MUC5B is the prominent mucin in human gallbladder and is also expressed in a subset of colonic goblet cells

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Van Klinken, B. J. an-Willem, J. an Dekker, Sandy A. van Gool, J. an van Marle, Hans A. Buller, and Alexandra W. C. Einerhand. MUC5B is the prominent mucin in human gallbladder and is also expressed in a subset of colonic goblet cells. Am. J. Physiol. 274 (Gastrointest. Liver Physiol. 37): G871–G878, 1998.—To elucidate the roles of human gallbladder mucin (HGBM), such as in gallstone formation and cytoprotection, it is essential to identify HGBM and study its expression. This was performed by metabolic labeling, Western blotting, immunohistochemistry, and RT-PCR. In a large number of individuals, antibodies against purified HGBM and against MUC5B detected a mucin precursor (~470 kDa) in the gallbladder and colon, but not in the small intestine. In the gallbladder, Western blotting using specific anti-MUC5B antibodies showed that this mucin precursor represented an identical mucin, MUC5B. RT-PCR experiments demonstrated a similar tissue distribution pattern of MUC5B mRNA. Immunohistochemistry with anti-HGBM and anti-MUC5B showed staining in gallbladder epithelial cells and colonic goblet cells in the crypt base, but not in the small intestine; double labeling showed that HGBM was located in small granules within goblet cells, colocalizing to MUC2-containing goblet cells. Metabolic labeling demonstrated the secretion of mature MUC5B in the colon. Conclusively, MUC5B is identified as the prominent HGBM and is also expressed and secreted in the colon.

METHODS

Tissues. Samples of the gallbladder fundus (10 patients, 2 with gallstones) and proximal jejunum (2 patients) were obtained from patients undergoing a Whipple operation for carcinoma of the pancreas or papilla Vater or from patients undergoing hemihepatectomy for liver carcinoma. Biopsies of the duodenum (10 patients), ascending colon (4 patients), transverse colon (1 patient), or sigmoid (23 patients) were taken by endoscopy from healthy tissue from patients admitted for reflux esophagitis, polypectomy (nonpolyposis coli), control after successful eradication of Helicobacter pylori, control for colon carcinoma, diverticulosis, irritable bowel syndrome, or constipation. Use of tissue was approved by the ethics committee of the Academic Medical Center.

Antibodies. Monoclonal anti-MUC5B was raised against purified deglycosylated tracheobronchial mucin and recognizes the tandem repeat of MUC5B (8, 27). Monoclonal antibody WE9 [anti-MUC2(1)] recognizes a peptide epitope in the unique terminals of MUC2 (36). Anti-HCM [anti-MUC2(2)] was raised against purified human colonic mucin and recognizes the unique non-O-glycosylated terminals of human MUC2 (35, 36). Anti-HGBM was raised against purified HGBM and recognizes unique non-O-glycosylated terminal carbohydrate moieties of HGBM (21, 36). Anti-BGBM antibody (anti-BGM) was raised against deglycosylated BGM (26). Anti-M3P (anti-MUC3) was raised against a synthetic peptide representing the tandem repeat of MUC3 (16). Anti-MUC6.1 (anti-MUC6) was raised against a synthetic peptide representing the tandemly repeated amino acid sequence of MUC6 and purified by peptide affinity chromatography (5). Anti-MUC4 antibody was raised against the synthetic peptide with the sequence TSSASTGHATLPVTDTSSASC, representing the tandemly repeated amino acid sequence of MUC4, and affinity chromatography purified (not shown). The methods used for antibody production and purification are described elsewhere (21–23).
precursors, labeled with 35S-labeled amino acids, were used, 3–4% reducing SDS-PAGE as described previously (35, 40).

Gastrointestinal mucins
Antibodies used to identify Table 1.

Gel electrophoresis. Radiolabeled mucins were analyzed on 3–4% reducing SDS-PAGE as described previously (35, 40). For molecular weight markers, unreduced rat gastric mucin precursors, labeled with 35S-labeled amino acids, were used, with molecular masses of 300 kDa for the monomer and 600 kDa for the dimer (6). Prestained high molecular weight markers with molecular masses between 49.5 and 205 kDa (Bio-Rad, Richmond, CA) were used. Gels were either Western blotted onto nitrocellulose membranes (35) or fixed in 10% methanol and 10% acetic acid, incubated for 10 min with Amplify (Amersham), and dried. Alternatively, reduced immunoprecipitated mature mucins were analyzed by 0.8% agarose gel electrophoresis in the presence of 0.1% SDS and run for 14 h at 20 mA in buffer containing 0.04 M Tris-borate (Sigma Chemical, St. Louis, MO), pH 8.0, and dried according to Thornton et al. (33). All gels were exposed to X-ray film (Biomax MR, Kodak) from 1 to 4 wk at −70°C.

Immunohistochemistry. Tissues were fixed in 4% (wt/vol) paraformaldehyde (Merck), embedded in paraffin (Sherwood Medical, St. Louis, MO), cut in 7-µm sections, and mounted. After deparaffination, sections were boiled in 0.01 M citrate buffer, pH 6.0, for 10 min and treated with 1% (vol/vol) hydrogen peroxide (BDH, Poole, UK) for 30 min to reduce endogenous peroxidase activity. Sections were then incubated for 30 min in 10 mM dithiothreitol, 500 mM random hexamers, 1 µg RNA, and 500 µM of each dATP, dCTP, dGTP, and dTTP. After 1 h, digestion with RNase H (GIBCO BRL, Breda, The Netherlands) in a total volume of 20 µl according to the manufacturer’s suggested protocol. The final reaction condition was 20 mM Tris·HCl, pH 8.4, 50 mM KCl, 2.5 mM MgCl2, 0.01% BSA, 10 mM dithiothreitol, 500 mM random hexamers, 1 µg RNA, and 500 µM of each dATP, dCTP, dGTP, and dTTP. After 1 h, digestion with RNase H (GIBCO BRL) for 10 min at 42°C was carried out. This was followed by a PCR reaction in a total volume of 20 µl using 1 µl cDNA as a template in combination with either β-actin primers (5′-CAAGGCCAAC-CGGAGAAAG-3′ and 5′-CAGGGTACATGGTGGTGCC-3′) or MUC5B-specific primers (5′-TGGGCTCTCGAGTCCCCGTG-3′ and 5′-CACCGGGATCTTGTCCGCC-3′) based on a non-repetitive sequence within MUC5B (14). Final PCR reaction conditions were 10 mM Tris·HCl, pH 8.4, 50 mM KCl, 5 mM MgCl2, 0.01% gelatin, 0.2 U Taq polymerase, 200 nM of each primer, cDNA template, and 200 µM each of dATP, dCTP, dGTP, and dTTP. The PCR reaction was carried out as follows: 5 min at 95°C, 30-1 min cycles at 95°C, 1 min at 57°C, and 1 min at 72°C, followed by a 10-min extension step at 72°C. The PCR products were analyzed on a 2% agarose gel following standard procedures (29). The amplified MUC5B sequence was verified by digestion with restriction enzyme PstI (Boehringer).

RESULTS

Biosynthesis of HGBM precursors in gallbladder and intestine. To identify HGBM, human gallbladder biopsies were pulse labeled with 35S-labeled amino acids, followed by immunoprecipitations and analysis on SDS-PAGE. Using this method, we previously showed that mucin precursors, synthesized by the colonic cell line LS 174T or by human gastrointestinal tissues, have distinct molecular masses on SDS-PAGE and can thus be discriminated electrophoretically (38, 40). HGBM precursor (apparent molecular mass 470 kDa) was immunoprecipitated from radiolabeled gallbladder homogenates by anti-HGBM and displayed a similar apparent molecular mass as the mucin precursors immunoprecipitated by anti-MUC5B and anti-BGBM (Fig. 1). Anti-MUC3 antibody immunoprecipitated MUC3 precursor from gallbladder homogenate, displaying a different apparent molecular mass (~550 kDa) than MUC5B precursor (Fig. 1). In gallbladder homogenates, no MUC4 or MUC6 precursors were detectable (Fig. 1). In addition, no MUC2 precursors were detectable in gallbladder homogenates, as demonstrated by immunoprecipitations with the antibodies anti-MUC2(1) (Fig. 1) and anti-MUC2(2) (not shown).

| Table 1. Antibodies used to identify gastrointestinal mucins |
|---------------------------------|----------------|----------------|---|
| Specificity                    | Name in Study | Original Name | Ref. |
| Non-O-glycosylated region of MUC2 | Anti-MUC2(1) | WE9            | 36 |
| Non-O-glycosylated region of MUC2 | Anti-MUC2(2) | Anti-HCM       | 35, 36 |
| Tandem repeat region of MUC3   | Anti-MUC3     | Anti-M3P       | 16 |
| Tandem repeat region of MUC4   | Anti-MUC4     | Anti-MUC4      | 38 |
| Tandem repeat of MUC5B         | Anti-MUC5B    | Anti-MUC5B     | 8, 27 |
| Non-O-glycosylated region of HGBM | Anti-HGBM     | Anti-HGBM      | 21, 36 |
| Deglycosylated region of BGBM  | Anti-BGBM     | Anti-BGBM      | 26 |
| Tandem repeat of region of MUC6 | Anti-MUC6     | Anti-MUC6,1    | 5 |

HGBM, human gallbladder mucin. BGBM, bovine gallbladder mucin.
To further identify the 470-kDa HGBM precursor, we investigated its tissue-specific expression. From duodenal homogenates, MUC2 precursor (apparent molecular mass 600 kDa), but not HGBM precursor, could be immunoprecipitated (Fig. 1). Interestingly, from human sigmoid (Fig. 1), as well as ascending and transverse colon (not shown), HGBM precursor could be immunoprecipitated by anti-HGBM as a band with an apparent molecular mass of 470 kDa. When immunoprecipitations with anti-MUC5B (Fig. 1) or anti-BGBM (not shown) were performed on sigmoidal homogenates, a very similar 470-kDa band was detected, further indicating that anti-BGBM, anti-HGBM, and anti-MUC5B immunoprecipitated identical mucin precursors. An extra 600-kDa band was observed in immunoprecipitations with anti-MUC5B from sigmoidal homogenates (Fig. 1). This extra band was attributed to cross-reactivity of anti-MUC5B to MUC2 precursor, since the apparent molecular mass of this extra band was similar to MUC2 precursor immunoprecipitated with anti-MUC2(2) (Fig. 1). This cross-reactivity of anti-MUC5B to MUC2 precursor was observed in all (n = 20) colonic homogenates investigated.

To determine whether a similar tissue-specific expression of the 470-kDa HGBM precursor could be detected in different individuals, immunoprecipitations with anti-HGBM, anti-MUC5B, and anti-BGBM were performed on metabolically labeled biopsies of the gallbladder and intestine of a large number of individuals. After immunoprecipitations with anti-HGBM, the 470-kDa precursor was detected in all gallbladder homogenates (n = 9) and in all homogenates of ascending colon (n = 4), transverse colon (n = 1), and sigmoid (n = 15), but never in homogenates of the duodenum (n = 9) or jejunum (n = 2).

Identification of HGBM as MUC5B by Western blot analysis. Western blotting was used to establish the identity of the mucin precursors. First, mucin precursors were immunoprecipitated from radiolabeled gallbladder homogenate by anti-HGBM, anti-MUC5B, and anti-BGBM (anti-BGBM not shown), followed by Western blotting of these mucin precursors with anti-MUC5B (Fig. 2, left). The Western blot showed that anti-MUC5B recognized the precursors immunoprecipitated by anti-HGBM, anti-MUC5B, and anti-BGBM (anti-BGBM not shown) antibodies from gallbladder homogenate. Moreover, the precursors all displayed similar apparent molecular masses of 470 kDa. When the blot was exposed to X-ray film, the radiolabeled bands completely coincided with the bands detected by anti-MUC5B on Western blot (Fig. 2, right). This demonstrated that the immunoprecipitated radiolabeled mucin precursors are identical to the bands that were detected by anti-MUC5B on Western blot.

MUC5B mRNA expression. To determine MUC5B mRNA expression in the gallbladder and the small and large intestine, an RT-PCR experiment was carried out using RNA from these tissues as templates (Fig. 3). First, cDNA was synthesized from the RNA using random primers, followed by a PCR reaction using either MUC5B primers derived from a nonrepetitive MUC5B region (8) or human β-actin primers (Fig. 3). When the latter primers were used (Fig. 3, lanes a, c, and e), equal amounts of the β-actin PCR fragments were observed of the expected size (587 bp), indicating that equal amounts of template RNA derived from the gallbladder and the small and large intestine were used and that RT-PCR proceeded equally efficiently. Interestingly, MUC5B PCR fragments of the expected length (148 bp) could be amplified from gallbladder and colonic cDNA, but not from jejunal cDNA. The identity of the MUC5B PCR product was further checked by digestion with Pst I, which yielded the expected 88- and 60-bp products (not shown).

Immunolocalization of MUC5B. To localize MUC5B, sections of human gallbladder, duodenum, and sigmoid were examined using anti-HGBM, anti-MUC5B, or anti-BGBM. In the sigmoid, a granular staining pattern was observed in goblet cells when stained by anti-HGBM, with highest intensity basally in the crypts (Fig. 4A), whereas in the gallbladder an intense stain-
ing of mucous granules in all epithelial cells was observed with anti-HGBM (Fig. 4F). In the duodenum, no staining with anti-HGBM was found (Fig. 4E). In the colon, anti-MUC5B showed a perinuclear staining in goblet cells, with highest intensity in the basal crypt region, while staining was absent from the mucous granules (Fig. 4B). The gallbladder also showed a high staining intensity with anti-MUC5B in all epithelial cells (Fig. 4G), whereas no staining was detected in the duodenum (Fig. 4D). Anti-BGBM showed a similar staining pattern as anti-MUC5B in gallbladder epithelial cells (Fig. 4H) and in colon (not shown).

Because both MUC2 and MUC5B are expressed in goblet cells of the colon, double immunofluorescence labeling, using confocal laser-scanning microscopy, was performed to study possible colocalization of these mucins. In gallbladder epithelial cells, a granular staining pattern was observed using anti-HGBM (Fig. 5a), whereas no cells stained with anti-MUC2(1) (not shown). In the duodenum, a staining of very large mucous granules in the goblet cells was observed with anti-MUC2(1) (Fig. 5b), but no staining was observed with anti-HGBM (not shown). Staining of sigmoid sections with both anti-HGBM (in green) and anti-MUC2(1) (in red) showed that small granules in goblet cells stained either single (i.e., green) or double (i.e., white), showing granules containing only MUC5B or both MUC5B and MUC2, respectively (Fig. 5, c and d). Anti-MUC2(1) stained the colonic goblet cells (Fig. 5, c and d), similar to the staining of the goblet cells of the duodenum (Fig. 5b). This shows that MUC2 is generally contained in large mucous granules of the goblet cells of the colon and duodenum. In longitudinal sections of the sigmoid crypt, a high intensity of white (Fig. 5c) was found basally (indicating colocalization of MUC2 and MUC5B), whereas red staining (indicating MUC2) was detected in all goblet cells of the crypt (Fig. 5c). At a higher magnification of colonic crypt cells, MUC5B was localized within small granules (Fig. 5d), some solely containing MUC5B or both MUC5B and MUC2.

Comparison of the biosynthesis and secretion of mature MUC5B and MUC2 in the colon. To compare the biosynthesis and secretion of mature MUC5B to MUC2...
in the human colon, pulse labeling with $^{35}$S-labeled amino acids, followed by chase incubations, was performed. After pulse labeling, MUC2 precursors (apparent molecular mass 600 kDa) and MUC5B precursors (apparent molecular mass 470 kDa) were immunoprecipitated from colonic homogenate (Fig. 6A). After a 4-h chase incubation an additional diffuse band representing mature mucin was detected, with electrophoretic mobility corresponding to 550 kDa, in immunoprecipitations performed with both anti-MUC2(2) and anti-HGBM on tissue homogenate (Fig. 6A). Precursor bands of MUC5B and MUC2 were weak after 4-h chase incubation, as a result of their conversion into mature mucins. The diffuse 550-kDa bands were also detected in the medium after immunoprecipitations with anti-MUC2(2) and anti-HGBM (Fig. 6A), indicating that mature MUC2 and MUC5B were secreted.

To further study mature mucins, pulse labeling with $^{35}$S-sulfate, which is incorporated in the last steps of the biosynthesis of mucins (6, 32), was performed. Anti-MUC2(2) and anti-HGBM immunoprecipitated mature MUC2 and MUC5B, respectively, after 0- and 4-h chase incubations from tissue homogenate of sigmoid (Fig. 6B). This indicates that MUC2 and MUC5B are secreted from the goblet cells, similar to the results shown in Fig. 6A.

Because mature colonic MUC2 and MUC5B have similar mobilities on SDS-PAGE, we performed analysis of $^{35}$S-sulfate-labeled mucins by agarose gel electrophoresis. This method was previously shown to distinguish electrophoretically between mature mucins (33). For comparison, LS 174T colon carcinoma cells, in which we previously showed the biosynthesis of MUC2 and MUC5B (40), were labeled and analyzed similarly. In both sigmoid and LS 174T cells, anti-MUC2(2) and anti-HGBM antibodies immunoprecipitated mature MUC2 and MUC5B, respectively, which were detected on agarose gel as diffuse bands (Fig. 7). From gallbladder, mature MUC5B (Fig. 7), but no mature MUC2, was immunoprecipitated (not shown). In immunoprecipitations from the sigmoid and LS 174T cells, mature

![Fig. 5. Immunofluorescence double labeling of human gallbladder, duodenum, and sigmoid. Anti-HGBM is shown in green and anti-MUC2(1) in red. Colocalization is rendered in shades of white, as shown in inset in c. Bars, 20 µm. a: Gallbladder, anti-HGBM. b: Duodenum, anti-MUC2(1). c: Sigmoid, anti-HGBM and anti-MUC2(1). d: Sigmoid sectioned through the bottom of a crypt, anti-HGBM and anti-MUC2(1).](http://appiophysiology.org/)
MUC2 (Fig. 7A) had a lower mobility on agarose gel than mature MUC5B (Fig. 7B).

**DISCUSSION**

The prominent HGBM is identical to MUC5B. The biosynthesis of the 470-kDa HGBM precursor and its maturation, secretion, and abundance in human bile has been demonstrated previously (21). Using the same antiserum raised against purified HGBM, we aimed to identify HGBM, because its identification is essential in studying the role of this mucin in disease. In the present study, we showed that MUC2, MUC4, and MUC6 precursors were not detectable in the gallbladder, whereas MUC3 precursors were detectable, displaying a different molecular mass on SDS-PAGE than HGBM precursors. Similarly, we previously demonstrated that, for Caco-2 and LS 174T cells, HGBM and MUC3 precursors displayed different molecular masses on SDS-PAGE (40). MUC1 was also not a likely candidate for the secretory mucin HGBM, since MUC1 is a relatively small membrane-bound mucin (11). Studies (3, 4, 16, 17, 19, 22, 40) previously indicated that MUC1, MUC3, MUC5B, and MUC6 are present in the gallbladder epithelium, whereas little or no MUC2, MUC4, or MUC5AC was detected. Moreover, MUC1, MUC3, and HGBM were also detected in bile (1, 21). Our immunoprecipitation data are thus in line with reported steady-state levels of mucin mRNA or protein, except that we could not immunoprecipitate MUC6 precursor from gallbladder homogenate. Most likely, the amount of MUC6 biosynthesized in the gallbladder is very low, because we were able to detect MUC6 precursors in LS 174T cells (40) and in human stomach (38), showing that the anti-MUC6 antibody functions well in immunoprecipitations. Finally, we showed that the mucin precursors immunoprecipitated from human gallbladder, by antibodies directed against HGBM, MUC5B, and BGBM, all recognized mucin precursors with similar apparent molecular masses (470 kDa). Moreover, by means of Western blotting with anti-MUC5B it was confirmed that these mucin precursors represented an identical mucin, namely MUC5B. In conclusion, the mucin detected by anti-HGBM antiserum is MUC5B and this mucin is biosynthesized in high amounts in the gallbladder, showing that MUC5B is the prominent HGBM. However, this does not exclude the presence of other mucins, such as MUC3, in human gallbladder.

We also showed that MUC5B was biosynthesized in the large, but not the small, intestine by performing immunoprecipitations with anti-MUC5B, anti-HGBM, and anti-BGBM antibodies. These antibodies all detected a similar tissue expression pattern, which was consistent in a large number of individuals. This gives further evidence that these antibodies recognize an identical mucin precursor, namely MUC5B.

To further substantiate the tissue-specific expression pattern of MUC5B, we analyzed MUC5B mRNA expression. By means of RT-PCR, we showed that MUC5B PCR fragments of the expected length (148 bp) could be amplified from gallbladder and colonic cDNA, but not from jejunal cDNA, indicating that the MUC5B mRNA and protein expression correlate.

Cellular localization of MUC5B in human gallbladder and intestine. To study the cellular localization of MUC5B, immunohistochemistry was performed. Klomp and co-workers (21) previously demonstrated an intense staining of mucous granules in gallbladder epithelial cells with anti-HGBM (21). The staining patterns of BGBM and MUC5B antibodies were similar to that of anti-HGBM: staining of all epithelial cells in the gallbladder, staining of goblet cells in the deeper crypt region of colon, and no staining in the small intestine. However, the cellular staining pattern was different: BGBM and MUC5B antibodies detected perinuclear antigens of colonic goblet cells, suggesting that rough endoplasmic reticulum-localized precursors were detected. This was anticipated because these antibodies recognize tandemly repeated amino acid sequences of BGBM and MUC5B, respectively (26, 27). These sequences are known to become masked on O-glycosylation (32), explaining the inability of anti-BGBM and anti-MUC5B to recognize mature MUC5B, which is stored in granules. In contrast, anti-HGBM recognizes the non-O-glycosylated sequences of HGBM and thus also detects mature mucin (21, 36), which explains the staining of the mucous granules by this antibody. In addition, MUC2 was contained in large mucous granules of the goblet cells of the colon and duodenum. Previously, it was demonstrated (3, 4, 16, 35) that MUC2 is expressed in all colonic goblet cells. However, MUC5B demonstrated a different distribution in colonic goblet cells. Double immunofluorescence labeling showed that MUC5B is typically expressed at a high level in the base of the colonic crypts, colocalizing to MUC2-containing goblet cells, while MUC5B expression ceases during migration of the goblet cells to the luminal side of the crypt. In addition, MUC5B is...
contained within small granules of the colonic goblet crypt cells. Mature MUC5B is secreted in the colon and can be discriminated from mature MUC2 by agarose gel electrophoresis. Previously, we showed that MUC2 is the prominent secretory mucin of the human colon (35). In the present study, we demonstrated that MUC5B is also a colonic secretory mucin. Strikingly, we found that mobilities on SDS-PAGE of mature MUC2 and MUC5B are much higher than expected. Because of extensive O-glycosylation, the molecular mass of the mature fully glycosylated mucin is expected to be much larger than 550 kDa. In fact, it was demonstrated that mobilities of mature mucins on SDS-PAGE are aberrant and depend highly on intrinsic negative charge (37). We therefore used agarose gel electrophoresis, which was previously shown to distinguish between mature mucin glycoproteins (33). For comparison, we studied the biosynthesis of mature MUC5B and MUC2 in the human LS 174T colon carcinoma cell line; in this cell line we previously showed the biosynthesis of both MUC2 and MUC5B (40). In the present study it is shown that mature MUC2 and MUC5B, synthesized by both sigmoid and LS 174T cells, can be discriminated by agarose gel electrophoresis, demonstrating unequivocally that mature MUC2 and MUC5B represent different secretory human colonic mucins.

The possible roles of MUC5B in disease. In this study we showed that MUC5B is expressed in the colon, but not in the small intestine, and this suggests a specific function for this mucin in the colon. Among other mucins, MUC5B serves a role in cytoprotection (10), for instance, in protecting the gallbladder from the deterrent effects of bile acids. It is tempting to speculate that MUC5B also protects the colonic epithelial cells from bile acids. For instance, MUC5B may protect the colon from unconjugated, hydrophobic bile acids, which are formed by the action of bacteria on bile acids present in the colon (18) and are potent inducers of mucin secretion (20). Colonic bile acids are causally related to the development of colorectal cancer (15, 24). Also, increased bile acid content of the stool is found in patients with inflammatory bowel disease (9). Therefore, in view of our data, it is of interest to study colonic MUC5B expression and function in these patients.

Of particular interest is the role of MUC5B in the development of gallstones. Now that we have identified the prominent HGBM as MUC5B, this will further aid in investigating the process of gallstone formation.

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