Identification of a human gastric mucin precursor: N-linked glycosylation and oligomerization

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Gastric mucin plays an important role in the protection of the stomach wall from chemical, microbiological and mechanical damage. We have previously isolated human gastric mucus glycoproteins and raised a polyclonal antiserum against these macromolecules. This antiserum specifically reacted with gastric mucins in immunoblotting experiments and stained mucous granules at the apical side of gastric surface epithelial cells. A similar staining pattern was obtained after incubation with an antiserum against rat gastric mucin. Next we used the antiserum in pulse-chase experiments of human stomach tissue explants. After short labelling periods with [³⁵S]methionine and [³⁵S]cysteine, the antiserum reacted with a polypeptide with an apparent molecular mass of approx. 500 kDa as determined by

SDS/PAGE, which was converted after 90 min into a heterogeneous high-molecular-mass glycoprotein. This high-molecular-mass form, but not the 500 kDa polypeptide, was detectable in the culture medium after 2 h. This strongly suggests that the 500 kDa polypeptide is the precursor of the purified gastric mucin. Analysis of pulse-chase experiments by non-reducing SDS/PAGE revealed that the precursors form disulphide-linked oligomers early in biosynthesis, before the addition of O-linked sugars. After preincubation with the N-glycosylation inhibitor, tunicamycin, the apparent molecular mass of the precursor decreased marginally but consistently, indicating that N-linked glycan chains are present on the mucin precursor.

INTRODUCTION

The epithelium of the alimentary tract is protected from mechanical and chemical damage by a layer of mucus. The stomach wall is particularly challenged by the low pH and the high pepsin content of the gastric lumen. This requires special properties of the stomach mucus compared with the mucus of other organs. Specialized epithelial cells synthesize and secrete mucous glycoproteins or mucins, which are responsible for the viscous nature of the mucous gel [1]. Mucins are very large glycoproteins (molecular mass > 1000 kDa) characterized by a high amount of glycan chains, O-glycosidically linked to the polypeptide backbone. These O-linked glycans usually account for more than 50% of the molecular mass of the protein. The acceptors for O-linked glycans, the amino acid residues serine and threonine, are located mainly in tandemly repeated amino acid sequences [2]. We have previously isolated mucins from human stomach (designated HGM) and characterized their chemical composition. The monosaccharide and amino acid compositions are typically mucin-like, with high amounts of galactose, *N*-acetylglucosamine and *N*-acetylgalactosamine as well as serine, threonine and proline. Moreover, it was found that rat gastric mucin (RGM) and HGM are synthesized and secreted as oligomeric molecules [3,4]. The oligomeric configuration is essential for the viscoelastic properties of mucins [5–7]. Electron-microscopic imaging of gastric mucins showed that the oligomers, which consist of head–tail-linked monomeric mucins, are filamentous molecules extending more than 1 µm in length [3,4].

In order to understand the molecular structure of HGM, we have investigated how it is synthesized in the cell. We provide evidence that it contains N-linked glycan chains and forms oligomers early in its biosynthesis. In addition, we studied the kinetics of maturation and secretion of the molecule.

EXPERIMENTAL

Stomach tissue

Stomach tissue (fundus) was obtained from male and female patients with oesophageal carcinoma undergoing partial stomach resection in the Academic Hospital of Utrecht, The Netherlands. The stomach was healthy as judged by macroscopic criteria.

Immunoblotting analysis

Gastric tissue (approx. 1 g) was homogenized in buffer containing 6 M guanidinium hydrochloride, 50 mM Tris/HCl, 5 mM sodium EDTA and 1 mM phenylmethanesulphonyl fluoride (PMSF) (pH 7.5) using a Potter–Elvehjem homogenizer at 4 °C. Insoluble material was pelleted by a 30 min centrifugation in a Sorval SS34 rotor at 20000 *g*. CsCl was added to a density of 1.45 g/ml and the homogenate was subjected to CsCl gradient ultracentrifugation at 150000 *g* for 66 h in a Beckman Ti50 rotor. Eleven fractions were collected and portions were weighed using a calibrated pipette to determine the density. Aliquots of the fractions were dialysed for 16 h against water and analysed by SDS/PAGE on 3–5% and 7.5% gels. The gels were stained with periodic acid/Schiff (PAS) reagent or Coomassie Brilliant Blue. The dialysed fractions were diluted 1:100 in PBS and 200 µl samples were blotted on nitrocellulose, using a dot-blot apparatus (Bio-Rad, Richmond, CA, U.S.A.). The filters were blocked with BLOTTO (5% milk powder, 50 mM Tris/HCl, 2 mM CaCl₂, 0.01% antifoam, 0.05% Nonidet P40, 0.05% NaN₃; pH 7.8) for 2 h. Blots were subsequently incubated for 1.5 h with a 1:500 dilution of anti-HGM serum or preimmune serum in TBS/BSA (20 mM Tris/HCl, 130 mM NaCl, 1% BSA; pH 7.8). Filters were rinsed for 30 min in several changes of BLOTTO and incubated with a 1:2000 dilution of goat anti-

rabbit IgG conjugated to alkaline phosphatase (Boehringer, Mannheim, Germany) in TBS/BSA for 1 h. Excess second antibody was removed by two 10 min washes with TBS/BSA and two 5 min washes with TBS. 5-Bromo-4-chloro-3-indolyl phosphate and 4-Nitroblue tetrazolium chloride were used as the substrates for the conjugated enzyme; staining was quantified by scanning with a laserscan densitometer (Ultrosan XL, LKB, Stockholm, Sweden).

Immunofluorescence staining of stomach tissue

After removal from the patients, small strips (approx. 3×15 mm) of fundus tissue were rinsed in PBS and fixed for 20 h at 4 °C in 2% formaldehyde/0.2% glutaraldehyde. The fixed tissue was infused with 2.3 M sucrose as a cryoprotectant for 2 h at 20 °C. Sections of 0.5 μ m were prepared on a Reichert FCS cryotome as described by Tokuyasu and Singer [8] and placed on microscope slips coated with 1% gelatin. The sections were subsequently incubated in PBS containing 0.02 M glycine (5 min), 0.1% NaBH_4 (2.5 min), 1% BSA and an appropriate dilution of anti-serum (16 h at 4 °C), and 1% BSA and a 1:100 dilution of fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit antibody (Nordic) (1 h). Antisera were used in the following dilutions: anti-HGM [3], 1:50; anti-RGM [9], 1:200; pre-immune, 1:100. The sections were enclosed in citifluor B and examined under a Reichert fluorescence microscope.

Metabolic labelling and analysis of radiolabelled stomach mucin

Labelling of newly synthesized proteins in human gastric explants with [^{35}S]methionine and [^{35}S]cysteine was performed exactly as described for rat stomach tissue explants [9] except that 66 MBq/ml $\text{TRAN}^{[35}\text{S]}$ (40 TBq/mmol; ICN) was used. For [^3H]galactose labelling, tissue explants were preincubated at 37 °C for 30 min in Eagle's minimal essential medium containing 10 mg/ml glucose instead of 1 g/l. D-[1- ^3H]Galactose (0.8 TBq/mmol; Amersham) was added to 37 MBq/ml. After the chase period, the explants were individually homogenized in 1 ml of homogenization buffer [50 mM Tris/HCl, 5 mM EDTA, 1% SDS, 1% Triton X-100, 0.5% sodium deoxycholate, 1 mM PMSF, 10 mM iodoacetamide, 100 $\mu\text{g}/\text{ml}$ pepstatin (Sigma, St. Louis, MO, U.S.A.), 50 $\mu\text{g}/\text{ml}$ leupeptin (Bachem, Bubendorf, Switzerland), 0.1% NaN_3 ; pH 7.5]; 200 μl portions of the homogenates or 500 μl of the media were subjected to immunoprecipitation with 20 μl of anti-mucin serum and 100 μl of a 10% suspension of *Staphylococcus aureus* (IgG-Sorb; The Enzyme Company, Boston, MA, U.S.A.) as described [9]. Immunoprecipitates were analysed on SDS/3–5% gradient polyacrylamide gels with 3% stacking gels using piperazine diacrylamide (Bio-Rad) as the cross-linker [10]. ^{14}C markers (Boehringer) and metabolically labelled RGM mono-, di- and tri-meric precursors with apparent molecular masses of respectively 300, 600 and 900 kDa [11] were used as molecular-mass markers.

RESULTS

Immunochemical and morphological characterization of the anti-HGM serum

In previous work from our laboratory [3] the isolation of intact HGM from intracellular storage granules was described. A polyclonal antiserum against this mucin was raised in rabbits and immunoblotting experiments revealed that the antiserum reacted primarily with peptide epitopes of the purified HGM [3]. To establish whether the antiserum specifically reacts with mucin, a gastric tissue homogenate was fractionated by CsCl gradient centrifugation (Figure 1). Most of the material that stained

Figure 1 Characterization of anti-HGM serum

CsCl gradient fractions of stomach homogenates were prepared and analysed by (a) SDS/PAGE on 3–5% gels stained with PAS, (b) SDS/PAGE on 7.5% gels stained with Coomassie Brilliant Blue and (c) scanning densitometry after immunoblotting. Δ , Anti-HGM serum; $\blacktriangle$, anti-RGM serum. The inset in (c) indicates the density of the fractions. Fraction 1 represents the bottom of the gradient. Molecular-mass standards (kDa) are indicated in (a) and (b). HGM migrates between the two arrowheads; the lower arrowhead indicates the top of the running gel.

Figure 2 Anti-HGM and anti-RGM sera specifically stain stomach surface mucous cells

Fluorescence micrographs of 0.5 μ m stomach sections incubated with anti-HGM (a) or anti-RGM (b) followed by goat anti-rabbit FITC conjugate. Magnification $\times 2375$.

intensely with PAS fractionated at a buoyant density of approx. 1.4 g/ml, which is characteristic of mucins [1,2]. This material hardly entered the running gel and appeared very heterogeneous, with similar electrophoretic mobility to the purified HGM. In these fractions, only a very small amount of contaminating proteins could be detected by Coomassie Brilliant Blue staining, whereas the bulk of the non-mucin (glyco)proteins were present in the top fractions of the gradient.

Immunoreaction of the anti-HGM serum peaked in the fractions containing the PAS-positive high molecular-mass glycoproteins and was virtually absent in the fractions containing the majority of the gastric proteins. Strikingly, incubation with anti-RGM serum produced a similar pattern, with a clear peak in fractions 7 and 8. No immunoreaction was detected on incubation with preimmune sera nor on preincubation of the antisera with 45 μ g of HGM (not shown). Furthermore, the antisera immunoprecipitated similar high-molecular-mass glycoproteins (not shown), suggesting that they specifically react with HGM in gastric tissue homogenates.

Using immunohistochemistry, anti-HGM serum mainly stained apical granules present in gastric surface epithelial cells and in cells lining the top segment of the gastric pits (Figure 2a). This region in the gastric wall is known to be comprised of mucin-producing cells. Specificity of staining was ascertained by

Figure 3 Immunoprecipitation of stomach homogenates yields a discrete 500 kDa polypeptide and a heterogeneous high-molecular-mass glycoprotein

Stomach segments were labelled with [35 S]methionine and [35 S]cysteine (lanes 1–4) or [3 H]galactose (lane 5) for 4 h. The homogenates were pooled and 200 μ l portions were subjected to immunoprecipitation with anti-HGM (lanes 1 and 5), anti-RGM (lane 2), anti-RGP (lane 3) or preimmune (lane 4) sera. Immunoprecipitates were analysed on a reducing 3–5% gradient gel. Molecular-mass markers (kDa) are indicated. The upper arrowhead indicates the top of the running gel; the high-molecular-mass glycoprotein migrates between the two arrowheads. The 500 kDa band is indicated by the arrow.

the absence of fluorescence after incubation with the preimmune serum and with an antibody against human gallbladder mucin (not shown). No immunoreaction with anti-HGM serum was observed in gallbladder sections and in lower regions of the gastric pits, where an anti-(rat pepsin) serum (raised in rabbits in our laboratory) and an anti-(pig H^+/K^+ -ATPase) serum [a generous gift from Dr. De Pont (Nijmegen, the Netherlands)] were used as morphological markers to stain chief cells and parietal cells respectively (not shown). This suggests that the antiserum does not react with other glycoproteins, including gallbladder mucins. It also suggests that the antiserum does not react with mucins produced in the neck region of the gastric pits. A similar staining pattern was seen using anti-RGM serum (Figure 2b), suggesting that HGM and RGM have epitopes in common. Also, a weak perinuclear staining was noticed, probably indicative of staining of rough endoplasmic reticulum and Golgi. This immunoreaction, thus visualized in a morphological context, indicates that anti-HGM and anti-RGM sera specifically recognize HGM.

Biosynthesis of HGM

Next, we tested the specificity of the anti-mucin antibodies in immunoprecipitation experiments with labelled stomach tissue homogenates (Figure 3). A 4 h labelling period with [35 S]methionine and [35 S]cysteine was chosen to obtain a steady-state labelling of proteins in the biosynthetic pathway. A heterogeneous very high-molecular-mass band was most obviously present using an antiserum raised against protease-digested RGM (anti-RGP; lane 3) [3,9], but was also seen after immunoprecipitation with anti-HGM and anti-RGM sera. The electro-

Figure 4 Biosynthesis and secretion of HGM

Stomach segments were pulse-labelled with [35 S]methionine and [35 S]cysteine for 30 min and chased for the indicated times. The explants were homogenized and samples of the homogenates and media were subjected to immunoprecipitation with anti-RGM serum and analysis by reducing SDS/PAGE on 3–5% gradient gels. Labelled HGM migrates between the two arrowheads and the tentatively identified 500 kDa mucin precursor is indicated by the arrow. The 300 kDa molecular-mass marker is indicated.

phoretic mobility of this band was similar to that of purified and reduced HGM stained with PAS [3] (Figure 1). This suggests that this band represents newly synthesized HGM. In our experiments a significant portion of this band was usually retained in the stacking gel, which may partly explain the weak appearance of this product in the running gel.

A number of bands were visible after immunoprecipitation with anti-HGM serum. The most prominent represents a protein with an apparent molecular mass of roughly 500 kDa (Figure 3, lane 1). Strikingly, this protein, but not the lower-molecular-mass polypeptides, was also precipitated with anti-RGM serum (Figure 3, lane 2), but only very weakly with anti-RGP serum. The presence of radiolabelled proteins after immunoprecipitation with anti-HGM and the absence of these bands when the preimmune serum was used indicates that these proteins specifically react with anti-HGM antibody. This therefore suggests that these lower-molecular-mass products probably represent degradation products of the 500 kDa or high-molecular-mass protein. This is further substantiated by the absence of immunoreaction of anti-HGM serum with the top fractions in Figure 1. In further experiments (Figures 3–5) the anti-RGM serum was used to immunoprecipitate HGM. Similar results were obtained after immunoprecipitation with anti-HGM serum, although we consistently observed more degradation products (not shown).

Anti-RGP serum, which recognizes almost exclusively epitopes on oligosaccharides of mature rat gastric mucin [9], reacts mainly with the heterogeneous high-molecular-mass protein. Moreover, the 500 kDa polypeptide is not labelled with [3 H]galactose. Therefore it is unlikely that the 500 kDa band contains these oligosaccharide epitopes. In contrast, the antisera yielding an intense band at approx. 500 kDa after [35 S]methionine-labelling (anti-RGM and anti-HGM) were previously shown to react primarily with polypeptide epitopes of purified mucin [3,9,11]. This suggests that the 500 kDa band is a form of the high-molecular-mass protein that does not contain O-linked oligosaccharides, probably an early biosynthetic intermediate. How-

ever, the faint band at 500 kDa may indicate that the anti-RGP serum has a low titre for peptide epitopes.

The relationship between the 500 kDa polypeptide and the high-molecular-mass product was investigated by pulse-chase incubation of gastric explants. Newly synthesized proteins were pulse-labelled with TRAN[35 S] for 30 min and chased in medium containing excess unlabelled methionine and cysteine (Figure 4). The polypeptide of approx. 500 kDa was visible immediately after pulse-labelling. The high-molecular-mass protein appeared only after 90 min of chase. After 2 h, this product, but not the 500 kDa polypeptide, was detectable in the medium. Moreover, only the high-molecular-mass polypeptide was labelled by pulse-chase incubation with [3 H]galactose (Figure 3), and the [3 H]galactose-labelled mucin was detectable in the medium as well (not shown). These results are consistent with the concept that the 500 kDa polypeptide is the precursor of the high-molecular-mass product. The latter represents newly synthesized mature HGM and is exclusively secreted into the culture medium.

Oligomerization and N-glycosylation

On analysis of the immunoprecipitated HGM precursors by non-reducing SDS/PAGE, a discrete additional polypeptide was detected (Figure 5). The apparent molecular mass of this protein was estimated to be at least 1500–2000 kDa. This polypeptide appeared after 30 min of chase, which is about 1 h earlier than the first appearance of the glycosylated mature HGM. After 60 min of chase, this band was more prominent than the 500 kDa polypeptide and the relative amount of radioactivity incorporated into it increased with longer chase times. This suggests that the 500 kDa polypeptide is converted into the 1500–2000 kDa product during the chase. Since the latter polypeptide was not observed when 2-mercaptoethanol was present in the sample buffer, we propose that it is a disulphide-linked HGM precursor oligomer, formed early in the mucin-biosynthetic process. Moreover, this

Figure 5 Oligomerization of the HGM precursor occurs early in biosynthesis

Immunoprecipitated HGM precursors were prepared as described in Figure 3 and analysed by non-reducing SDS/PAGE on 3–5% gels. Molecular-mass markers (kDa) are indicated. The 500 kDa precursor is indicated by the small arrow; the putative 1500–2000 kDa oligomer is indicated by the large arrow.

Figure 6 Gastric mucin precursors contain N-linked glycan chains

Stomach segments were preincubated for 4 h and labelled with [^{35}S]methionine and [^{35}S]cysteine for 90 min in the presence of the indicated concentrations of tunicamycin. HGM and precursors were immunoprecipitated from samples of the homogenates with anti-RGM serum and analysed by reducing SDS/PAGE on a 3–5% gradient gel. The 500 kDa mucin precursor is indicated by an arrow.

putative oligomeric precursor was not secreted into the medium (not shown). The apparent molecular mass of the oligomer suggests that at least three or four mucin monomers are linked.

Next, we determined whether the HGM precursor contained N-linked oligosaccharides. Tissue explants were preincubated and labelled in the presence of two concentrations of tunicamycin, an inhibitor of N-linked glycosylation. The apparent molecular mass of the precursor decreased slightly after treatment with 50 $\mu\text{g/ml}$ tunicamycin (Figure 6). In this experiment, 10 $\mu\text{g/ml}$ tunicamycin did not affect the electrophoretic mobility of all the precursors, resulting in the concomitant appearance of both the unshifted as well as the shifted precursors. Although the decrease in apparent molecular mass of the HGM precursor after tunicamycin treatment was small, it was consistently observed in all the experiments performed with tunicamycin. We conclude

that the HGM precursor contains N-linked oligosaccharides, consistent with the detection of mannose in the monosaccharide composition of HGM [3]. Attempts to confirm these results by digestion of the immunoprecipitated HGM precursors with endoglycosidase H were hampered by complete degradation of the precursors during the incubations.

DISCUSSION

As a first step in understanding the structural features of mucins in the protection of the stomach epithelium, gastric mucus glycoproteins were isolated and characterized [3]. Recently, two mucin mRNAs (MUC5C and MUC6) were shown to be expressed in the stomach [12,13]. Our results do not give any indication as to whether HGM is a product of either of these genes or another as yet unestablished MUC gene. However, although both the anti-RGM and anti-HGM sera used in this study were raised against mucins with a heterogeneous appearance on SDS/PAGE, they immunoprecipitated one discrete HGM precursor in tissue from all patients tested. Therefore our results suggest that anti-HGM and anti-RGM sera react with a single homogeneous gastric mucin precursor. The heterogeneity of HGM most probably results from differences in O-linked glycosylation. Mature HGM had similar mobility on SDS/PAGE to the majority of PAS-stained high-molecular-mass glycoproteins and the antiserum recognized CsCl-gradient fractions containing almost solely these macromolecules. In addition, all surface epithelial cells of the fundus were stained with these antisera. Therefore it is likely that anti-HGM serum reacts with a considerable proportion of the mucins synthesized in the stomach.

The apparent molecular mass of the HGM precursor was estimated at approx. 500 kDa by interpolation of the marker proteins in several different experiments. The estimated apparent molecular mass of the backbones of RGM (300 kDa) [9] and the predicted molecular mass of the MUC2 gene product (approx. 550 kDa) [14,15] are in a similar range. Also the kinetics of maturation and secretion of HGM and RGM are comparable. Maturation of these mucins (defined as migration of newly synthesized mucin with the same electrophoretic mobility as purified mucin) takes approx. 2 h and the mucin is detectable in the medium within 30 min of maturation.

The efficient formation of RGM precursor oligomers is dependent on the presence of N-linked glycan chains [11]. N-linked oligosaccharides form approx. 10% of the apparent molecular mass of the RGM precursors [11]. Also, the predicted amino acid sequence of the MUC2 gene product contains 31 potential N-glycosylation sites [14,15]. Here we show that HGM also contains N-linked glycans (Figure 6), suggesting a pivotal role for N-linked glycan chains in the biosynthesis of secreted mucins.

Biochemical and morphological experiments indicated that RGM and HGM as well as human tracheobronchial and cervical mucins form end-to-end disulphide-linked oligomers [16–21]. The MUC2 cDNA sequence [15] suggests that the MUC2 gene product also forms oligomers, although this still remains to be demonstrated. The oligomerization event occurs shortly after synthesis and most likely takes place in the rough endoplasmic reticulum of mucin-producing cells. RGM precursors form a fixed-ratio mixture of dimers, trimers and tetramers [11]. In contrast, HGM precursors seem to consist of a unique type of oligomer with a fixed number of monomers (presumably three to four). Dimeric HGM precursors (putative intermediates in the formation of higher oligomers) were not observed, suggesting that the formation of oligomers *per se* is a very rapid process. The presence of N-linked glycans on the backbone may facilitate the

generation of an oligomerization-competent configuration. However, whether HGM precursor oligomerization is impaired when N-linked glycosylation is prevented, as described for the RGM precursor [11], is an as yet unanswered question. Although we observe a discrepancy in the stoichiometry of precursor monomer assembly between rat and human stomach, the oligomerization event appears to be meticulously regulated in both species. This strict control suggests that correct conformation of mucin oligomers is important for the functional characteristics of the mucin and is reflected in the quality of the mucous gel. It will be interesting to determine, using the assays of biosynthesis and oligomerization described here, whether patients with stomach ulcer have altered stoichiometry or kinetics of mucin precursor oligomerization.

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