 M-NaCl/0.1 M-sodium acetate, pH 5.6, at a concentration of 2 mg/ml. H_2O_2 (Fisher Scientific Co., Fair Lawn, NJ, U.S.A.; 30% solution) was added to the mucin during vortex-mixing (50 μl/ml of mucin solution) and the mixture was then incubated at 37 °C. At various times between 15 and 180 min, portions of the digest were removed, diluted with 10 vol. of distilled water, adjusted to 10 mM with Na_2EDTA, dialysed for 4 h against distilled water and freeze-dried. Dried mucin digests were redissolved in phosphate-buffered saline at 2 mg/ml before analysis. Control digests were incubated in the absence of H_2O_2 for 180 min, but were otherwise treated identically with test samples.

Trypsin digestion

Mucin (2 mg/ml in phosphate-buffered saline, pH 7.4) was digested with bovine pancreatic trypsin (type III; Sigma Chemical Co., St. Louis, MO, U.S.A.) at an enzyme/substrate ratio of 1:1000 for up to 72 h at 37 °C. After digestion, phenylmethanesulphonyl fluoride was added to a final concentration of 1 mM to inhibit the enzyme, and the solution was incubated for 10 min at 37 °C and then dialysed against distilled water for 4 h. Digested mucin samples were stored at −80 °C before analysis. Control digests (for 72 h) were incubated concurrently and processed identically but in the absence of trypsin.

Thiol reduction

Mucin was dissolved at a concentration of 2 mg/ml in 0.18 M-NaCl/0.02 M-sodium barbital buffer, pH 8.6, containing 0.1% SDS. To portions of the solution, 2-mercaptoethanol was added to a final concentration of 5, 10, 50, 100, 200 or 400 mM and the mixtures were incubated for 1 h at room temperature (23 °C). Iodoacetamide was then added to a final concentration of 0.5 M and, after adjusting the pH to 8.5, the solution was incubated overnight at 4 °C in the dark. Reduced and alkylated mucin was dialysed against 0.01% SDS for 4 h at room temperature and stored at −80 °C before analysis.

Analysis of mucin digests

Digested or reduced and alkylated mucin samples (200–300 μg) were applied to Sepharose 2B columns [1.5 cm (diameter) × 45 cm] and eluted by upward flow with 0.2 M-NaCl containing 0.01% SDS at a rate of 5 ml/h. The absorbance of the eluate at 206 nm was monitored continuously by a 2158 Uvicord SD and plotted on a 2210 recorder (LKB-Produkter AB, Bromma, Sweden). The areas of the void-volume peak (fractions 14–18, containing polymeric mucin) and the partially included peak (fractions 19–29, containing monomeric mucin) were determined from the chromatograms, and the distribution of material eluted in each of the peaks was calculated as a percentage of the total recovered from the column to evaluate the degree of mucin depolymerization. Fractions (2 ml) of the column eluate were collected and analysed by slot-blot assays on nitrocellulose sheets. In one assay, blots were stained with periodic acid/Schiff (PAS) reagent (Mantle & Stewart, 1989) to identify those fractions containing mucin (polymer or monomer), whereas in a duplicate assay blots were probed with rabbit anti-(118 kDa glycoprotein) antibody, as described for Western-blot analyses (see below), to identify fractions containing 118 kDa glycoprotein. Blots were scanned on a laser densitometer. The intensity of staining of each band on the blot (as measured by the densitometer) was taken as an indication of the amount of material (mucin or 118 kDa glycoprotein) present in the sample applied to the blot (Mantle & Stewart, 1989).

Mucin samples (containing 350 μg of mucin) were also analysed by SDS/PAGE by the method of Laemmli (1970) under reducing and non-reducing conditions (incubation at 100 °C for 5 min with or without 200 mM-2-mercaptoethanol). Gels were stained with Coomassie Blue. Duplicate gels, to which 50 μg of mucin was applied, were processed for electrophoretic transfer on to nitrocellulose sheets (Western-blot analysis) by the method of Towbin *et al.* (1979). Antigenic bands were detected by the Immun-blot assay using rabbit anti-(118 kDa glycoprotein) antibody (1:1000 dilution) followed by goat anti-(rabbit IgG) antibody conjugated to horseradish peroxidase (1:2000 dilution) and peroxidase substrate (as described by Bio-Rad Laboratories, Richmond, CA, U.S.A.). Gels and Western blots were also scanned on the densitometer (as above).

Reduced and alkylated mucin samples (800 μg) were analysed for their disulphide-bond content (Mantle *et al.*, 1990). In some experiments, reduced and alkylated mucin (2 mg) was chromatographed on a Sepharose 2B column and fractions 14–18 (the void-volume peak), 19–29 (first partially included peak) and 30–36 (second partially included peak) were pooled. After dialysis against distilled water for 4 h, each peak was freeze-dried, reconstituted in 700 μl of distilled water and analysed for mucin by PAS slot-blot (50 μl) for disulphide bonds (as above; 600 μl) and on SDS/PAGE and Western blots (50 μl). Polyacrylamide gels were silver-stained by the Bio-Rad Laboratories procedure.

Cross-reactivity studies

Purified rat, rabbit and human intestinal mucins were subjected to Western-blot analysis, and nitrocellulose sheets were probed with either rabbit anti-(human fibronectin) antibody (Calbiochem, San Diego, CA, U.S.A.) or rabbit anti-(human secretory component) antibody (Sigma Chemical Co.). Antigenic bands were detected as described above by the Immun-blot assay. Controls for these experiments included purified human fibronectin (Boehringer Mannheim, Laval, Quebec, Canada) and human secretory IgA (ICN Immunobiologicals, Lisle, IL, U.S.A.). In addition, Western blots of human fibronectin, human secretory IgA and microvillus membranes from rat mammary tumour cells of the 13762 adenocarcinoma line (kindly provided by Dr. K. Carraway, University of Miami, Miami, FL, U.S.A.) were probed with the rabbit anti-(118 kDa glycoprotein) antibody.

RESULTS AND DISCUSSION

To gain further insight into the role of the putative 'link' glycoprotein of rat intestinal mucin, the present studies were designed to examine mucin breakdown under mild degradative conditions to determine: (*a*) whether release or destruction of the 118 kDa glycoprotein always paralleled dissociation of the macromolecule; or (*b*) whether selective release/destruction of the 118 kDa glycoprotein could be achieved without complete disruption of the mucin polymer. To this end, purified mucin was treated with H_2O_2, trypsin or 2-mercaptoethanol, and release or destruction of the 118 kDa glycoprotein was monitored by SDS/PAGE and Western-blot analysis, and mucin depolymerization was monitored by gel filtration on Sepharose 2B. The latter analyses were carried out in the presence of SDS to eliminate non-covalent interactions and ensure maximum separation of mucin degradation products on the columns.

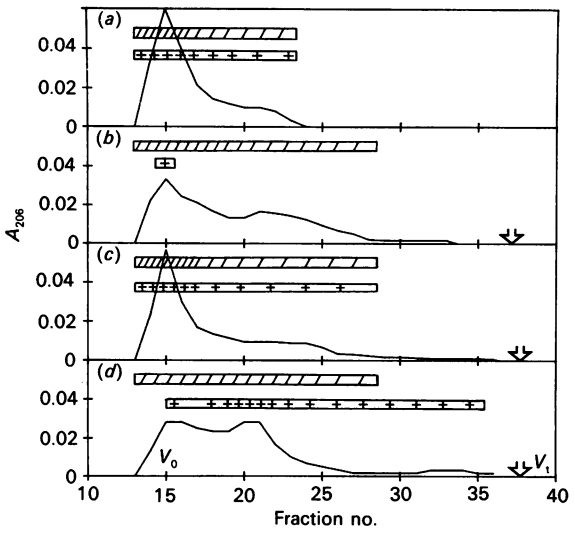

Fig. 1. Sepharose 2B chromatography of rat intestinal mucin before and after H_2O_2 treatment, trypsin digestion or thiol reduction

(*a*) Purified untreated mucin (250 μg); (*b*) mucin after H_2O_2 digestion (1.5% solution) at pH 5.6 and 37 °C for 3 h (300 μg); (*c*) mucin after trypsin digestion (enzyme/substrate ratio of 1:1000) at 37 °C for 72 h (250 μg); (*d*) mucin after reduction with 400 mM-2-mercaptoethanol for 1 h at room temperature and alkylation with iodoacetamide (0.5 M) overnight at 4 °C in the dark (300 μg). Digested or reduced and alkylated mucins were applied to Sepharose 2B columns eluted with 0.2 M-NaCl containing 0.01% SDS, and the A_{206} of the eluate was monitored continuously and plotted on a recorder (——). Fractions (2 ml) were collected and analysed by slot-blot assays on nitrocellulose sheets. In one assay, blots were stained with PAS reagent to identify fractions containing mucin (polymer or monomer) while duplicate blots were probed with rabbit anti-(118 kDa glycoprotein) antibody followed by goat anti-(rabbit IgG) antibody conjugated to horseradish peroxidase and peroxidase substrate to identify fractions containing 118 kDa glycoprotein. V_0 and V_t are the void and total volumes of the column respectively; arrow indicates the salt peak; ▭ indicates PAS-reactive fractions; ▭ indicates fractions reacting with anti-(118 kDa glycoprotein) antibody; spacing of markers (/ and +) in the boxes indicates intensity of staining on the blots.

H_2O_2 digestion

Digestion with H_2O_2 resulted in progressive breakdown of polymeric molecules of rat intestinal mucin into smaller components eluted partially included on a Sepharose 2B column (Fig. 1*b*). Analysis of digests after 15, 30, 45, 60, 90 and 180 min revealed that, at each time point, the void-volume peak became smaller and less PAS-reactive, whereas the partially included peak became larger and more PAS-reactive. From the size of the two peaks, it was calculated that the amount of material eluted in the void volume gradually decreased from 80% at zero time (Fig. 1*a*) to 51% after 180 min (Fig. 1*b*), whereas the amount of material in the partially included peak correspondingly increased from 20% to 49% of the total. Slot-blots probed with the anti-(118 kDa glycoprotein) antibody demonstrated loss of immunoreactive material from the void-volume fractions, but the partially included peak contained no detectable immunoreactivity (Fig. 1*b*).

On SDS/PAGE and Western-blot analysis under non-reducing conditions, no protein-staining bands were observed on the gels and no 'free' 118 kDa glycoprotein or its degradation products were detected on the blots (Fig. 2, tracks 2 and 11). When mucin samples were reduced before electrophoresis, the staining intensity of the 118 kDa glycoprotein on both gels and Western blots became progressively fainter with increasing time of digestion (Fig. 2, tracks 4–9 and tracks 13–18) until, after 180 min, virtually all of the 118 kDa glycoprotein had been destroyed (Fig. 2, tracks 9 and 18). Although no discrete breakdown products of the 118 kDa glycoprotein could be identified by SDS/PAGE, an immunoreactive band of 20–24 kDa was detected on Western blots during the early stages of digestion but, at 180 min, this too was barely detectable. Since the 20–24 kDa band was only observed in reduced samples, it must have been disulphide-bound to large mucin fragments in the digests. From the faint staining intensity of the 20–24 kDa band, it obviously did not contain all of the immunoreactivity originally present in the 118 kDa glycoprotein, suggesting that H_2O_2 destroyed antigenic determinants. Control digestions without H_2O_2 showed none of the above changes on Sepharose 2B gel filtration, SDS/PAGE or Western-blot analysis.

The above results indicate that H_2O_2 treatment of rat intestinal mucin slowly degraded the polymer into smaller components of similar size to mucin monomers released by thiol reduction (since they are eluted in the same position from a Sepharose 2B column) and also progressively destroyed the 118 kDa glycoprotein. Identical results were obtained when human intestinal mucin was digested with H_2O_2 (M. Mantle, unpublished work). Previous studies showed that dilute H_2O_2 cleaves histidine residues in ovarian-cyst mucin, converting them into aspartic acid with the simultaneous scission of the histidyl-peptide bond (Creeth *et al.*, 1983; Cooper *et al.*, 1985). Chemical analyses of the 118 kDa glycoproteins from rat and human intestinal mucins (Fahim *et al.*, 1987*a*; Mantle *et al.*, 1984*b*) have shown that both their peptide moieties contain 3–4 mol% of histidine. Since the peptide represents approx. 50% by weight of the molecule, then (from its amino acid composition) the 118 kDa glycoprotein

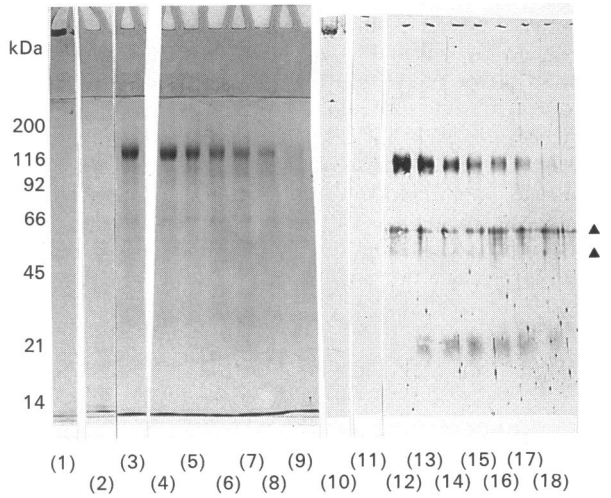

Fig. 2. SDS/PAGE and Western blots of rat intestinal mucin after H_2O_2 digestion

Purified mucin was digested with H_2O_2 (1.5% solution) at pH 5.6 and 37 °C for times between 15 and 180 min. Portions of the digests (350 μg) were subjected to SDS/PAGE on 10% separating gels with 5.7% stacking gels under non-reducing and reducing conditions (100 °C, 5 min, with or without 0.2 M-2-mercaptoethanol). Gels were stained with Coomassie Blue. Duplicate gels, to which 50 μg of mucin was applied, were processed for Western-blot analysis. Blots were treated with anti-(118 kDa glycoprotein) antibody (1:1000 dilution) and antigenic bands were detected by the Immun-blot assay. (1) Control digest incubated without H_2O_2 for 180 min, non-reduced; (2) H_2O_2-digested mucin after 180 min, non-reduced; (3) control digest, reduced; (4)–(9) H_2O_2-digested mucin after 15, 30, 45, 60, 90 and 180 min respectively, reduced; (10)–(18) Western blots of tracks (2)–(10) respectively. Overstaining of blots leads to the appearance of 2-mercaptoethanol artifacts (▲).

could contain on average 9–12 residues of histidine. On the basis of these estimates, it is not unexpected that H_2O_2 treatment resulted in extensive destruction of the 118 kDa glycoprotein, leading to the absence of discernible bands on SDS/polyacrylamide gels, since such small fragments may be lost on dialysis or may migrate at the solvent front. Because digests treated with H_2O_2 for 180 min contained almost no detectable 118 kDa glycoprotein or its 20–24 kDa breakdown product, even though about 50% of the mucin was still eluted in the void volume of the Sepharose 2B column, it would appear that the 118 kDa glycoprotein may be selectively destroyed without complete depolymerization of the macromolecule. These findings are not consistent with the 118 kDa glycoprotein serving as a centrally located 'link' component holding together all the mucin monomers in the native polymer.

Trypsin digestion

Mild trypsin digestion at an enzyme-to-substrate ratio of 1:1000 resulted in very little breakdown of polymeric rat intestinal mucin over the course of 72 h (Fig. 1*c*). Gel-filtration profiles of digests analysed after 4, 8, 16, 24, 48 and 72 h demonstrated only small decreases in the size and PAS reactivity of the void-volume peak and correspondingly small increases in the partially included peak. Immunoreactivity with the anti-(118 kDa glycoprotein) antibody remained largely associated with the void-volume material. After 72 h, approx. 70% of the mucin was still eluted in the high-molecular-mass void-volume fractions of the column (Fig. 1*c*).

SDS/PAGE and Western-blot analyses under non-reducing conditions revealed no protein-staining bands on the gels and no 'free' 118 kDa glycoprotein or its breakdown products on the

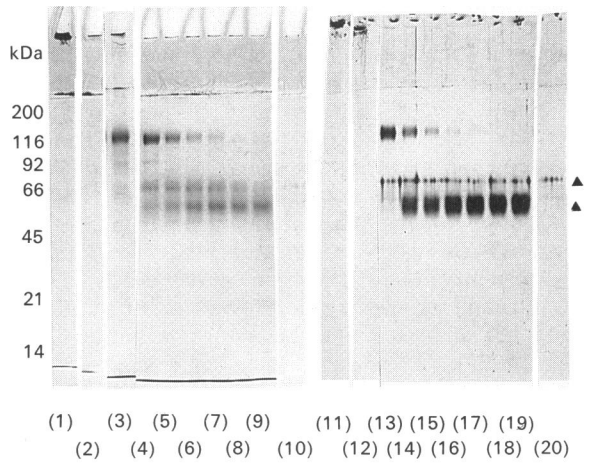

Fig. 3. SDS/PAGE and Western blots of rat intestinal mucin after trypsin digestion

Purified mucin was digested with trypsin (enzyme/substrate ratio 1:1000) at 37 °C for times between 4 and 72 h. Portions of the digests were subjected to SDS/PAGE and Western-blot analysis as described in Fig. 2. (1) Control digest incubated without trypsin for 72 h, non-reduced; (2) trypsin-digested mucin after 72 h, non-reduced; (3) control digest, reduced; (4)–(9) trypsin-digested mucin after 4, 8, 16, 24, 48 and 72 h respectively, reduced; (10) control incubation containing enzyme only; (11)–(20) Western blots of tracks 1–10 respectively. ▲, 2-Mercaptoethanol artifacts.

Table 1. Thiol reduction of rat intestinal mucin

Mucin was reduced in the presence of 0.1% SDS for 1 h at room temperature with 2-mercaptoethanol at the concentrations shown. After alkylation with iodoacetamide (0.5 M) and dialysis against 0.01% SDS, portions were analysed for their disulphide-bond content by the method of Mantle *et al.* (1990). Additional samples were chromatographed on Sepharose 2B columns eluted with 0.2 M-NaCl containing 0.01% SDS, and the A_{206} of the eluate was monitored continuously and plotted on a recorder. The areas of the void-volume peak (fractions 14–18) and the partially included peak (fractions 19–29) were determined from the chromatograms. The distribution of material eluted in each of the peaks is presented as a percentage of the total recovered from the column.

Mercaptoethanol concn. (mM)	Disulphide bonds (nmol/mg)	Area (%)	
		Fractions 14–18	Fractions 19–29
0	60.0	80	20
5	47.3	69	31
10	34.5	63	37
20	31.3	60	40
50	31.0	55	45
100	27.8	55	45
200	22.0	52	48
400	20.8	49	51

blots (Fig. 3, tracks 2 and 12). When mucin samples were examined after reduction, the 118 kDa glycoprotein band on SDS/polyacrylamide gels became progressively fainter with increasing time of digestion and had disappeared by 48 h (Fig. 3, tracks 4–9). Two smaller protein-staining bands of 60–66 kDa and 54–56 kDa appeared on the gels after 4 h of trypsin treatment. The staining intensity of these two bands together accounted for the majority of stain lost from the 118 kDa band, suggesting that both proteins were derived from the 118 kDa

glycoprotein. With prolonged digestion, the larger protein decreased in staining intensity while the smaller protein increased in intensity (Fig. 3, tracks 4–9), consistent with further cleavage of the 60–66 kDa component to yield a 54–56 kDa component. Western blots also demonstrated the disappearance of the 118 kDa glycoprotein (Fig. 3, tracks 14–19) and the appearance of a single broad band of 52–56 kDa, which increased in intensity as the 118 kDa band faded during digestion. Since all of the immunoreactivity lost from the 118 kDa band on Western blots was recovered in the 52–56 kDa band, it appears that trypsin (unlike H_2O_2) did not destroy antigenic determinants. The 60–66 kDa protein seen on SDS/polyacrylamide gels was not detected on Western blots, suggesting that this band represented a region of the 118 kDa glycoprotein that was devoid of antigenic determinants. However, although unlikely, it has yet to be confirmed that the 60–66 kDa protein was not derived from the peptide core of the mucin monomers. In the absence of trypsin, Sepharose 2B, SDS/PAGE and Western-blot profiles were unchanged.

Previous experiments carried out by Fahim *et al.* (1987*a*) indicated that brief digestion of rat intestinal mucin with small amounts of trypsin or Pronase may release intact 118 kDa glycoprotein under conditions which would not be expected to cause extensive degradation of the mucin polymer. The present studies confirm that very low concentrations of trypsin (enzyme/substrate ratio of 1:1000) indeed resulted in little breakdown of the mucin macromolecule. However, release of intact 118 kDa glycoprotein from the mucin complex was not observed, even when lower or higher enzyme concentrations and/or shorter digestion times were used. Replacement of trypsin with Pronase similarly did not selectively release the 118 kDa glycoprotein (M. Mantle, unpublished work). Instead, trypsin split the 118 kDa glycoprotein into two components of 54–56 kDa and 60–66 kDa and then further cleaved the latter, decreasing its size to 54–56 kDa. Despite this proteolytic cleavage, the breakdown products of 118 kDa glycoprotein and the mucin monomers remained as a disulphide-bound high-molecular-mass complex. Trypsin digestion of human intestinal mucin yielded similar results (M. Mantle, unpublished work). These data suggest that there may only be two lysine–arginine cleavage sites in the 118 kDa glycoprotein. One site is centrally located, producing the 54–56 and 60–66 kDa fragments, whereas the other, less sensitive, site is located in the larger fragment, leading to further breakdown into a 54–56 kDa band after longer periods of digestion. Because the 118 kDa glycoprotein (or its degradation products) was not actually released from the mucin complex by trypsin, these experiments could neither confirm nor refute its role as a link region in the mucin macromolecule. Nonetheless, the data indicate that proteolysis of the 118 kDa glycoprotein can occur without dissociation of the mucin polymer and hence emphasize the importance of disulphide bonds in holding together mucin components, even when the 118 kDa glycoprotein is no longer intact.

Thiol reduction

Incubation of purified mucin for 1 h at room temperature with increasing concentrations of 2-mercaptoethanol caused a progressive loss of disulphide bonds (Table 1). At the same time, Sepharose 2B eluates revealed a gradual decrease in the size and PAS reactivity of the void-volume peak and an increase in the partially included peak (Table 1, Fig. 1*d*), consistent with cleavage of the polymer into monomers. A very small peak eluted closer to the total volume of the column was also detected by the absorbance monitor, but this was not PAS-reactive. Slot-blots probed with the anti-(118 kDa glycoprotein) antibody demonstrated the successive loss of immunoreactive material

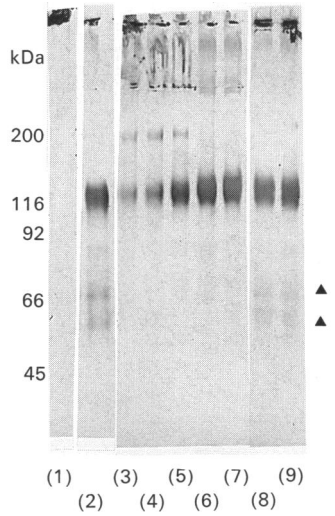

Fig. 4. Western blots of rat intestinal mucin after thiol reduction

Mucin was reduced with different concentrations of 2-mercaptoethanol for 1 h at room temperature and alkylated with iodoacetamide (0.5 M) overnight at 4 °C in the dark. Portions of the samples were subjected to SDS/PAGE on 7% separating gels with 5.7% stacking gels and Western-blot analysis as described in Fig. 2. Western blots of: (1) native untreated mucin, non-reduced; (2) mucin after complete reduction; (3)–(9) mucin after partial reduction with 5 mM-, 10 mM-, 20 mM-, 50 mM-, 100 mM-, 200 mM- and 400 mM-2-mercaptoethanol respectively. ▲, 2-Mercaptoethanol artifacts.

from the void-volume peak and its appearance in both partially included peaks. When mucin was reduced with 400 mM-2-mercaptoethanol, it appeared that the void-volume fractions contained almost no 118 kDa glycoprotein (Fig. 1*d*).

On SDS/PAGE and Western blots, increasing concentrations of 2-mercaptoethanol were seen to release progressively greater amounts of 118 kDa glycoprotein (Fig. 4, tracks 3–9). At the same time, staining in the stacking gel diminished, consistent with more extensive mucin depolymerization at higher 2-mercaptoethanol concentrations. Lower concentrations of reducing agent also gave rise to a faintly staining band of 200 kDa (Fig. 4, tracks 3–5). However, when the concentration increased to 50 mM and above, the 200 kDa band was no longer detectable (Fig. 4, tracks 6–9). In earlier studies, Fahim *et al.* (1987*b*) also observed a 200 kDa component that appeared to be a dimer of the 118 kDa glycoprotein in rat intestinal mucin. Although the origin of the 118 kDa glycoprotein dimer is not yet known, the present experiments suggest that it may be formed by incomplete reduction, possibly as a result of rapid disulphide exchange. By comparison with fully reduced mucin (Fig. 4, track 2), it appeared that all of the 118 kDa glycoprotein had been released by treatment with 200 mM- and 400 mM-2-mercaptoethanol.

Thus increasingly harsher conditions of reduction resulted in progressive breakdown of the mucin polymer into monomers, cleavage of disulphide bonds and release of the 118 kDa glycoprotein. However, after treatment with 400 mM-2-mercaptoethanol, a substantial number of disulphide bonds remained in the mucin preparation, and a large amount of material (apparently devoid of 118 kDa glycoprotein) was eluted in the void volume of a Sepharose 2B column. To establish the location of these disulphide bonds and to confirm the presence/absence of 118 kDa glycoprotein in the Sepharose 2B peaks, mucin was reduced with 400 mM-2-mercaptoethanol for 1 h at room temperature and the void-volume and two partially included peaks from the Sepharose 2B column were harvested.

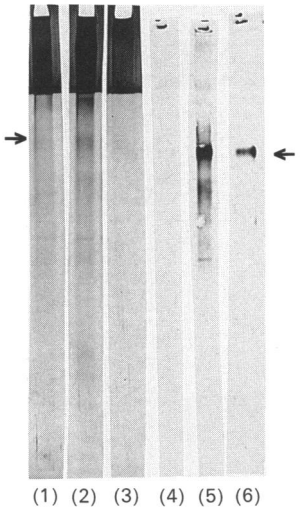

Fig. 5. SDS/PAGE and Western blots of reduced rat intestinal mucin after Sepharose 2B chromatography

Mucin was reduced with 400 mM-2-mercaptoethanol for 1 h at room temperature, alkylated with iodoacetamide (0.5 M) overnight at 4 °C in the dark and then chromatographed on a Sepharose 2B column. The void-volume peak (fractions 14–18), first partially included peak (fractions 19–29) and the second partially included peak (fractions 30–36) were pooled. After dialysis against distilled water, each peak was freeze-dried, reconstituted in 700 μl of distilled water, and 50 μl portions were subjected to SDS/PAGE and Western-blot analysis as described in Fig. 2, except that gels were silver-stained. (1) Void-volume peak (50 μg of mucin); (2) first partially included peak (70 μg of mucin); (3) second partially included peak; (4)–(6) Western blots of tracks (1)–(3) respectively; arrows indicate 118 kDa glycoprotein.

These peaks were designated A, B and C respectively. Slot-blots indicated that peak A (void volume) contained approx. 40% of the PAS-reactive material recovered from the column and had a disulphide-bond content of 15.5 nmol/mg of PAS-reactive material. On SDS/PAGE and Western blots, no 118 kDa glycoprotein was detected in this peak (Fig. 5, tracks 1 and 4). Since many previous studies have shown that complete reduction (using longer times or higher temperatures) totally dissociates the mucin polymer into Sepharose 2B-partially included monomers (Mantle *et al.*, 1981, 1984*b*), and since a significant number of disulphide bonds were present in the incompletely reduced peak A material, it would seem reasonable to conclude that these high-molecular-mass fractions consisted of mucin monomers that were still covalently linked by disulphide bonds. Peak B (first partially included peak) contained the remaining 60% of the PAS reactivity and had a disulphide-bond content of 17.0 nmol/mg of PAS-reactive material. Significant quantities of 118 kDa glycoprotein were present in this peak (Fig. 5, tracks 2 and 5). The number of manipulations required to carry out this experiment frequently resulted in some degradation of the 118 kDa glycoprotein, as seen from the appearance of multiple bands on both gels and blots of peak B. These results indicate that peak B contained both mucin monomers and 118 kDa glycoprotein. Peak C (second included peak) was not PAS-reactive. Owing to the small size of this peak, accurate disulphide-bond analysis was not possible. On SDS/PAGE only very faint traces of 118 kDa glycoprotein were detected in peak C, but on Western blots the band was more clearly visible (Fig. 5, tracks 3 and 6). Thus peak C appeared to contain only 118 kDa glycoprotein. Taking into account the volumes used for electrophoresis and the intensity of the 118 kDa bands on Western blots of peaks B and C, it was estimated that less than 10% of the 118 kDa glycoprotein present in the initial mucin sample was recovered in peak C.

The above data illustrate that the 118 kDa glycoprotein can be separated from mucin with a large molecular mass under conditions of partial reduction. These findings suggest that the 118 kDa glycoprotein may not function as a 'link' component for all of the mucin monomers in the native polymer.

Cross-reactivity studies

If the 118 kDa glycoprotein does not function as a 'link' component in intestinal mucins, then what purpose does it serve? In an attempt to address this question, cross-reactivity experiments were carried out. Initially, Western blots of microvillus membranes from rat mammary tumour cells were probed with the anti-(118 kDa glycoprotein) antibody. These membranes contain ASGP-2 (for ascites sialoglycoprotein-2) which has a similar molecular mass (120 kDa) and similar chemical composition to the 118 kDa glycoprotein and is involved in the intracellular transport and membrane attachment of large, extracellular, mucin-like glycoproteins (Hull *et al.*, 1990; Sheng *et al.*, 1990). However, the anti-(118 kDa glycoprotein) antibody did not react with any component in these membrane preparations (results not shown).

In a second series of experiments, Western blots of purified rat, rabbit and human intestinal mucins were probed with anti-(human secretory component) antibody and, conversely, blots of human secretory IgA were probed with the anti-(118 kDa glycoprotein) antibody. Secretory IgA depends on secretory component for intracellular transport and secretion into the gut lumen (Brandtzaeg, 1984; Tomasi, 1989). Mature membrane-bound secretory component (120 kDa) and its secreted portion (approx. 83 kDa) are glycoproteins with amino acid profiles resembling that of the 118 kDa glycoprotein (Eiffert *et al.*, 1984; Mostov *et al.*, 1984). However, secretory component and the 118 kDa glycoprotein are immunologically distinct since the anti-(human secretory component) antibody did not react with rat, rabbit or human 118 kDa glycoprotein and the anti-(118 kDa glycoprotein) antibody did not recognize any component of secretory IgA (results not shown).

Finally, Western blots of the three intestinal mucins were probed with anti-(human fibronectin) antibody, and blots of human fibronectin were probed with the anti-(118 kDa glycoprotein) antibody. It has been proposed that the 118 kDa glycoprotein may be a proteolytically degraded fragment of fibronectin present in mucous secretions (Okazaki *et al.*, 1990). However, cross-reactivity studies did not support this hypothesis, since the anti-(118 kDa glycoprotein) antibody did not react with purified fibronectin and anti-(fibronectin) antibody failed to recognize rat, rabbit or human 118 kDa glycoprotein. It seems likely that the anti-(fibronectin) antibody used by Okazaki *et al.* (1990) recognized a similar short peptide or oligosaccharide present in both the 118 kDa glycoprotein and fibronectin, but the remainder of the molecules (recognized by the antibodies used in the present experiments) differ significantly. In all of the above studies, the antibodies used reacted appropriately with their own respective antigens (results not shown).

Cross-reactivity studies therefore demonstrated that the 118 kDa glycoproteins from rat, rabbit and human intestinal mucins share common antigenic determinants that are not found in other glycoproteins associated with or present in mucous secretions. Although these studies do not preclude the possibility that the 118 kDa glycoprotein may play a role in mucin packaging/transport, secretion or adhesion to epithelial-cell surfaces, the experiments failed to provide immediate insight into alternative functions for the molecule.

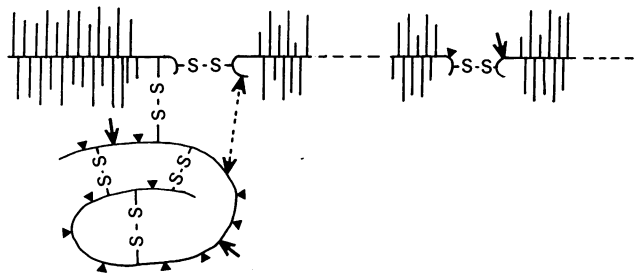

Fig. 6. Possible models for the attachment of the 118 kDa glycoprotein to rat intestinal mucin monomers

Solid arrows indicate the sites of cleavage by trypsin; ▲ indicates H_2O_2-cleavage sites; S-S are disulphide bonds; dashed arrow indicates that the 118 kDa glycoprotein may be attached to more than one monomer by disulphide bonds.

Model

Since it is not yet known whether all polymeric molecules in a given preparation of rat intestinal mucin contain a 118 kDa glycoprotein and, in those that do, what the ratio of 118 kDa glycoprotein to mucin monomers is, it is not yet possible to put together a complete structural model of the mucin. However, on the basis of the experimental findings reported herein, it seems likely that mucin monomers polymerize by disulphide bonds between their peptide cores, whereas the 118 kDa glycoprotein is attached to one (or just a few) of the monomers, as illustrated schematically in Fig. 6. In the proposed model, H_2O_2 would cleave the 118 kDa glycoprotein into small components without completely dissociating the mucin polymer. Scission of some histidine residues in the peptide core of the mucin monomers would account for the partial depolymerization observed in the present experiments. Similarly, limited thiol reduction may release the 118 kDa glycoprotein before separation of all of the mucin monomers. In addition, trypsinolysis of the 118 kDa glycoprotein could occur without breaking down the polymer. Although trypsin-sensitive sites may be present in the peptide core of the mucin monomers, these may be relatively less accessible to the enzyme because of the numerous oligosaccharide chains. Hence, harsher digestion conditions than those used in the present experiments would be required to cleave these sites and depolymerize the mucin. As yet, it is not clear whether the Sepharose 2B void-volume fractions generated after partial H_2O_2 digestion, trypsinolysis or reduction contain mucin with a full complement of monomers. It is possible that the material is composed of only a few monomers linked in a structure still large enough to be excluded by the Sepharose matrix. Further studies are necessary to clarify this issue. Finally, the proposed model would allow for variability in the addition of 118 kDa glycoprotein to the polymeric mucin complex. This would account for previous observations on human intestinal mucin, showing that the 118 kDa glycoprotein content of individual preparations, which are still polymeric, varies and can be very low (Mantle & Stewart, 1989), suggesting that not all molecules contain the 118 kDa glycoprotein. Therefore, although the present studies do not definitively eliminate the possibility that the 118 kDa glycoprotein may function as a 'link' component for some mucin monomers, they nonetheless clearly demonstrate that it is not required for the bridging of all monomers. Further studies are now required to elucidate where the 118 kDa glycoprotein is attached in the mucin complex and what function(s) it serves.

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