The oligomeric structure of rat and human gastric mucins

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Intact oligomeric gastric mucins were isolated from the fundic part of rat and human stomach. Physicochemical properties of the oligomeric mucins from both species, such as buoyant density, molecular mass, proteinase-resistance, amino acid composition and monosaccharide composition were similar. Biochemical analysis showed that the oligomeric mucins from both species consist of disulphide-linked mucin monomers exclusively: no other covalently attached proteins were detected in purified monomeric mucin. Four major differences were found between the monomeric mucins of these species: (1) the human monomer is larger, (2) the proteolytic-digest peptides derived from proteinase-sensitive portions of the polypeptide backbone displayed no sequence similarity, (3) the human mucin was less sulphated compared with rat mucin, and (4) the proteinase-sensitive part of the human mucin was relatively larger. However, analyses of [³H]galactose-labelled mucin from both species on gel filtration revealed that both gastric mucins were exclusively synthesized as oligomers. The results indicate that the oligomeric structures of human and rat gastric mucin are similar and their biosyntheses are not affected by the differences in the subunits.

INTRODUCTION

The adherent mucus layer in the stomach protects the gastric mucosa against endogenous agents such as HCl and pepsin and against exogenous substances that have been swallowed [1–3]. Mucus glycoproteins (mucins) are the most important structural components of this mucus. Reconstituted mucus gels of isolated intact gastric mucins formed *in vitro* at physiological concentrations (about 50 mg/ml) display rheological properties similar to those of intact mucus gel [3–5]. Therefore it is generally accepted that the mucins are responsible for the unique viscoelastic properties of the gastric mucus gel. One of the features of gastric mucin molecules, essential to gel formation, is their disulphide-bound oligomeric structure [1–8]. Chemical reduction of the disulphide bonds of oligomeric gastric mucins results in complete loss of gel-forming properties [2–7].

In 1981 Allen proposed a model for pig gastric mucin, in which four monomeric mucin subunits were linked to one central link protein [7]. Recently, we showed that the oligomerization of rat gastric mucin occurs via disulphide-bond linkage of mucin precursors within the rough endoplasmic reticulum [9]. Furthermore, this study indicated that the mucin oligomer does not contain covalently bound non-mucin proteins. In the present study we have analysed the monomeric and oligomeric structures of both human and rat gastric mucins. Biochemical and immunochemical analyses shows that the monomeric mucins differ markedly. However, analysis of the newly synthesized mucin oligomers reveals that their structures are similar.

METHODS

The gastric mucins were isolated by a previously described method [10,11], adapted to isolation of mucin oligomers with intact disulphide bonds. The stomach mucosa was homogenized in a buffer containing 6 M-guanidinium chloride as described previously [10,11], in the presence of 100 mM-iodoacetamide to preserve the disulphide bonds. The mucin was isolated by three subsequent CsCl/guanidinium chloride density gradients as described previously [10,11], with 100 mM-iodoacetamide present throughout the procedures. When the subunit structure of the

mucin was studied by reduction and carboxymethylation, no further purification was carried out before analysis.

The following procedures were performed as previously described [10,11]: isolation of high-molecular-mass glycopeptide of gastric mucins, density measurement, mucin iodination with ¹²⁵I, preparation of antisera, immunoblotting of mucins and glycopeptides, metabolic labelling of gastric mucin *in vitro* and subsequent analysis by immunoprecipitation, amino acid composition assays and proteolytic digestion of purified [³H]galactose- or ¹²⁵I-labelled mucin.

Hexose was assayed by the orcinol method of François *et al.* [12], with galactose and fucose as standards (ratio 2:1, w/w). Sulphate was determined by the method of Silvestri *et al.* [13]. Buoyant density analysis with CsCl gradients and gel filtration on a Sepharose CL-2B (Pharmacia) column (100 cm × 1.6 cm) were by the methods of Carlstedt *et al.* [14]. Reduction and carboxymethylation of proteins by dithiothreitol and iodoacetamide respectively were performed as described in ref. [15]. SDS/PAGE was performed as described by Laemmli [16]. Samples were applied to the gel after boiling for 3 min in sample buffer containing 1% (w/v) SDS, with or without 5% (v/v) 2-mercaptoethanol. The gels were stained with periodic acid/Schiff reagent [17], with Coomassie Blue or with silver stain (Silver Stain Kit; Bio-Rad Laboratories, Richmond, CA, U.S.A.). Radiolabelled glycoproteins on SDS/PAGE were detected by exposure to Kodak SB-5 X-ray films. The proteins detected on SDS/PAGE and autoradiography were quantified with a laser densitometer (Ultrosan XL; LKB, Stockholm, Sweden). Quantitative analysis of monosaccharide composition was done as described by Kamerling *et al.* [18]. ³H and ¹²⁵I radioactivities were measured in a liquid-scintillation counter and a γ -radiation counter respectively.

RESULTS AND DISCUSSION

It is generally accepted that gastrointestinal mucins are composed of disulphide-linked oligomers [1,3,6]. Whereas these mucins were shown to contain both mucin and non-mucin proteins [1,3], studies of the biosynthesis of rat gastric mucin

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Fig. 1. Gel-filtration analysis of mucins

Panels (a) and (b). Native rat and human mucins were labelled with ^{125}I and analysed on Sepharose CL-2B in buffer containing 6 M-guanidinium chloride before and after reduction and carboxymethylation (a). Gastric tissue from rat and man was labelled for 15 min with ^3H galactose and homogenized and mucin was immunoprecipitated with specific antisera [10]. The immunoprecipitated mucin was divided into two portions, one of which was reduced and carboxymethylated. The mucin was analysed as described above (b). ○ and ●, Rat mucin before and after reduction respectively; □ and ■, human mucin before and after reduction respectively. Panels (c) and (d). ^3H Galactose-labelled monomers (c) and ^{125}I -labelled monomers (d) were digested with trypsin and analysed by gel filtration as described above. ○ and ●, Rat monomer before and after digestion respectively; □ and ■, human monomer before and after digestion respectively. The white and black arrowheads indicate the void volume and total volume of the column respectively.

indicated that the oligomers of this mucin are devoid of non-mucin proteins [9–11]. Therefore we isolated intact oligomeric mucin from both rat fundus and human fundus in the presence of 100 mM-iodoacetamide using identical procedures [10,11], and analysed the structure and composition of these mucins. For

Fig. 2. Effect of reduction on the native oligomeric gastric mucins

The effect of reduction was determined by incubating the isolated mucin and ^{125}I -labelled mucin with 2-mercaptoethanol and analysis on SDS/PAGE (10% running gel, 3% stacking gel). Panel (a), periodic acid/Schiff staining; panel (b), silver staining; panel (c), autoradiograph of ^{125}I -labelled mucin. Lanes 1 and 2, human mucin before and after reduction respectively; lanes 3 and 4, rat mucin before and after reduction respectively. Molecular-mass standards are indicated (kDa). The white and black arrowheads indicate the top of the stacking gel and the border of the stacking and running gel respectively.

analysis of the oligosaccharide moieties of the isolated oligomeric mucins, we labelled the mucins metabolically with ^3H galactose before isolation. To enable detection of the protein moieties of the isolated mucins, the mucin oligomers were labelled on the tyrosine residues with ^{125}I . The components of the mucins, released by reduction and carboxymethylation from the oligomeric mucin, were identified without further purification on gel filtration, SDS/PAGE and CsCl-density-gradient centrifugation. The mucin-type glycoproteins released from the oligomers by chemical reduction are defined as mucin monomers.

Native, ^{125}I -labelled and ^3H galactose-labelled mucins, analysed by gel filtration, migrated primarily in the void volume (Figs. 1a and 1b). Upon reduction, both the ^{125}I -labelled and ^3H galactose-labelled mucins from both species were completely included on gel filtration. The elution patterns of the intact and reduced ^{125}I -labelled and ^3H galactose-labelled mucins from either species were identical (Figs. 1a and 1b). Fig. 2 shows that the native mucin from both species did not enter the stacking gel on SDS/PAGE, whereas on reduction the native mucins from both species were converted into mucin-type glycoproteins with an enhanced mobility on SDS/PAGE. The mucins on the gels shown in Figs. 2(a) and 2(b) were quantified by densitometric analysis. About 95% and 85% of the human and rat mucins respectively remained on top of the stacking gel, whereas the reduced mucins from both species entered the stacking gel virtually completely. About 15% of the oligosaccharide-containing material in the native rat mucin preparation migrated to the top of the running gel on SDS/PAGE (lanes 3 in Figs. 2a and 2b), whereas this material was not labelled with ^{125}I (lane 3 in Fig. 2c). We showed previously that all tyrosine residues of rat gastric mucin were lost on proteolytic digestion [11]. Therefore this material probably represented degradation products, originating from peptic digestion of secreted mucin in the stomach lumen.

Table 1. Amino acid and monosaccharide compositions of rat and human gastric mucins

Reduced and carboxymethylated rat (rGM) and human gastric mucin (hGM) were purified [11] and analysed. The human gastric monomer was digested by proteinase K, and the proteinase-resistant glycopeptide (hGP) was purified by gel filtration. The amino acid and monosaccharide compositions were expressed as mol of residue per 100 mol of amino acid residues and as molar proportions respectively. Cysteine was detected as its derivative, carboxymethylated by iodoacetamide. Sulphate was expressed as percentage dry weight. Abbreviation: N.D. not determined.

	rGM	hGM	hGP
Amino acid			
Asx	7.4	5.4	3.2
Thr	15.7	21.1	23.0
Ser	14.8	13.6	17.5
Glx	10.5	7.4	5.9
Pro	8.9	11.6	11.6
Gly	10.2	6.2	7.9
Ala	7.3	7.4	7.9
Cys	1.2	4.1	0.0
Val	5.6	4.6	3.3
Met	0.3	0.0	0.0
Ile	1.7	2.5	2.0
Leu	3.6	3.7	2.4
Tyr	0.3	1.2	0.0
Phe	1.4	1.7	0.0
His	3.2	2.9	2.3
Lys	3.8	2.5	2.1
Arg	0.9	4.1	0.0
Monosaccharide			
Fucose	1.35	0.90	1.01
Mannose	0.06	0.20	0.23
Galactose	2.35	1.18	1.36
GalNAc	1.00	1.00	1.00
GlcNAc	3.03	0.87	0.93
Sialic acid	0.07	0.04	0.05
Sulphate	3.20	1.60	N.D.

We conclude that mucin isolation by CsCl/guanidinium chloride-density-gradient centrifugation in the presence of iodoacetamide resulted in mucin preparations with intact intermolecular disulphide bonds. Besides mucin-type monomers, no other non-mucin proteins were detected after reduction of the native mucin oligomers by the following criteria. (1) No ^{125}I -labelled polypeptides were found in the total volume of the gel-filtration column after reduction of either of the mucins (Fig. 1b). (2) Analysis by SDS/PAGE of native ^{125}I -labelled mucins by silver staining and autoradiography respectively revealed no release of non-mucin proteins from either preparation (Figs. 2b and 2c). (3) Reduction of isolated oligomeric mucin before analysis on CsCl-density-gradient centrifugation had no effect on the buoyant density of the mucins from both rat and human (1.34–1.43 and 1.28–1.38 g/ml respectively). All oligosaccharide-containing material detected during CsCl-density-gradient centrifugation was present in mucin-type glycoproteins, whereas analysis of all CsCl-density-gradient fractions by reducing SDS/PAGE and subsequent silver staining revealed no release of other proteins (results not shown). (4) After metabolic labelling of rat gastric mucin with [^{35}S]methionine or [^{35}S]cysteine we showed previously that immunoprecipitated mature mucin did not contain any other (non-mucin) proteins [9,10]. Therefore our conclusion is that isolated gastric mucins of both species are homo-oligomers consisting exclusively of mucin-type glycoproteins.

As gel-filtration analysis distinguishes oligomeric and monomeric mucins, the experiments described in Figs. 1(a) and 1(b) showed that mucous cells exclusively synthesize oligomeric mucin. The [^3H]galactose-labelled mucins from both species displayed similar elution profiles before and after reduction when compared with the ^{125}I -labelled mucin native (Figs. 1a and 1b). Chase incubation for 6 h in the presence of unlabelled galactose after 15 min labelling with [^3H]galactose had no effect on the elution profiles of the intact and reduced mucins from either species (results not shown). We conclude that [^3H]galactose is exclusively incorporated into oligomers, as no ^3H -labelled subunits were detected on gel filtration. As the 300 kDa monomeric mucin precursors oligomerize in the rough endoplasmic reticulum, and do not reach the trans cisternae of the Golgi complex in monomeric form [9], the gastric mucous cells of both species most likely synthesize and secrete only oligomeric mucin.

Table 1 shows the amino acid and monosaccharide compositions of both mucins after reduction and carboxymethylation. The amino acid compositions, comprising mainly serine, threonine, proline, alanine and glycine, and the monosaccharide compositions, showing principally galactose, *N*-acetylgalactosamine, *N*-acetylglucosamine and fucose, are characteristic for mucins, and indicate a high abundance of *O*-linked glycans in both mucins [1,3,21–23]. The amounts of sialic acid are low. Mannose residues are detected in both mucins, indicating the occurrence of *N*-linked glycans. The amino acid and carbohydrate compositions of the oligomeric mucins from both species were identical with the compositions of the monomeric mucins.

The *O*-linked oligosaccharides are known to render the molecule resistant to proteolysis [1,3,11]. To examine the extent of protection, human monomer was digested by proteinase K and the remnant glycopeptide was purified by gel filtration. The amino acids serine, threonine, proline, alanine and glycine were relatively enriched on proteolytic digestion, indicating that these are the most abundant amino acids in the protected glycopeptide. All tyrosine, phenylalanine and cysteine residues of the human monomer were lost, which was earlier reported for rat monomer [11]. The carbohydrate residues of the human mucin, including mannose, were recovered in the glycopeptide fraction, indicating that all oligosaccharides, including *N*-linked glycans, are present in this glycopeptide (Table 1).

Differences were observed between the mucins of rat and human with respect to molecular mass of the monomer and the relative sizes of the proteinase-sensitive parts of the protein moieties. On gel filtration, the human monomer displayed a higher molecular mass than the rat monomer, whereas the glycopeptides of both mucins (resulting from proteolytic digestion) were virtually identical in size (Fig. 1c). Thus, although the sizes of the *O*-glycosylated regions of both mucins are probably similar, the proteinase-susceptible part of the polypeptide moiety of the human mucin is larger. This was also suggested by metabolic labelling of human tissue *in vitro*. Analysis of immunoprecipitated [^{35}S]cysteine-labelled mucin precursors from tissue segments of three patients by reducing SDS/PAGE showed specific precipitation of a discrete 900 kDa protein band (J. Dekker, L. Klomp & G. J. Strous, unpublished work). This tentative protein backbone of the human gastric mucin is about 3 times larger than the 300 kDa precursor of rat gastric mucin [10]. Taken together, these experiments strongly indicate that human gastric mucin has larger unprotected peptides than rat mucin. One consequence could be that the human mucin is more prone to peptic digestion than the rat mucin.

Despite the similarities in amino acid compositions (Table 1), major differences were found between the peptide structures of

Fig. 3. Proteolytic peptide analysis of rat and human monomeric mucin

Panel (a) shows the autoradiograph of ^{125}I -labelled rat (R) and human (H) monomers digested separately by four different proteinases and analysed by SDS/PAGE (10–20 % gradient gel, 3 % stacking gel). Panel (b) shows the SDS/PAGE analysis (7 % running gel, 3 % stacking gel) of the digestion of unlabelled monomers by trypsin and subsequent staining with periodic acid/Schiff reagent. Tr, trypsin; C, chymotrypsin; E, elastase; Th, thermolysin. Molecular-mass standards are indicated (kDa). The white and black arrowheads indicate the top of the stacking gel and the border of the stacking and running gels respectively. All ^{125}I -labelled tyrosine-containing peptides were smaller than 14 kDa. This was in accordance with the complete loss of tyrosine residues from human and rat mucin after proteolysis with proteinase K (ref. [11] and Table 1).

the mucins from rat and man. Specifically, the proteinase-susceptible parts of the protein backbones show little sequence similarity. As seen in Fig. 3(a), analysis of peptides obtained from ^{125}I -labelled mucins after proteolytic digestions with various enzymes showed no similarity of tyrosine-containing peptides. This lack of similarity is also illustrated by the absence of cross-reactivity of mucin-specific antisera. Table 2 shows that antisera raised against intact mucins are species-specific: no cross-reactivity towards the intact monomer or the glycopeptides from the other species was observed. As the antisera against intact mucins were primarily directed against peptide epitopes [10],

there was little immunochemical resemblance between the human and rat mucins in the peptide epitopes. On the other hand, antiserum raised against the rat glycopeptide, devoid of extending peptides, which was directed primarily against carbohydrate epitopes [10], showed cross-reactivity towards the human glycopeptide. Therefore we conclude that amino acid sequences in non-glycosylated regions differ considerably among human and rat mucins.

The rat mucin is probably more negatively charged than the human mucin. The glycopeptide of the rat mucin carries an intrinsic highly negative charge, due to oligosaccharide sulphation [10,11,24]. As sialic acid content is very low in both mucins, sulphate is the major charged residue on the carbohydrate moieties (Table 1). The rat mucin was shown by direct analysis to contain twice as much sulphate as the human mucin (Table 1). Moreover, the protein-resistant glycopeptide of rat mucin, which displayed an identical molecular mass on gel filtration (Fig. 1d), had a much higher mobility on SDS/PAGE (Fig. 3b), probably due to a higher negative charge.

We showed that the oligomeric structures of gastric mucins from human and rat are similar. Mucin oligomerization in the rat was shown to be an *N*-glycosylation-dependent process [9]. The presence of mannose in human gastric mucin indicates that *N*-linked glycans are also present on this mucin, and we speculate that these may have a similar function in oligomerization. The major differences in monomer structure do not affect the oligomeric structure. Both mucins form homo-oligomers, without non-mucin proteins present. Recorded electron micrographs of intact and reduced human and rat gastric mucins showed that the filamentous monomeric subunits were linked together end-to-end in the oligomers [11,19]. This conformation of oligomeric mucin was also found for pig gastric mucin and human cervical and bronchial mucins [6,8,20]. Although the major differences between the mucins may reflect interesting function-related mucin specializations, they do not influence the quaternary structure of the gastric mucins.

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Table 2. Immunochemical characterization of rat and human gastric mucins

The rat and human mucins were immunochemically characterized with three antisera directed against (1) intact rat monomer (anti-rGM), (2) intact human monomer (anti-hGM) and (3) rat proteinase-resistant glycopeptide (anti-rGP). The tested substances were (1) intact rat monomer (rGM), (2) rat proteinase-resistant glycopeptide (rGP), (3) intact human monomer (hGM) and (4) human proteinase-resistant glycopeptide (hGP). Antiserum specificity and incubation procedures were described previously [10]. –, no binding detected; +, weak binding; ++, intermediate binding; +++, strong binding.

Mucin (ng)...	rGM			rGP			hGM			hGP		
	1	10	100	1	10	100	1	10	100	1	10	100
Anti-rGM	+	++	+++	–	–	–	–	–	–	–	–	–
Anti-rGP	–	+	++	–	+	++	–	–	+	–	–	+
Anti-hGM	–	–	–	–	–	–	+	++	+++	–	–	–

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