Biochemical characterization of a rat oncofetal colonic antigen defined by a monoclonal antibody raised against gastric surface epithelium

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The 660 epitope was defined by a monoclonal antibody raised against rat gastric surface epithelium scrapings. This epitope, a marker of goblet cell differentiation, shows oncofetal behaviour in the colonic mucosa. We found that it co-purified with gastric mucin glycoproteins. We isolated rat gastric mucus glycoproteins using standard techniques: gastric scrapings in PBS were submitted to isopycnic density gradient centrifugation in CsCl in the presence of proteinase inhibitors. Fractions of relative density 1.4–1.45 with a high neutral sugar/protein ratio were chromatographed on an Ultrogel A4 column. According to the usual criteria, the high-molecular mass glycoproteins recovered in the excluded volume were purified mucins; when stained with periodic acid/Schiff reagent, they showed little migration on 4–15% gradient gel acrylamide electrophoresis. Serine+threonine+proline residues accounted for 35% of the total amino acids; the carbohydrate composition consisted of galactose, fucose, *N*-acetylgalactosamine and *N*-acetylglucosamine. These mucus glycoproteins carried the 660 epitope. After disulphide bond reduction, the remaining high-molecular-mass subunits were retained by the Ultrogel A4 column; amino acid and saccharide compositions were generally similar to those of the unreduced fraction. Trypsin digestion of the 660 epitope

glycoprotein carrier did not modify its chromatographic and electrophoretic patterns, nor its chemical composition. The 660 epitope was still present after these treatments. However, trypsin digestion of subunits gave rise to smaller components that were retained by an Ultrogel A4 column. The saccharide composition of these fragments was unchanged, but the proportion of serine+threonine+proline residues rose to 46% of the total. These digested subunits had lost nearly all reactivity with monoclonal antibody 660. Our results fit well with the macromolecular model of Carlstedt, Lindgren and Sheehan [(1983) *Biochem. J.* 213, 427–435]: mucin glycoproteins are homopolymers of subunits assembled end-to-end via disulphide bonds into very large linear macromolecules. After disulphide bond reduction, proteolytic attack sites are uncovered and trypsin digestion results in glycopeptides bearing the typical oligosaccharidic units and with enhanced amounts of serine, threonine and proline, the characteristic amino acids of this hyperglycosylated region of the peptide core. These digested subunits have lost virtually all 660 epitope reactivity. We thus show that the 660 epitope, a determinant of a mucin molecule, is probably associated with the peptide core of the glycoprotein.

INTRODUCTION

In the gut, the mucus forms a continuous layer that covers the epithelium and protects it from endogenous and exogenous substances. The mucus is a viscoelastic gel that is synthesized and secreted by specialized mucous cells. Mucus glycoproteins are the most important structural components of the mucus, and are responsible for the rheological properties of the gel (Allen et al., 1984). Mucus glycoproteins are large polydispersed molecules containing 70–90% (by wt.) carbohydrate, occurring as oligosaccharide units. *N*-Acetylgalactosamine residues attach the sugar chains to the central protein core via O-glycosidic linkages to serine or threonine residues (Allen, 1981). Another characteristic feature of these glycoproteins is their oligomeric structure, which is maintained by disulphide bonds (Snary et al., 1970; Pearson et al., 1980; Mantle et al., 1981; Carlstedt et al., 1983).

A number of tumour-associated and differentiation antigens are expressed on the carbohydrate chains of mucins (Feizi et al., 1984), whereas others are associated with the peptide core (Bara et al., 1991). In a previous immunohistological study (Decaens et al., 1988), we characterized a new oncofetal antigen. Defined by a monoclonal antibody called 660 (MAb 660), this epitope is expressed in 100% of chemically induced rat colonic carcinomas. Moreover, it is a marker of early carcinogenesis, being expressed

by precancerous colonic mucosa long before tumour appearance. It is present in intestinal and colonic goblet cells of rat fetuses, but is absent from normal adult colonic mucosa. Previous experiments seemed to indicate that the epitope recognized by MAb 660 might be associated with a mucin-type glycoprotein.

The aim of the present work was to show that the 660 epitope is carried by rat gastric mucus glycoproteins. We also attempted to define the peptide or saccharide nature of this epitope.

MATERIALS AND METHODS

Mucin purification

Stomachs of adult female Wistar rats were scraped at 4 °C in PBS containing proteinase inhibitors (1 mM phenylmethanesulphonyl fluoride, 5 mM *N*-ethylmaleimide, 0.1 mM di-isopropyl fluorophosphate, 5 mM EDTA and 0.02% Na₂S₂O₃). After 12 h of stirring at 4 °C, the supernatant was dialysed against distilled water, concentrated and submitted to isopycnic density gradient centrifugation with CsCl in the presence of proteinase inhibitors at 4 °C for 66 h using an SW 41 TI rotor at 150 000 *g* in a Beckman ultracentrifuge. The starting relative density (*d*) was 1.42. Fractions with relative densities ranging from 1.40 to 1.45 and a high neutral sugar/protein ratio (determined by the Bio-Rad protein assay and orcinol sulphuric dosage for assay of

Abbreviations used: MAb, monoclonal antibody; PAS, periodic acid/Schiff reagent; Lotus-PO, *Lotus tetragonolobus* labelled with peroxidase.

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neutral sugars) were pooled. These mucin fractions, which reacted with MAb 660 (as determined by dot-blots on nitrocellulose), were dialysed and digested with DNAase II and RNAase A (Sigma) in 0.1 M sodium phosphate, 0.01 M MgCl_2 , 0.02 % NaN_3 , pH 7.4, and proteinase inhibitors for 24 h at 20 °C. They were then chromatographed on Ultrogel A4. Proteins and carbohydrates were estimated by dot-blots of eluted fractions and staining with Coomassie Blue and periodic acid/Schiff reagent (PAS). The high-molecular-mass mucins that eluted in the excluded volume and that contained immunoreactivity towards the 660 epitope were dialysed against distilled water and stored at -20 °C.

Disulphide bond reduction

Purified mucins were treated with 0.4 M β -mercaptoethanol for 5 h at 37 °C in the presence of proteinase inhibitors. Then, 1 M iodoacetamide was added and incubation was carried out overnight at 4 °C in the dark; the fraction was then dialysed and chromatographed on Ultrogel A4. Aliquots of eluted fractions were blotted on nitrocellulose and stained with PAS, *Lotus tetragonolobus* labelled with peroxidase (Lotus-PO) and MAb 660. As a control, an aliquot was processed as described, but without β -mercaptoethanol.

Trypsin digestion

Trypsin type XI (from bovine pancreas, diphenylcarbamyl chloride-treated; Sigma) was added to the mucin (enzyme/substrate ratio of 1:10) in 0.1 M Tris buffer, pH 8, for 4 h at 37 °C, followed by dialysis and chromatography on Ultrogel A4. Staining with PAS, Lotus-PO and MAb 660 was carried out on blotted aliquots of column fractions. As a control, an aliquot was processed as described, but without trypsin.

Dot-blots

Mucin fractions (1–3 μl , containing 20–50 ng of protein), were blotted on to a nitrocellulose sheet which was then saturated with 2 % BSA in PBS for 2 h. Incubation was carried out overnight at 4 °C with MAb-660-containing ascitic fluid. After rinsing in 0.5 % Tween/PBS, anti-mouse IgG(H + L) alkaline phosphatase-conjugated antibodies (Promega) were added for 1 h and, after rinsing, detection was performed with Nitroblue Tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as substrates in 100 mM Tris/HCl buffer, pH 9.5. Lotus-PO was incubated overnight and, after rinsing, peroxidase was revealed using 0.01 % H_2O_2 and chloronaphthol as chromogen. The dot stainings were then scanned on a Chromoscan 3 apparatus (Joyce-Loebl).

Acrylamide electrophoresis and Western blotting

Electrophoresis was performed in 4–15 % acrylamide gradient gels in 0.025 M Tris/0.192 M glycine buffer, pH 8.4; gels were then stained with Coomassie Blue, PAS and silver stain (Bio-Rad kit) or electrophoretically transferred to poly(vinylidene difluoride) (PVDF) Problott sheet (Applied Biosystems) or 0.45 μm nitrocellulose at 100 mA for 12 h in the same buffer. The sheets were then incubated with MAb 660 or an MAb against blood group A (Bara et al., 1986), and then processed as for the dot-blots.

Ultrogel A4 chromatography

Analytical gel filtration was run on a 48 cm $\times$ 1 cm column of Ultrogel A4, eluted in 0.02 % NaN_3 /PBS. Fractions of 1 ml were collected and aliquots were analysed by dot-blots with Coomassie Blue, PAS and immunochemical detection.

Amino acid composition

Purified mucins were hydrolysed with 6 M HCl in an N_2 atmosphere at 105 °C for 24 h, then derivatized with phenyl isothiocyanate. Amino acids were analysed by reverse-phase h.p.l.c. on a 5 μm Ultrasphere ODS C-18 column (Beckman) using a modified Gupta technique (Gupta and Jentoft, 1989) in order to distinguish amino acids and hexosamines.

Monosaccharide composition

Purified mucins were hydrolysed in methanolic 0.75 M HCl and derivatized according to the method of Chaplin (1982), and then analysed on a 0.530 mm $\times$ 30 m HP-1 column (Hewlett-Packard) on a Hewlett-Packard gas chromatograph. The internal standard used was *meso*-inositol.

MAb 660

As previously described (Decaens et al., 1988), MAb 660 was prepared against high-molecular-mass glycoproteins isolated from the rat gastric surface epithelium. Briefly, following nuclease digestion, the supernatant from the gastric scrapings was chromatographed on Sepharose CL-4B. The void volume was then rechromatographed on Ultrogel A4, and the excluded fraction of this second chromatography was used for immunization. After immunohistological screening and cloning, MAb 660 reacted only with the mucous cells of the surface gastric epithelium in normal adult rat gut. Purified 660 IgG that was immobilized on a Sepharose 4B column retained the 660 reactivity present in the high-molecular-mass components of rat gastric scrapings, as indicated by dot-blots and Western blotting.

Lotus-PO

Fucose-binding protein from the *Lotus tetragonolobus* (Miles) was bound to horseradish peroxidase (Sigma, type IV) using glutaraldehyde (Avrameas and Ternynck, 1969). Labelled lectin was used for testing fucose terminal reactivity in aliquots of mucin fractions. L-Fucose at 0.2 M completely inhibited staining.

RESULTS

Mucin purification

All purification steps were followed by protein and neutral sugar assays, as well as by testing of reactivity on dot-blots with MAb 660 and Lotus-PO.

On centrifugation in a CsCl gradient, the gastric scrapings gave a peak of d 1.42 that contained most of the carbohydrates; this is a standard pattern in mucin purification. In dot-blots, the distribution of reactivity with MAb 660 was similar to that of neutral sugars (Figure 1). The high-density fraction ($d > 1.5$) contained nucleic acids (as indicated by absorbance at 260 and 280 nm; results not shown). The low-density fraction ($d < 1.35$) contained a major proportion of the proteins.

The nucleic acids were digested with nucleases, and removal of contaminating proteins was carried out by filtration on an Ultrogel A4 column (Figure 2). The excluded volume contained

Figure 1 CsCl density gradient centrifugation of rat gastric scrapings in PBS/proteinase inhibitors

For details, see the Materials and methods section. Curve A, relative density measured by weighting (○); curve B, neutral sugars (●). The horizontal bar indicates epitope-660-positive fractions, as tested by dot-blots.

Figure 2 Ultrogel A4 chromatography of the d 1.42 fraction

Scan profiles of 1 µl portions of eluted fractions blotted on nitrocellulose (integrated intensity in arbitrary units). ×, PAS staining; ●, reactivity with MAb 660; ▲, Coomassie Blue staining. Abbreviations: V_0 , excluded volume; V_t , total volume; T, thyroglobulin elution volume; BSA, bovine serum albumin elution volume.

> 60% of the carbohydrates, but accounted for < 5% of total proteins eluted on the column. This fraction showed immuno-reactivity with MAb 660; the neutral sugar/protein ratio determined by chemical assays was 3.5. The major fraction of the contaminating proteins (non-reactive with MAb 660) was recovered in the included volume.

Figure 3 Electrophoresis and Western blotting of purified mucin after β -mercaptoethanol reduction and trypsin digestion

After Ultrogel A4 chromatography of treated mucin, the eluted fractions reacting with PAS and/or MAb 660 on dot-blots were pooled, dialysed, concentrated and run on 4–15% acrylamide gels. Gels were stained with PAS or electrophoretically transferred, then incubated with MAb 660 or a MAb with anti-(blood group A) specificity. Lanes a, control purified mucin; lanes b, β -mercaptoethanol-treated mucin; lanes c, trypsin-digested mucin after previous reduction; lanes d, trypsin-digested mucin.

Table 1 Amino acid composition

Residue	Composition (residues/100 amino acids)			
	Purified mucin	Purified mucin + trypsin	Reduced mucin	Reduced mucin + trypsin
Asp	1.7	6.7	1.4	1.5
Glu	4.0	8.0	2.8	4.2
Ser	13.6	22.0	16.8	19.0
Gly	9.0	10.6	13.4	10.6
His	1.6	5.6	6.2	4.1
Arg	9.2	7.0	5.2	3.1
Thr	16.0	14.0	19.0	22.5
Ala	11.0	6.5	7.7	10.7
Pro	6.0	4.9	5.2	4.8
Tyr	1.5	1.3	3.0	2.6
Val	6.4	5.7	9.0	3.4
Met	3.0	0.77	2.4	3.4
Cys	3.2	1.8	1.4	0.26
Ile	2.9	2.2	2.3	2.0
Leu	9.5	3.2	3.1	1.85
Phe	2.0	2.1	1.5	1.65
Lys	3.5	1.0	3.4	2.2
Ser + Thr + Pro	35.6	40.9	41.0	46.3
GlcNAc/GalNAc ratio	2.3	1.85	1.95	1.8

Upon electrophoresis, this purified carbohydrate fraction barely entered the acrylamide gel. PAS staining showed a band at the top of the gel (Figure 3, lanes a). Silver staining was identical to PAS, and Coomassie Blue staining was very faint (results not shown). No other proteins or glycoproteins were stained, even with sensitive silver staining, which indicates that the isolated mucin was pure. The high-molecular-mass component obtained reacted with MAb 660 (Figure 3, lanes a).

Table 1 shows the amino acid composition of the isolated mucin. It can be seen that serine, threonine and proline residues accounted for 35.6% of the total amino acids. The *N*-acetylglucosamine/*N*-acetylgalactosamine ratio was 2.5 as determined by carbohydrate g.l.c. analysis, and 2.3 as determined by amino acid h.p.l.c. analysis. The monosaccharide composition of the

Table 2 Monosaccharide composition

The data are expressed as molar ratios relative to the amount of *N*-acetylgalactosamine

	Composition			
	Purified mucin	Mucin + trypsin	Reduced mucin	Reduced mucin + trypsin
<i>N</i> -Acetylgalactosamine	1.00	1.00	1.00	1.00
<i>N</i> -Acetylglucosamine	2.50	2.35	2.35	1.90
Galactose	1.95	1.95	1.85	1.67
Fucose	1.15	1.10	1.08	1.12
Sialic acid	0.11	0.11	0.10	0.10
Mannose	0.22	0.26	0.20	0.07

mucin fraction (Table 2) was characterized by large amounts of galactose, fucose, *N*-acetylgalactosamine and *N*-acetylglucosamine, with trace amounts of sialic acid and mannose.

The high-molecular-mass glycoproteins obtained after purification showed amino acid and monosaccharide compositions and an electrophoretic pattern typical of mucin glycoproteins. Since reactivity towards MAb 660 was associated with this fraction, the 660 epitope can be considered as a mucin determinant.

The purified mucin fraction accounted for 0.2% of proteins and 15–20% of neutral sugars present in the initial scrapes. The 660 epitope was purified at least 50-fold, as estimated by dot-blots of aliquots of all purification steps, tested at increasing dilutions of MAb 660.

Disulphide bond reduction

The occurrence of subunits linked by disulphide bonds was tested by β -mercaptoethanol treatment of the purified mucin fraction, followed, after dialysis, by filtration on Ultrogel A4. The elution profile showed that the PAS-positive material was included in the column and eluted near the thyroglobulin elution volume. This indicates a continuing high molecular mass for the subunits, which also reacted with MAb 660 and Lotus-PO, as shown by dot-blot scanning of Ultrogel A4 fractions (Figure 4b).

Acrylamide electrophoresis of the subunits gave a broad band from the top of the gel to regions corresponding to 300 kDa when stained with PAS or an MAb against blood group A (Figure 3, lanes b) and silver staining (results not shown). When stained with MAb 660 after transfer, the band had migrated to 700 kDa (Figure 3, lanes b). No other band was seen, even with silver staining.

The amino acid composition gave a serine + threonine + proline residue content of 41% of the total residues. The monosaccharide composition was similar to that of the non-reduced fraction. The *N*-acetylglucosamine/*N*-acetylgalactosamine ratio was 2.35 as obtained by g.l.c., and 1.95 as obtained by h.p.l.c.

Reduction of purified mucin led exclusively to an Ultrogel A4-included subunit fraction containing all PAS staining and MAb 660 reactivity (Figure 4b). We verified that the eluted fractions corresponding to the void volume and fractions corresponding to the total volume were devoid of mucin-like components (no PAS or silver staining or MAb 660 reactivity after electrophoresis of these two concentrated fractions).

Figure 4 Ultrogel A4 chromatography of purified mucin after β -mercaptoethanol reduction and trypsin digestion

Portions of 2–3 μ l of each fraction were blotted on nitrocellulose and treated with PAS and MAb 660. Scan profiles show reactivity in arbitrary units of integrated intensity. ▲, PAS staining; ●, reactivity with MAb 660; ○, reactivity with Lotus-PO. Abbreviations: V_0 , excluded volume; V_t , total volume; T, thyroglobulin elution volume. (a) Control purified mucin (30 μ g of mucin protein and 105 μ g of neutral sugar); (b) mucin after reduction with 0.4 M β -mercaptoethanol for 5 h at 37 °C and alkylation with 1 M iodoacetamide overnight at 4 °C in the dark (30 μ g of mucin protein); (c) mucin after trypsin digestion at 37 °C for 4 h (3 μ g of enzyme/30 μ g of mucin protein); (d) mucin first reduced by β -mercaptoethanol and then digested by trypsin (30 μ g of mucin protein).

Trypsin digestion of purified mucin with and without previous reduction

Trypsin treatment of purified mucin without previous reduction did not modify the elution profile on Ultrogel A4 (Figure 4c); most of the PAS-positive material was present in the void volume, as was MAb 660 and Lotus-PO reactivity. The acrylamide electrophoretic pattern was similar to that obtained with untreated mucin: a PAS-positive band with MAb 660 reactivity and blood group A specificity barely entered the gel (Figure 3, lanes d). The amino acid composition of trypsin-treated mucin gave a serine + threonine + proline content of 40.9% of total residues; the monosaccharide composition was unchanged com-

pared with the untreated mucin (Table 2). The *N*-acetylglucosamine/*N*-acetylgalactosamine ratio was 2.35 (1.85 by g.l.c.).

Trypsin digestion of previously reduced purified mucin led to a PAS-positive fraction being included on an Ultrogel A4 column with very faint MAb 660 reactivity (Figure 4d). Upon electrophoresis, a trailing band from the deposit to 300 kDa was stained for carbohydrates. Virtually no reaction was observed with MAb 660 after transfer, but blood group A reactivity was unchanged (Figure 3, lanes c). The monosaccharide composition was similar to that of untreated mucin, with an *N*-acetylglucosamine/*N*-acetylgalactosamine ratio of 1.9 (1.8 by h.p.l.c.). However, the mannose content decreased. Serine + threonine + proline residues accounted for 46.3 % of total amino acid residues.

DISCUSSION

We have described the purification of a new oncofetal antigen associated with a mucin-type glycoprotein. The mucin glycoproteins of gastric scrapings were purified according to their properties by isopycnic density gradient centrifugation and gel chromatography (Carlstedt and Sheehan, 1984). We obtained carbohydrate-rich high-molecular-mass components with a relative density of 1.42 which were excluded from the Ultrogel A4 column. On acrylamide electrophoresis, a band showing very low migration was stained with PAS and silver, but not with Coomassie Blue.

Serine + threonine + proline residues accounted for 35 % of total amino acids; in addition, the four characteristic saccharides, *N*-acetylgalactosamine, *N*-acetylglucosamine, galactose and fucose, were found (Neutra and Forstner, 1987). In addition to these residues, indicative of the presence of O-linked saccharides, mannose residues were detected which indicated the presence of N-linked oligosaccharides; this has been described previously in the rat (Dekker et al., 1989b).

We purified mucin glycoproteins from rat gastric surface epithelium according to biochemical criteria. All immuno-reactivity with MAb 660 was associated with these high-molecular-mass glycoproteins: we showed that 660 reactivity was linked with mucin glycoproteins during CsCl gradient and Ultrogel A4 chromatography. Thus the 660 epitope can be considered to be a mucin determinant.

Dekker et al. (1991) showed that the gastric mucins they isolated from rat fundus were sulphated. By histochemical means, we have shown that our starting material was scraped from the most superficial surface epithelial cells, containing largely neutral mucins (Sheehan and Jervis, 1976; Spicer et al., 1978); MAb 660 stained only these upper mucous cells. For these reasons we did not carry out sulphate analyses, as sulphate was unlikely to be important in recognition of the 660 epitope.

Dekker and Strous (1990) studied rat gastric mucin biosynthesis by pulse-chase experiments with radiolabelled amino acids, and were able to detect peptide precursors of about 300 kDa. However, we could not demonstrate these precursor molecules. One possible explanation is that CsCl ultracentrifugation has eliminated non-glycosylated material; another explanation could be that MAb 660 lacks reactivity against the peptide precursors for conformational reasons, as is frequently observed with deglycosylated mucins that are not recognized by antibodies raised against native mucins. Labelling and pulse-chase experiments would be very useful to study the appearance of 660 reactivity during mucin biosynthesis.

The reduction of disulphide bonds in purified mucin gave rise to subunits; although included in the column, they eluted near

the thyroglobulin used for calibration, which indicated a molecular mass of about 700 kDa. This molecular mass is only approximate, since there is extensive heterogeneity of carbohydrate chains in mucins (Devine and McKenzie, 1992), which is confirmed by the fact that subunit PAS staining gave a broad smearing band from the site of deposit to 300 kDa. Since reduction was performed in the presence of proteinase inhibitors, we can conclude that, in native mucin, subunits are linked by disulphide bonds and are not proteolysis fragments. The subunits have amino acid and saccharide compositions similar to those of the unreduced mucin. The reduced mucin amino acid composition is very similar to that described by Dekker et al. (1989a), who found that serine, threonine, proline, alanine and glycine accounted for 56.9 % of total amino acid residues (compared with 62 % for the subunits we isolated). However, we found a higher number of threonine residues (19 versus 15.7) but a lower number of proline residues (5.2 versus 8.9). Likewise, the proportions of aspartic acid and glutamic acid in our fractions were lower. The *N*-acetylglucosamine/*N*-acetylgalactosamine ratio averaged 2.1 (as determined after carbohydrate and amino acid analyses), with only slight variations caused by the different treatments. Dekker et al. (1991) obtained a higher value of 3.03. These discrepancies might indicate that the glycoproteins are not exactly the same: our starting material was from fundus and antrum scrapings, whereas that of Dekker was from fundus only. The 660 epitope carriers might be more heterogeneous, particularly with regard to the carbohydrate chains.

Subunits linked by disulphide bridges are a very common feature of mucins isolated from mucous gels (Carlstedt et al., 1985). The polymeric structure would permit further intermolecular interactions necessary for gel formation *in vivo*. Two different structural models have been proposed to account for the mucin polymer structure. On the one hand, Allen (1981) described, for a gastric mucin structure, a polymer with four glycopeptide subunits connected to a link-peptide by disulphide bridges; this was called the 'windmill model'. The link-peptide was found to be present in rat goblet cell mucin (Fahim et al., 1987) and porcine gastric mucin (Allen, 1981). On the other hand, Carlstedt and Sheehan (1984) suggested that subunits are linked together end-to-end via S-S bonds, giving rise to a long, partly coiled thread. In our experiments, no component except the subunits were detected on Ultrogel A4 chromatography after reduction (based on absorption at 280 nm, PAS staining and MAb 660 and Lotus-PO reactivity). In the same way, only subunits were silver-stained in acrylamide electrophoresis; these observations rule out the presence of a link-peptide in rat gastric mucin. Our results are in agreement with those of Dekker et al. (1991), who showed that the oligomeric rat gastric mucin consists exclusively of disulphide-linked mucin-type monomers, without other proteins. Recently, Slomiany et al. (1991) compared the 118 kDa 'link-peptide' of human and rat intestinal mucins, and a 118 kDa fragment of fibronectin, and concluded that they were identical. Fibronectin interacts covalently with mucin and can split off according to the techniques used for purification.

In Western blots, MAb 660 reacted with the subunits; migration showed a trailing band from the deposit to 700 kDa (according to calibrating proteins). Reduction did not suppress 660 reactivity, indicating that the 660 epitope is not a conformational one.

Digestion with trypsin seemed to have no effect on the purified mucin using our detection methods. We obtained the same elution profile and electrophoretic pattern, the same reactivity with PAS and MAb 660, and the same amino acid and carbohydrate composition. Mucins can contain two distinct peptide portions, one bearing a large number of oligosaccharide chains,

called T domains, and therefore protected from proteolytic attack; and a second, called the 'naked region', which has few oligosaccharidic chains and is more susceptible to enzymes. Our results suggest that we are dealing with the naked region of the rat gastric mucin peptide core which is also protected from proteolysis by trypsin. However, we cannot rule out the possibility that some proteolysis is giving rise to still large components, which are eluted in the excluded volume of Ultrogel A4 and have poor electrophoretic migration. Whatever the case may be, trypsin digestion had no effect on the 660 epitope carried by the mucin.

In contrast, trypsin digestion of previously reduced mucin gave rise to smaller components that were included in the Ultrogel A4 column and migrated from the deposit to about 300 kDa in electrophoresis and were stained by PAS. These components had the same monosaccharide composition as purified mucin, except for a decrease in the mannose content, perhaps associated with small fragments of the subunits that are susceptible to trypsin proteolysis. However, the content of serine + threonine + proline increased to 46 % of the total residues (cf. 35 % for native mucin). These results agree well with the macromolecular architecture described for the mucins (Carlstedt et al., 1985): after fractionation and depolymerization of mucin by β -mercaptoethanol, trypsin gained access to its proteolytic sites on the naked region of the peptide core, giving rise to subunits with an enriched content of serine + threonine + proline residues, amino acids that are characteristic of the highly glycosylated region of the peptide core. A large decrease in the cysteine content was observed; hydrolysis was carried out in the absence of an internal standard, and possibly the trypsin-treated reduced mucin had lost more cysteine residues. This residue is prone to destruction during acid hydrolysis and phenyl isothiocyanate derivatization, so the variations observed in cysteine ratios could reflect this property. The saccharide chain composition remained unchanged.

The digested subunits had lost most of their reactivity towards MAb 660, whereas blood group A reactivity was unchanged; these results are in favour of the 660 epitope having a peptide nature, confirming previous experiments on a less purified gastric mucin (Decaens et al., 1988).

Numerous tumour-associated antigens described in human and animal mucins are present on the carbohydrate chains, with most of them being blood group antigens (Feizi, 1985); others may be associated with the peptide core (Bhushana et al., 1973; Mantle et al., 1981; Shochat et al., 1982; Bara et al., 1991). The 660 epitope, which shows oncofetal behaviour in the colonic mucosa, was considered to be a marker of goblet cell differentiation. The peptide nature of the 660 epitope may be important in understanding differentiation during colonic carcinogenesis. It may be the result of re-expression of the fetal or gastric peptide core mucin as a consequence of regulatory gene failure. Fearon and Vogelstein (1990) described a genetic model for colorectal tumorigenesis occurring as a result of an accumulation of genetic

alterations. Bara et al. (1991) have described a family of oncofetal epitopes associated with human colonic mucins during carcinogenesis. The detection of early-stage colorectal tumours is of paramount importance in humans, where a predisposition to form these tumours can be inherited. Mortality would be prevented if tumours could be detected at an early stage through identification of mutant gene products secreted into the blood or faeces. The study of differentiation markers such as the 660 epitope is therefore useful in screening high-risk populations for colon cancer.

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