Biosynthesis of a human gall-bladder mucin

Leo W. J. KLOMP,* Arie Jan DE LELY and Ger J. STROUS

Laboratory of Cell Biology, AZU H02.314, University of Utrecht, 3584 CX Utrecht, The Netherlands

Mucin glycoproteins play an important role in the initial stages of gall-stone formation by a currently largely unknown mechanism. Understanding the structure of gall-bladder mucin is necessary to comprehend the mechanism by which cholesterol monohydrate crystals aggregate. Three successive CsCl-gradient-ultracentrifugation steps were used to purify human gall-bladder mucin from gall-bladder tissue. The isolated macromolecules had a typical mucin-like monosaccharide composition and appeared as heterogeneous high- M_r glycoproteins on SDS/PAGE. A polyclonal antiserum was raised against these molecules and the specificity of the antiserum was ascertained by immunoblotting. The antiserum specifically stained mucous granules at the apical side of all gall-bladder epithelial cells in neck, fundus and body. The antibody was subsequently used to immuno-

precipitate the mucin and biosynthetic intermediates from gall-bladder-tissue homogenates. An early biosynthetic precursor of the isolated mucin was identified by SDS/PAGE as a single polypeptide with an apparent M_r of approx. 470000. This precursor protein was converted after 1 h into a heterogeneous high- M_r glycoconjugate with an electrophoretic mobility similar to that of the purified mucin. The mature mucin, but not the precursor, was secreted into the culture medium, starting at 1 h. As shown by SDS/PAGE under non-reducing conditions, the precursors form disulphide-linked oligomers. Using the N-glycosylation inhibitor tunicamycin, the apparent M_r of the precursor was decreased to approx. 410000, indicating that N-linked glycan chains are attached to the precursor polypeptide.

INTRODUCTION

Although considerable progress has been made in the comprehension of the structure, function and biosynthesis of mucin-type glycoproteins in general (reviewed recently by Strous and Dekker [1]), information about human biliary mucin remains scarce. Mucin is the most important structural and functional component of mucus, a viscous gel that covers and protects epithelia of the gastrointestinal tract. Primary structures of mucin polypeptide backbones expressed in different organs differ considerably. However, they have several structural aspects in common. Mucins are very-high- M_r glycoproteins which are characterized by their tandemly repeated amino acid sequences with large numbers of glycan chains, O-linked to serine and threonine residues. This feature, together with their ability to form homo-oligomers by intermolecular disulphide bonds, accounts for their visco-elastic properties [2,3]. The O-linked sugar chains often terminate with the negatively charged residues sialic acid or sulphate and cause an extended conformation of the molecules, giving the mucins their filamentous appearance [4].

Several lines of evidence suggest that high- M_r mucin glycoproteins play an important role in the onset of gall-stone formation. Mucin concentration is increased in bile obtained from patients suffering from cholesterol gall-stones, as well as in bile from patients with biliary sludge, a putative precursor stage of gall-stones [5]. An important observation is that the synthesis of gall-bladder mucins is inhibited by the cyclo-oxygenase inhibitors indomethacin [6] and aspirin both *in vitro* and *in vivo* [7,8], with a concomitant inhibition of gall-stone formation [9,10]. In addition, the secretion of gall-bladder mucins in prairie dogs (*Cynomys ludovicianus*) is stimulated upon addition of arachidonate, presumably by an increase in prostaglandin concentration [6,11]. Moreover, it was shown that the unglycosylated portions of human gall-bladder mucin bind phosphatidylcholine

and cholesterol in model bile, thereby promoting the cholesterol crystal nucleation [12]. In conclusion, the current hypothesis of the mechanism of gall-stone formation is based on the assumption that mucins play a key role in this process by acting as a scaffold for the aggregation of cholesterol monohydrate crystals. In addition, once a precipitate has formed, the mucus gel may assist the retention of the crystals in the gall-bladder [12–16].

The significance of these studies in the understanding of the mechanisms preceding gall-stone formation is greatly hampered by the lack of detailed knowledge of the structure of gall-bladder mucins. It is currently unknown how gall-bladder mucins are synthesized and whether the structural characteristics of mucins as described above apply to gall-bladder mucins and indeed are factors in gall-stone pathogenesis. Here we describe the isolation of a mucin from human gall-bladder (here after called 'HGBM') and the preparation of a specific antiserum against this mucin. This antiserum was subsequently used to localize the site of HGBM synthesis. Moreover, it enabled the identification of a polypeptide precursor of HGBM, which appeared to be provided with N-linked glycans and to form oligomers early in biosynthesis.

MATERIALS AND METHODS

Tissue and bile samples

Gall-bladder tissue and bile were obtained from patients undergoing laparoscopic cholecystectomy in the Academic Hospital of Utrecht, Utrecht, The Netherlands. Inflamed gall-bladders were excluded from the experiments.

Isolation of HGBM

Mucin was isolated from 24 g of gall-bladder tissue, derived from six female patients. Immediately after removal, the tissue was

Abbreviations used: FITC: fluorescein isothiocyanate; GalNAc, N-acetylgalactosamine; HGBM, human gall-bladder mucin; MEM, minimal essential medium; PAS, periodic acid/Schiff; PMSF, phenylmethanesulphonyl fluoride; RER, rough endoplasmic reticulum; TBS, Tris-buffered saline.

* To whom correspondence should be addressed.

extensively washed in PBS until no more mucus could be removed from the tissue, the mucosa was stripped from the muscle layer, dropped into liquid N_2 and stored at -70°C until homogenization. The combined mucosal tissue was homogenized in ice-cold 6 M guanidinium chloride/50 mM Tris/HCl/5 mM NaEDTA/1 mM phenylmethanesulphonyl fluoride (PMSF), pH 7.5, using a Potter–Elvehjem homogenizer. Mucin was then isolated on three successive CsCl gradients in the presence of guanidinium chloride as described previously [17,18]. From all gradients, 18 fractions were collected; small aliquots were weighed to determine the density and were analysed by SDS/3–10% PAGE. The gels were stained with periodic acid/Schiff (PAS) reagent, as well as with Coomassie Brilliant Blue. PAS-positive material, migrating very slowly on the gel, was detected in three fractions with an average buoyant density (ρ) of 1.46 g/ml. Most of the PAS-positive material entered the running gel, but a small amount of PAS-stained material was sometimes observed in the stacking gel in the same fractions. The positive fractions were pooled and re-centrifuged twice. This procedure yielded 10 mg of pure mucin. The oligosaccharide composition of the purified mucin was determined as described by Kamerling et al. [19]. The purified tissue mucins (100 μg) were used to immunize a New Zealand White rabbit according to standard protocols. Preimmune serum was obtained from the same rabbit before immunization.

Immunoblotting

Serial dilutions of human bile or purified HGBM were prepared in PBS. In the fractionation experiment, 10 μl of bile was mixed with 1 ml of 4 M guanidinium chloride/50 mM Tris/HCl/5 mM NaEDTA/1 mM PMSF/2.84 M CsCl, pH 7.5 (ρ 1.45 g/ml). Isopycnic density-gradient centrifugation was performed in a Beckman TLA 120.2 rotor for 18 h at 175000 g . The gradient was fractionated, and 5 μl aliquots were diluted to 1 ml with PBS. Aliquots of the dilutions (200 μl) were blotted in duplicate 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 CaCl_2 , 0.01% anti-foam/0.05% Nonidet P40/0.05% NaN_3 , pH 7.8) for 1.5 h. Subsequently, filters were incubated for 1.5 h with a 1:500 dilution of anti-HGBM antiserum or the preimmune serum in Tris-buffered saline (TBS)/BSA (20 mM Tris/HCl/130 mM NaCl/1% BSA, pH 7.8). Anti-HGBM (5 μl) was preincubated with 45 μg of purified HGBM or 45 μl of human bile for 1 h at 4°C . Filters were rinsed for 30 min in several changes of BLOTTO and incubated with a 1:3000 dilution of ^{125}I -goat anti-rabbit IgG in TBS/BSA for 1 h. The excess second antibody was removed by four 3-min washes with BLOTTO and four 3-min washes with PBS. The bound radioactivity was scanned with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, U.S.A.) and analysed using ImageQuant software (Molecular Dynamics). Goat anti-rabbit IgG (100 μg ; produced and affinity-purified in our laboratory) was iodinated to a specific radioactivity of approx. 150 MBq/mg with 18.5 MBq of ^{125}I (The Radiochemical Centre, Amersham, Bucks., U.K.) and five iodobeads (Pierce, Rockford, IL, U.S.A.) in 200 μl of PBS. Free ^{125}I was removed by dialysis against PBS and the ^{125}I -IgG was stored at -20°C .

Histochemical and Immunohistochemical staining of gall-bladder tissue

Small segments of gall-bladder tissue were fixed in Bouin fixative and embedded in glycol methacrylate (Technovit 7100; Kulzer,

Wehrheim, Germany). Sections (5 μm thickness) were treated with 1% HIO_4 for 30 min at 55°C , rinsed with water, then incubated with Schiff reagent for 30 min at room temperature. Sections were rinsed and counterstained with haematoxylin for 5 min at room temperature and subsequently examined under a Nikon photomicroscope. To prepare gall-bladder tissue for immunofluorescence, small strips were fixed in 2% formaldehyde/0.2% glutaraldehyde for 16 h at 4°C . The tissue was embedded in 10% gelatin and incubated for 2 h in 2.3 M sucrose as a cryoprotectant. Semi-thin cryosections (0.5 μm) were prepared on a Reichert FCS cryotome as described by Tokuyasu and Singer [20]. The sections were successively incubated in PBS containing 0.02 M glycine (5 min), 0.1% sodium borohydride (2.5 min), 1% BSA and a 1:300 dilution of the anti-HGBM antiserum (16 h at 4°C), 1% BSA and a 1:100 dilution of fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Nordic, The Netherlands) (1 h). The sections were examined under a Reichert fluorescence microscope.

Metabolic labelling of gall-bladder explants

Immediately after the gall-bladder was taken out of the patient, it was opened, and a small piece was removed, rinsed and transported to the laboratory in Eagle's minimal essential medium (MEM). All incubations started within 10 min after removal from the patients. Small tissue segments of identical size (3 mm $\times$ 3 mm) were incubated in prewarmed MEM (37°C) under CO_2/O_2 (1:19) as described by Dekker et al. [21]. After a preincubation of 0.5 h in medium lacking methionine and cysteine, 15 MBq/ml of $\text{TRAN}^{[35]}\text{S}$ (40 TBq/mmol; ICN, CA, U.S.A.) was added. After a 30 min incubation the tissue segments were rinsed in prewarmed chase medium (MEM containing 100 $\mu\text{g}/\text{ml}$ methionine and cysteine) and incubated for various times in chase medium. To inhibit N-glycosylation, explants were preincubated for 4 h as well as labelled in the presence of tunicamycin (Boehringer, Mannheim, Germany).

Analysis of labelled gall-bladder mucin

After the chase period, the tissue explants were individually homogenized in 1 ml of ice-cold homogenization buffer [50 mM Tris/HCl/5 mM NaEDTA/1% SDS/1% Triton X-100/0.5% sodium deoxycholate/1 mM PMSF/10 mM iodoacetamide/100 $\mu\text{g}/\text{ml}$ pepstatin (Sigma)/50 $\mu\text{g}/\text{ml}$ leupeptin (Bachem, Bubendorf, Switzerland)/0.1% sodium azide, pH 7.5] by 20 strokes with a Potter–Elvehjem homogenizer. Cell debris and nuclei were removed by a 5 min 10000 g spin at 4°C , and the supernatant was immediately used or stored at -70°C until further analysis. Mucins were immunoprecipitated from 100 μl of the tissue homogenate or 500 μl of the chase medium using 10 μl of the anti-HGBM antiserum and 100 μl of a 10% suspension of *Staphylococcus aureus* (IgG-Sorb; The Enzyme Company, Boston, MA, U.S.A.) as described in [21]. If required, the immunoprecipitates were treated with endoglycosidase H (Boehringer) for 16 h at 37°C . Finally the immunoprecipitates were boiled for 3 min in SDS/PAGE sample buffer (60 mM Tris/HCl/10% glycerol/2% SDS) with or without 2% 2-mercaptoethanol, pH 6.8, and analysed on SDS/3–7%-PAGE gradient polyacrylamide gels with a 3% stacking gel. SDS/PAGE was performed as described by Laemmli [22] using piperazine diacrylamide (Bio-Rad) as the cross-linking agent. ^{14}C -labelled markers (Boehringer) and metabolically labelled rat gastric mucin mono-, di- and tri-meric precursors with apparent M_r values of respectively 300000, 600000 and 900000 were used as markers. Gels were dried and fluorographed as described previously [23],

and incorporation of radiolabel was quantified by a Laserscan densitometer (Ultrosan XL; LKB, Stockholm, Sweden).

RESULTS

Isolation and characterization of HGBM

To obtain mucin, which was not exposed to the lumen of the gall-bladder, mucus glycoproteins were isolated from gall-bladder tissue. In this way, a mucin was obtained which was synthesized in the gall-bladder epithelium. We used a method that had previously been shown to yield pure and intact mucin preparations [17,18]. To achieve complete purification, three successive isopycnic CsCl-gradient-ultracentrifugation steps were performed, two with a starting density of 1.42 g/ml in the presence of 4 M guanidinium chloride, and a third gradient with an initial density of 1.50 g/ml in the presence of 1 M guanidinium chloride to separate the mucins from nucleic acids. The gradients were fractionated, and aliquots of the fractions were weighed to determine the density; they were then analysed by SDS/PAGE, followed by PAS/Coomassie Brilliant Blue staining. HGBM-containing fractions were identified by the buoyant density of approx. 1.46 g/ml and a slow electrophoretic mobility of PAS-stained material. As shown in Figure 1, the HGBM was stained by PAS, but not by Coomassie Brilliant Blue. After the second gradient, no contaminating proteins were visible after Coomassie Brilliant Blue staining, and after the third gradient a pure HGBM preparation was obtained. Complete purification of the mucin was verified by silver staining of the final preparation (results not shown). The isolated HGBM appeared heterogeneous in M_r on SDS/PAGE analysis, as previously observed for other mucin preparations [17,18,24–26]. The oligosaccharide composition of the HGBM was characterized by h.p.l.c. (Table 1). The relative amount of total carbohydrate was 50%, and the composition is characteristic for mucins [1]. Moreover, the ratios of the individual sugars were in accordance with previously published results [5,27,28], although we detected a relative low amount of *N*-acetylgalactosamine.

A rabbit polyclonal antiserum was raised against the purified

Figure 1 Purification of HGBM

Aliquots of pooled fractions after subsequent CsCl-isopycnic-gradient centrifugation were reduced and analysed by SDS/3–10%-PAGE and stained with PAS or Coomassie Brilliant Blue (CBB). Amounts equivalent to 10 μ g of hexose were applied in each lane. Lanes 1, crude tissue homogenate; lanes 2, pooled fractions after the first CsCl gradient; lanes 3, purified HGBM. M_r markers are indicated on the left; the upper arrowhead indicates the top of the running gel; the HGBM migrates between the two arrowheads.

Table 1 Monosaccharide composition of HGBM

Values are expressed as molar ratios relative to the amount of galactose (ND, not detectable). The percentage of total carbohydrate was determined after extensive dialysis and freeze-drying of the mucin.

Monosaccharide	Molar ratio
Fucose	0.6
Mannose	ND
Galactose	0.2
<i>N</i> -Acetylglucosamine	1.00
<i>N</i> -Acetylgalactosamine	1.00
Sialic acid	0.10
Total carbohydrate (% w/w)...	50

Figure 2 Characterization of the anti-HGBM antiserum

(a) and (b) Increasing amounts of purified HGBM (a) or human bile (b) were blotted on nitrocellulose in duplicate. The blots were incubated with anti-HGBM (Δ), preimmune-serum ($\blacktriangle$), anti-HGBM preincubated with human bile ($\diamond$) or anti-HGBM preincubated with purified HGBM ($\blacklozenge$). The immune complexes were made visible with 125 I-goat anti-rabbit IgG and quantified by scanning with a Phosphorimager. (c) and (d) A sample of human bile was fractionated by CsCl-gradient ultracentrifugation, and aliquots of the fractions were blotted onto nitrocellulose. The filter was incubated with anti-HGBM and 125 I-goat anti-rabbit IgG and autoradiographed (c). The numbers indicate the fractions (1, bottom; 12, top of the gradient). The radioactivity was quantified after scanning with a Phosphorimager (d) and plotted as the percentage of the total radioactivity (Δ). $\blacktriangle$ Indicates the density of the fractions.

Figure 3 Anti-HGBM antiserum specifically stains granules in gall-bladder surface epithelial cells

Panel A, section incubated with PAS and counterstained with haematoxylin, magnification: $380\times$. Panel B, photomicrograph of gall-bladder section incubated with anti-HGBM followed by goat anti-rabbit FITC conjugate; magnification: $380\times$; panel C, as panel B; magnification: $1190\times$. Nuclei and basal side of the epithelial cells are depicted ('n' and 'b' respectively).

HGBM and characterized using immuno-dot-blotting. Therefore increasing amounts of HGBM were blotted on to nitrocellulose and detected with the anti-HGBM antiserum, followed by ^{125}I -

goat anti-rabbit IgG. A linear relationship between the amount of HGBM and radioactivity was observed when blots were incubated with the anti-HGBM antiserum. The immunoreaction was absent after incubation with the preimmune serum, and was completely quenched after preincubation of the antiserum with purified HGBM or human bile (Figure 2a). Additionally, the antiserum, but not the preimmune serum, reacts with (a) component(s) of human bile. Again, this reaction was totally abolished by preincubation with purified HGBM (Figure 2b). These results were consistently obtained in bile samples from different patients. The mucin-like character of the reactive component was further confirmed by blotting CsCl-gradient fractions of bile and subsequent immunodetection of HGBM (Figures 2c and 2d). The antiserum bound mainly to fractions with a density of approx. 1.4 g/ml , similarly to the purified HGBM. Almost no signal was detected in the top fractions of the gradient, where the vast majority of the (glyco)proteins fractionate. These results imply that the anti-HGBM antiserum specifically recognizes HGBM present in bile.

Localization of HGBM-producing cells

To further characterize the rabbit antiserum against HGBM, it was used in immunofluorescence experiments. Cryosections of the gall-bladder wall were incubated with the anti-HGBM antiserum and immunostained with fluorescein-conjugated goat anti-rabbit. Distinct staining was found in the epithelial cells, while no immunostaining was present in the cells and matrix of the underlying connective tissue and the smooth-muscle layer (Figure 3b, panel B). This immunostaining was absent when the sections were incubated with the preimmune serum, while a complete absence of the staining was observed when the antiserum was preincubated with $10\text{ }\mu\text{g}$ of the HGBM. In addition, no immunoreaction was observed after incubation of human gastric mucosal sections with the anti-HGBM antiserum (results not shown), indicating that the staining of the gall-bladder epithelial cells was not due to an aspecific reaction with other glycoproteins, including gastric mucin. The immunofluorescence in the epithelial cells was observed in cryosections obtained from different regions (neck, body and fundus) of the gall bladder, suggesting that mucin synthesis is not restricted to a particular region of the gall bladder. Histochemical staining of the sections with PAS revealed the same staining patterns in all regions (Figure 3a, panel A), indicative of a high concentration of mucins in the epithelial cells.

At higher magnification (Figure 3, panel C) the immune reaction yielded a granular-like staining almost exclusively localized to the apical side of all columnar epithelial cells. No staining was observed over the nucleus. The basal side of the cells stained very weakly. We conclude that the antiserum specifically reacts with the contents of mucous granules of all epithelial cells lining the gall bladder in neck, body and fundus.

Identification of the HGBM polypeptide precursor

The biosynthesis of HGBM was studied by immunoprecipitation experiments involving metabolically radiolabelled gall-bladder proteins. We therefore incubated gall-bladder explants in the presence of $\text{TRAN}^{[35\text{S}]}$ to label newly synthesized proteins. Because the immunofluorescence experiments demonstrated that HGBM was present in all epithelial cells, various regions of the gall-bladder were used in the experiments. To label the entire biosynthetic route, gall-bladder explants were labelled for 4 h. The total explant homogenate contained numerous different

Figure 4 Immunoprecipitations of gall-bladder homogenates with anti-HGBM reveals a discrete 470 000- M_r protein and a heterogeneous high- M_r glycoprotein

Gall-bladder segments were labelled with $\text{TRAN}^{[35\text{S}]}$ for 4 h. Labelled proteins were analysed on reducing 3–7% gradient gels without immunoprecipitation (lane 1) or after immunoprecipitation with preimmune serum (lane 2) or immunoprecipitation with anti-HGBM (lane 3). M_r markers are indicated. The upper arrowhead indicates the top of the running gel; the high- M_r glycoprotein migrates between the two arrowheads and co-migrates with PAS-positive material (results not shown). The 470 000- M_r protein is indicated by the arrow.

labelled polypeptides, of which only two proteins could be immunoprecipitated: a polypeptide with an apparent M_r of approx. 470 000 and a heterogeneous high- M_r protein (Figure 4). The high- M_r product had the same slow mobility and heterogeneous appearance on SDS/PAGE as HGBM (Figure 1), strongly suggesting that this labelled high- M_r protein represents newly synthesized HGBM. If no antiserum or if a non-specific antiserum was used, neither the high- M_r product nor the 470 000- M_r polypeptide was immunoprecipitated. Therefore the polyclonal antiserum reacts with HGBM in immunoprecipitation experiments of labelled gall-bladder tissue homogenates and it binds to an additional polypeptide of M_r approx. 470 000. Strikingly, if the total complement of labelled proteins were analysed by SDS/PAGE, both the high- M_r product and the polypeptide of M_r about 470 000 were also detected.

To study a possible precursor–product relationship between the two labelled products, newly synthesized proteins were pulse-labelled with $\text{TRAN}^{[35\text{S}]}$ for 30 min and subsequently chased in medium containing excess unlabelled precursors (Figure 5). After pulse-labelling, the 470 000- M_r protein was visible. On chase incubation the intensity of this band initially increased, but decreased after prolonged chase incubation. Concomitant with the disappearance of the 470 000- M_r protein, we observed the appearance of the high- M_r protein with the same electrophoretic mobility as HGBM in the cell homogenates and in the medium. The initial increase of the 470 000- M_r protein signal is probably due to inefficient replacement of the $\text{TRAN}^{[35\text{S}]}$ by the unlabelled precursors during the chase, resulting in a prolonged metabolic labelling period. The subsequent decrease of the 470 000- M_r band intensity is most likely caused by its conversion into the high- M_r product. After 1 h of chase incubation, this product, but not the 470 000- M_r polypeptide, was detectable in the medium. The secretion into the medium increased after prolonged chase incubations. From these results we conclude that the high- M_r

Figure 5 Synthesis and secretion of HGBM

Gall-bladder segments were pulse-labelled with $\text{TRAN}^{[35\text{S}]}$ for 30 min and subsequently chase-incubated for the indicated times. The explants were homogenized and aliquots of the homogenates and the media were subjected to immunoprecipitation with anti-HGBM and analysis on reducing 3–7% gradient gels. M_r markers are indicated. The upper arrowhead indicates the top of the running gel; the labelled HGBM migrates between the two arrowheads, and the 470 000- M_r HGBM precursor is indicated by the arrow.

protein is newly synthesized HGBM and that the 470 000- M_r protein is its intracellular precursor. During its biosynthesis, the HGBM precursor is converted into the heterogeneous high- M_r protein by the addition of O-linked glycan chains. Subsequently, only the mature HGBM is secreted into the culture medium.

N-glycosylation and oligomerization of the HGBM precursor

Next, we investigated whether HGBM contains N-linked glycans in addition to O-linked oligosaccharides. The gall-bladder tissue explants were incubated with different concentrations of tunicamycin, which inhibits the synthesis of the N-glycan precursor dolichyl phosphate. As shown in Figure 6(a), treatment with a high concentration of tunicamycin caused a decrease in the apparent M_r of the precursor. At a suboptimal concentration of tunicamycin (10 $\mu\text{g}/\text{ml}$), both the low- M_r and the high- M_r precursors were visible. An alternative way to demonstrate the presence of N-linked oligosaccharides is digestion with endoglycosidase H, which removes 'high-mannose' oligosaccharides from asparagine residues on the polypeptide backbone. Treatment of immunoprecipitated HGBM precursors with endoglycosidase H resulted in a decrease in M_r similar to that caused by tunicamycin. The apparent M_r of the unglycosylated precursor was estimated to be approx. 410 000, suggesting that the HGBM backbone contains at least 10% N-linked glycans (approx. 60 000 M_r). The sensitivity for digestion by endoglycosidase H confirms that the oligosaccharides of the HGBM precursor glycoprotein are in a high-mannose configuration and that it therefore is an early biosynthetic intermediate.

Previously we have shown that N-linked oligosaccharides facilitate oligomerization of rat gastric mucin precursors [29]. To

Figure 6 N-linked glycosylation and oligomerization of the HGBM precursor

(a) Gall-bladder segments were labelled with TRAN^[35S] for 4 h in the presence or absence of the indicated concentrations of tunicamycin (tm). HGBM and precursors were immunoprecipitated from aliquots of the homogenates with anti-HGBM and analysed on reducing 3–7% gradient gels. After immunoprecipitation, the samples in lanes 4 and 5 were incubated for 16 h at 37 °C in endoglycosidase H (endo H) digestion buffer with (+) and without (–) addition of endoglycosidase H. M_r markers are indicated. The upper arrowhead indicates the top of the running gel; the labelled HGBM migrates between the two arrowheads and the 470 000- M_r mucin precursor is indicated by the arrow. (b) Gall-bladder segments were pulse-labelled with TRAN^[35S] for 30 min in the presence or absence of 10 μ g of tunicamycin (tm) and subsequently chased for the indicated times. The explants were homogenized, and aliquots of the homogenates were subjected to immunoprecipitation with anti-HGBM and analysed on a non-reducing 3–7% gradient gel. M_r markers are indicated on the left. The labelled HGBM and the monomeric precursor are indicated as in (a). The putative HGBM precursor oligomer is indicated by the arrow on the right.

investigate whether N-linked glycan chains play a role in the maturation of the HGBM precursor, pulse-chase experiments were performed in the presence of a suboptimal concentration of tunicamycin. Again, the incubation with tunicamycin initially results in the presence of both the N-glycosylated and the unglycosylated precursors (Figure 6b). During the chase, the intensity of the (upper) glycosylated precursor band decreased, most likely due to its conversion into the mature HGBM,

Figure 7 Different individuals have similar HGBM precursor apparent M_r values

Gall-bladder explants were labelled with TRAN^[35S] for 30 min and immediately homogenized. The homogenates were kept at –70 °C until homogenates of four different patients were available. Aliquots of the homogenates were subjected to immunoprecipitation with anti-HGBM and analysis on reducing 3–7% gradient gels. The 300 000- M_r marker is indicated. The arrowhead indicates the top of the running gel and the 470 000- M_r HGBM precursor is indicated by the arrow.

similarly to what was shown in Figure 5. At the same time, the amount of radioactivity in the unglycosylated precursor remained remarkably constant during the chase period, suggesting that its conversion into the mature high- M_r form was delayed or prevented. The incorporation of radiolabel in the two precursor proteins was quantified by laserscan densitometry. The relative contribution of the unglycosylated precursor increased approx. 3-fold after 3.5 h and 5 h of chase, indicating that only the precursors containing N-linked oligosaccharide chains were efficiently converted into the mature HGBM.

To test whether the HGBM precursors form disulphide-linked oligomers, as previously reported for rat gastric mucin [29], the immunoprecipitated HGBM precursors were analysed under non-reducing conditions. A sharp polypeptide band, intermediate between the precursor and the mature HGBM, appeared after 30 min of chase and was prominently present after 1 h (Figure 6b). This band was not observed when samples were reduced before electrophoresis (cf. Figure 6b and Figure 5). This suggests that this band represents a HGBM oligomer which is formed before the addition of the O-linked sugar chains, most probably in the rough endoplasmic reticulum (RER). The apparent M_r of this band was estimated in six different experiments by extrapolation of the sizes of the marker proteins, and amounted to approx. 2000 000, presumably indicative of a tetrameric structure. Smaller oligomers (putative dimers and trimers) were not observed, even when the samples were reduced very mildly. In some of the experiments performed (e.g. Figures 6 and 7), a number of extra bands were observed on the gel. This may possibly be explained by variable degradation of the HGBM precursors during homogenization and immunoprecipitation.

It has been shown for MUC1 (a transmembrane protein with structural similarity to secreted mucins) and proposed for rat gastric mucin, that multiple alleles encode mucin protein backbones with different numbers of tandem-repeated amino acid sequences. Two of these alleles can be expressed simultaneously, giving rise to two mucin precursors. The different numbers of tandem repeats may lead to heterogeneity of precursor length

between several individuals [21,30,31]. We analysed immunoprecipitates of labelled gall-bladder tissue homogenates of four individuals in one experiment. No variation in the SDS/PAGE mobility of the precursors was observed, either in one patient or when the four patients were compared (Figure 7). This suggests that similar allelic variation of HGBM may not take place.

DISCUSSION

In this study we used a polyclonal antiserum directed against HGBM to investigate several aspects of its synthesis. Using this antiserum we have identified the HGBM polypeptide precursor and studied its conversion into mature mucin.

We isolated HGBM from gall-bladder tissue to avoid exposure to the potential degradative environment in the lumen of the gall-bladder and to avoid contamination with mucins synthesized in the biliary ducts or in the liver. The identity of the isolated mucin was confirmed by (1) its ability to react with PAS, (2) the typical mucin-like buoyant density of approx. 1.45 g/ml, (3) the slow and heterogeneous mobility on SDS/PAGE and (4) the typical mucin-like oligosaccharide composition, comparable with previously published gall-bladder mucin sugar compositions [5,27,28]. Attempts to characterize HGBM further by gel-permeation chromatography were unsuccessful, as the polypeptide portions of HGBM appeared highly liable to degradation.

The antiserum raised in a rabbit against the purified HGBM was characterized using dot-blotting, immunofluorescence and immunoprecipitation. The immunoblot experiments showed that the antiserum reacted with HGBM and a constituent of human bile in a concentration-dependent fashion. Preincubation with an excess of HGBM prevented binding of the antiserum. The antiserum precipitated two products from labelled tissue homogenates, one most probably being newly synthesized HGBM and the other its proposed intracellular precursor. In immunofluorescence studies, the anti-HGBM polyclonal antiserum stained the apical part of gall-bladder epithelial cells where mucin storage granules reside. The fluorescence co-localized with the PAS histochemical staining pattern. The immunostaining was absent in non-mucin-producing cells, but, in addition, the antiserum did not react with mucin granules in gastric epithelial cells, indicating that the reaction was specific for HGBM. The combined results of these experiments lead us to conclude that the antiserum specifically reacts with HGBM. They also indicate that the antiserum reacts with intracellular HGBM and HGBM present in bile, suggesting that they may be the same molecules.

The specific staining of all surface epithelial cells in the gall-bladder with the antiserum raised against the purified mucin species indicated that HGBM synthesis is not restricted to a certain region of the gall-bladder. This suggests that the mucosa of the gall-bladder is completely covered by a mucus gel and that HGBM presumably constitutes a major gall-bladder mucin. The distribution of HGBM-producing cells is probably unaffected by the presence of gall-stones, as we found that the HGBM precursor was synthesized in all regions of gall-stone gall-bladders and in a gall-bladder obtained after a Whipple operation (results not shown). This suggests that, in non-gall-stone patients too, HGBM is synthesized in each individual surface epithelial cell of the gall-bladder.

After short metabolic labelling periods and immunoprecipitation with the anti-HGBM antiserum, a discrete glycoprotein with an apparent M_r of approx. 470 000 was observed. We conclude that this peptide is the early intracellular precursor form of HGBM, since (1) it reacted specifically with the anti-HGBM antiserum, (2) it was labelled after short labelling periods

and disappeared gradually during chase, (3) it was not secreted and (4) its N-linked glycan chains were in the 'high-mannose' configuration. The reaction of the antiserum with this polypeptide suggests that the antiserum recognizes, at least in part, epitopes present on the polypeptide backbone of HGBM.

Disulphide-linked oligomerization of the precursor molecule was observed after 30 min, suggesting that this process takes place in an early biosynthetic compartment, probably in the RER. The putative oligomers were exclusively present when unreduced HGBM precursors were analysed, suggesting that the observed increase in M_r is not the result of *N*-acetylgalactosamine (GalNAc) addition, the first step of O-linked glycan formation. Moreover, on reduction of the oligomers, all radioactivity migrated at a mobility similar to that of the monomeric precursors, indicating that no other polypeptides participate in the formation of oligomers. We observed a striking difference in the stoichiometry of precursor oligomerization of HGBM as compared with rat gastric mucin. The latter forms dimers, trimers and tetramers in a fixed ratio [29], whereas we consistently detected only a single, discrete, HGBM precursor oligomer. Estimation of the apparent M_r of the HGBM oligomers suggested that they are at least larger than dimers. Smaller oligomeric precursors were not observed. The finding of this single oligomer species, in contrast with rat gastric mucin, may indicate that strict control of the number of covalently linked HGBM monomers is important. How this is regulated is as yet unknown.

The presence of N-linked oligosaccharides is regarded as a general feature of mucins [1]. In rat stomach, N-linked glycan chains are essential for efficient oligomerization of mucin precursors and for the exit from the RER, probably by inducing proper folding of the protein [29]. The same was observed for the HGBM precursor, which is provided with N-linked glycans that account for roughly 10% of its molecular mass. When the precursors were synthesized in the presence of tunicamycin and consequently were devoid of N-linked sugars, they were converted into mature mucin less efficiently as compared with the fully glycosylated precursors. Our results suggest that N-linked glycan chains facilitate the oligomerization of HGBM precursors. In conclusion, the biosynthesis of HGBM proceeds essentially similar to the synthesis of rat gastric mucin [21,29].

Although the isolated HGBM appeared heterogeneous on SDS/PAGE, a single and discrete polypeptide precursor was identified, suggesting that only one gall-bladder mucin is identified by our antiserum. The discrete mobility on SDS/PAGE of the precursor, if analysed both before as well as after immunoprecipitation (Figure 4), suggests that the heterogeneity of the mature HGBM completely results from the O-linked glycosylation, whereas the protein backbone is homogeneous. In contrast with these observations, several groups have reported that mucin mRNAs appear as polydisperse bands on Northern blots, comprising lengths between 1000 and 15000 nucleotides [33–36]. The discrete polypeptide backbone predicts that a HGBM mRNA population of at least 11 000 nucleotides encodes this polypeptide. Both the MUC2 and MUC6 mRNAs have been detected in gall-bladder tissue [35,37], but whether the HGBM backbone described here is a product of the MUC2 or MUC6 gene or yet another mucin gene remains to be determined. In addition, it is still unknown how many different mucins are synthesized in the gall-bladder. Since mucin glycoproteins are implicated in gall-stone formation [12–16,39,40], it will be important to determine in what manner the described structural features of HGBM contribute to this process.

We greatly acknowledge the patients and their surgeons of the Academic Hospital Utrecht for the gall-bladder tissue, Dr. G. van Berge Henegouwen for stimulating

discussions, Dr. J. Kamerling for the determination of the monosaccharide composition, Roel Dol and Dr. G. Posthuma for kindly helping in the localization studies, and Tom van Rijn, René Scriwanek and Maurits Niekerk for preparing the Figures. This work was supported by the Dutch Organization for Scientific Research (NWO; grant 900-522-085) and NATO (grant 870316).

REFERENCES

- 1 Strous, G. J. and Dekker, J. (1992) *Crit. Rev. Biochem. Mol. Biol.* **27**, 57–92
- 2 Allen, A. (1978) *Br. Med. Bull.* **34**, 28–33
- 3 Allen, A. (1981) in *Physiology of the Gastrointestinal Tract* (Johnson, R. L., ed.), vol. 1., pp. 617–639, Raven Press, New York
- 4 Shogren, R., Gerken, T. A. and Jentoft, N. (1989) *Biochemistry* **28**, 5525–5536
- 5 Lee, S. P. and Nicholls, J. F. (1986) *Gastroenterology* **90**, 677–686
- 6 LaMont, J. T., Turner, B. S., Dibenedetto, D., Handin, R. and Schaffer, A. I. (1983) *Am. J. Physiol.* **83**, G92–G98
- 7 Rhodes, M., Allen, A. and Lennard, T. W. J. (1992) *Gut* **33**, 1109–1112
- 8 Rhodes, M., Allen, A., Dowling, R. H., Murphy, G. and Lennard, T. W. J. (1992) *Gut* **33**, 1113–1117
- 9 Lee, S. P., Carey, M. C. and LaMont, J. Th. (1981) *Science* **211**, 1429–1431
- 10 Hood, K., Gleeson, D. and Rupp, D. C. (1988) *Lancet* **II**, 1223–1225
- 11 LaMorte, W. W., LaMont, J. Th., Hale, W., Booker, M. L., Scott, T. E. and Turner, B. (1986) *Am. J. Physiol.* **251**, G701–G709
- 12 Smith, B. F. (1987) *J. Lipid Res.* **28**, 1088–1098
- 13 Levy, P. F., Smith, B. F. and LaMont, J. Th. (1984) *Gastroenterology* **87**, 270–275
- 14 Lee, T. J. and Smith, B. F. (1989) *J. Lipid Res.* **30**, 491–498
- 15 Marks, J. W., Bonorris, G. G., Albers, G. and Schoenfield, L. J. (1992) *Gastroenterology* **103**, 566–570
- 16 Afdhal, N. H., Niu, N., Gantz, D., Small, D. M. and Smith, B. F. (1993) *Gastroenterology* **104**, 1515–1523
- 17 Carlstedt, I., Lindgren, H., Sheehan, J. K., Ulmsten, U. and Wingerup, L. (1983) *Biochem. J.* **211**, 13–22
- 18 Dekker, J., van Beurden-Lamers, M. O., Oprins, A. and Strous, G. J. (1989) *Biochem. J.* **260**, 717–723
- 19 Kamerling, F. J., Gerwig, G. J., Vliegthart, J. F. G. and Clamp, J. R. (1975) *Biochem. J.* **151**, 491–495
- 20 Tokuyasu, K. T. and Singer, S. J. (1976) *J. Cell Biol.* **71**, 894–906
- 21 Dekker, J., van Beurden-Lamers, W. M. O. and Strous, G. J. (1989) *J. Biol. Chem.* **264**, 10431–10437
- 22 Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- 23 Bonner, W. M. and Laskey, R. A. (1974) *Eur. J. Biochem.* **46**, 83–88
- 24 Dekker, J., Aelmans, P. H. and Strous, G. J. (1991) *Biochem. J.* **277**, 423–427
- 25 Mantle, M., Mantle, D. and Allen, A. (1981) *Biochem. J.* **195**, 277–285
- 26 Gupta, R., Jentoft, N., Jamieson, A. M. and Blackwell, J. (1990) *Biopolymers* **29**, 347–355
- 27 Pearson, J. P., Kaura, R., Taylor, W. and Allen, A. (1982) *Biochim. Biophys. Acta* **706**, 221–228
- 28 Harvey, P. R. C., Rupar, C. A., Gallinger, S., Petrunka, C. N. and Strasberg, S. M. (1986) *Gut* **27**, 374–381
- 29 Dekker, J. and Strous, G. J. (1990) *J. Biol. Chem.* **265**, 18116–18122
- 30 Swallow, D. M., Gendler, S., Griffiths, B., Corney, G., Taylor-Papadimitriou, J. and Bramwell, M. E. (1987) *Nature (London)* **328**, 82–83
- 31 Gendler, S., Taylor-Papadimitriou, J., Duhig, T., Rothbard, J. and Burchell, J. (1988) *J. Biol. Chem.* **263**, 12820–12823
- 32 Thornton, D. J., Davies, J. R., Kraayenbrink, M., Richardson, P. S., Sheehan, J. K. and Carlstedt, I. (1990) *Biochem. J.* **265**, 179–186
- 33 Gum, J. R., Byrd, J. C., Hicks, J. W., Toribara, N. W., Lampert, D. T. A. and Kim, Y. S. (1989) *J. Biol. Chem.* **264**, 6480–6487
- 34 Gum, J. R., Hicks, J. W., Swallow, D. M., Lagace, R. L., Byrd, J. C., Lampert, D. T. A., Siddiki, B. and Kim, Y. S. (1990) *Biochem. Biophys. Res. Commun.* **171**, 407–415
- 35 Jany, B. H., Gallup, M. W., Yan, P. S., Gum, J. R., Kim, Y. S. and Basbaum, C. B. (1991) *J. Clin. Invest.* **87**, 77–82
- 36 Porchet, N., Vancong, N., Dufosse, J., Audie, J. P., Guyonnet-Duperat, V., Gross, M. S., Deni, C., Degand, P., Bernheim, A. and Aubert, J. P. (1991) *Biochem. Biophys. Res. Commun.* **175**, 414–422
- 37 Toribara, N. W., Robertson, A. M., Ho, S. B., Kuo, W. L., Gum, E., Hicks, J. R., Byrd, J. C., Siddiki, B. and Kim, Y. S. (1993) *J. Biol. Chem.* **268**, 5879–5885
- 38 Gum, J. R., Hicks, J. W., Toribara, N. W., Rothe, E. M., Lagace, R. E. and Kim, Y. S. (1992) *J. Biol. Chem.* **267**, 21375–21383
- 39 Afdhal, N. H., Offner, G. D., Murray, F. E., Troxler, R. F. and Smith, B. F. (1990) *Gastroenterology* **98**, 1633–1641
- 40 Afdhal, N. H., Gong, D., Niu, N., Turner, B., LaMont, J. T. and Offner, G. D. (1993) *Hepatology* **17**, 693–700