Human gallbladder mucin binds biliary lipids and promotes cholesterol crystal nucleation in model bile

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Abstract The binding of phosphatidylcholine and cholesterol in model bile to human gallbladder mucin was studied by means of a rapid filtration binding assay and sucrose density gradient ultracentrifugation. Numerous low affinity binding sites for phosphatidylcholine and cholesterol were present on gallbladder mucin. Binding of phosphatidylcholine and cholesterol to mucin increased as a function of cholesterol saturation index. Proteolytic digestion of mucin disaggregated the native mucin polymer and removed hydrophobic domains on the mucin peptide core that bind 1-anilino-8-naphthalenesulfonic acid. Proteolytic digestion also resulted in a 91% and 78% decrease, respectively, in the binding of phosphatidylcholine and cholesterol to mucin. The ability of trypsin-treated and native mucin to promote the nucleation of cholesterol monohydrate crystals was compared in a model bile. The incidence of cholesterol monohydrate crystal nucleation with native mucin was significantly greater at 3 days than with trypsin-treated mucin or controls ($P < 0.001$). After 3, 6, and 9 days of incubation, samples containing native mucin contained significantly more crystals than controls or trypsin-digested mucin samples ($P < 0.0001$ for each). These data indicate that highly purified human gallbladder mucin binds phosphatidylcholine and cholesterol in model bile. Furthermore, this study demonstrates that structural integrity of the native mucin polymer and hydrophobic domains on the peptide core are essential for the nucleation of cholesterol monohydrate crystals by mucin in model bile.—**Smith, B. F.** Human gallbladder mucin binds biliary lipids and promotes cholesterol crystal nucleation in model bile. *J. Lipid Res.* 1987. **28:** 1088—1097.

Supplementary key words gallstone • phosphatidylcholine • cholesterol • hydrophobic domains

Supersaturation of gallbladder bile with cholesterol is almost universally present in individuals who develop cholesterol gallstones (1, 2). The common occurrence of cholesterol supersaturation in the gallbladder bile of normal individuals, however, indicates that additional factors are needed to promote the initiation and growth of cholesterol gallstones (3). Controversy currently exists as to whether pro- or anti-nucleating factors are responsible for the development of cholesterol cholelithiasis in humans. Low molecular weight proteins have been described in lithogenic bile which promote nucleation of cholesterol crystals (4), while other low molecular weight proteins, possibly apolipoproteins, have also been described which retard the nucleation of cholesterol crystals in cholesterol supersaturated but nonlithogenic bile (5). Characterization of these low molecular weight protein fractions of bile is currently incomplete.

Evidence obtained in experimental animals and in humans indicates that gallbladder mucin, a high molecular weight glycoprotein secreted by the gallbladder epithelium, is important for the initiation of cholesterol gallstone formation. The initial stage of gallstone formation, the nucleation of cholesterol monohydrate crystals, occurs in mucus gel adherent to the gallbladder epithelium (6, 7). Lee, LaMont, and Carey (8) demonstrated a marked hypersecretion of mucin resulting in the accumulation of mucus gel in the gallbladder lumen prior to the appearance of cholesterol crystals and stones in the cholesterol-fed prairie dog. Moreover, aspirin, in doses that suppressed mucin hypersecretion, prevented cholesterol crystal nucleation and stone formation while not altering the supersaturation of gallbladder bile with cholesterol (9). Recent prospective ultrasonographic studies (10) in humans have documented the formation of gallstones in gallbladder sludge, which contains mucus and bile pigments. Moreover, a mucin-bilirubin complex has been identified in the nonlipid matrix at the center of cholesterol gallstones which has a pigment composition

Abbreviations: ChMC, cholesterol monohydrate crystals; HGM, human gallbladder mucin; HMG-T, trypsin-digested human gallbladder mucin; BGM, bovine gallbladder mucin; BGM-T, trypsin-digested bovine gallbladder mucin; Ch, cholesterol; PC, phosphatidylcholine; NaTC, sodium taurocholate; PBS, phosphate-buffered saline; 0.1 M sodium phosphate, 0.15 M sodium chloride, 0.03% sodium azide, pH 7.0; CSI, cholesterol saturation index; ANS, 1-anilino-8-naphthalenesulfonate.

identical to biliary sludge (11). These observations taken together suggest that gallbladder sludge, consisting of mucus gel and bile pigments, may serve as the nidus for gallstone development in man.

Highly purified human gallbladder mucin accelerates the nucleation of cholesterol monohydrate crystals (ChMC) in supersaturated model bile (12). This nucleation-promoting effect is dependent on mucin concentration, total lipid concentration, and the cholesterol saturation index of model bile. The nucleation-promoting effect of mucin has also been demonstrated in supersaturated human gallbladder bile (13). However, the mechanism by which mucin promotes the nucleation of ChMC in model bile is unknown. A previous study of gallbladder mucin structure demonstrated protease-sensitive hydrophobic domains on the nonglycosylated portion of the mucin peptide core (14). This study examines the ability of mucin to bind biliary lipids and compares the pro-nucleating properties of native and protease-digested mucin. We report that highly purified human gallbladder mucin (HGM) binds phosphatidylcholine (PC) and cholesterol (Ch) in supersaturated model bile. Proteolytic digestion of mucin disaggregates the native mucin polymer and removes hydrophobic domains on the mucin peptide core. Furthermore, proteolytic digestion significantly reduces the binding of PC and Ch to mucin and impairs the ability of mucin to nucleate ChMC in supersaturated model bile. These findings suggest that the binding of biliary lipids and the nucleation of ChMC by gallbladder mucin are dependent on the structural integrity of hydrophobic domains on the mucin peptide core.

MATERIALS AND METHODS

Gallbladder mucin purification

Human gallbladder mucin (HGM) was purified from mucosal scrapings of gallbladders obtained at autopsy from patients without biliary tract disease as previously described (12). Bovine gallbladder mucin (BGM) was purified from normal cow gallbladders (14). Purified mucins were free of low molecular weight protein contaminants as gauged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and contained less than 0.2% by weight as lipid (12, 14).

Proteolytic digestion of gallbladder mucin

Initially, pronase (*Streptomyces gresius*, type 6, Sigma Chemical Co., St. Louis, MO) was used to remove the hydrophobic domains on the nonglycosylated portion of the mucin peptide core (14). Subsequently, highly purified trypsin (2 × crystallized containing TRSF, Worthington Biochemical Corp., Freehold, NJ) was found to be equally effective in removing the hydrophobic domains on gallbladder mucin (15) and was used in place of pronase because of its greater purity and enzymatic specificity. Proteolytic digestion of mucin was performed only after mucin had been completely purified (12, 14). All proteolytic digestion experiments were performed in duplicate with active and heat-killed enzyme. Both pronase and trypsin were boiled at 100°C for 5 min in their respective buffers to destroy enzymatic activity. Pronase digestion was performed in a 0.1 M sodium phosphate, 0.15 M NaCl, 0.03% sodium azide, pH 7.0 buffer (PBS). Proteolysis using trypsin was performed in a 0.46 M Tris, 0.0115 M CaCl, 0.03% sodium azide, pH 8.0 buffer. In both systems, lyophilized purified mucin was reconstituted in the appropriate buffer at a concentration of 4 mg/ml and either heat-killed or active enzyme was added. Mucin–enzyme mixtures were incubated at 37°C for 72 hr with addition of enzyme at 0, 24, and 48 hr at a mucin–enzyme ratio of 20 :1 (w/w). Following digestion, mucin was dialyzed for 24 hr against 0.2 M NaCl, 0.03% sodium azide and then chromatographed over a Sepharose 2B-CL column (87 cm × 2.6 cm) equilibrated in the same buffer. Column fractions (15 ml) were monitored for glycoprotein using the periodic acid/Schiff assay (16). Peak fractions of digested mucin were pooled, dialyzed against deionized water for 72 hr, lyophilized, and stored at −20°C until used.

Preparation of model bile

Cholesterol USP (Ch), egg yolk phosphatidylcholine type 5 (PC), and sodium taurocholate (NaTC) were obtained from Sigma Chemical Co., St. Louis, MO. Ch was purified by double recrystallization from hot 95% ethanol. NaTC was purified by recrystallization by the method of Pope (17) two times. PC was used as supplied in chloroform–methanol 9:1 (v/v). Lipids were stored at 4°C in the dark under N_2 to avoid oxidation prior to use. Cholesterol saturation indices (CSI) were determined from the critical tables of Carey (18). The molar ratio of PC/(NaTC and PC) in model bile was 0.2 and all samples were prepared with an initial total lipid concentration of 10 g/dl. This value was chosen to reflect the average concentration of lipids in lithogenic human gallbladder bile (2). Model biles were prepared by co-precipitation of the lipids from organic solvents, followed by resuspension in PBS (12). Model biles were then heated to 80°C for 1 hr to obtain an isotropic phase, filtered through a sterile 0.22 μm filter (Gelman Acrodisc, Gelman Sciences Inc., Ann Arbor, MI), and equilibrated at 37°C for 4 hr before use. For the binding experiments outlined below, [4-^{14}C]cholesterol and 1-palmitoyl,2-[9,10-^{3}H]palmitoyl-*sn*-glycerophosphocholine (New England Nuclear Research Products, Boston, MA) were added to model bile in tracer quantities prior to co-precipitation from organic solvents. Specific activities for both radioisotopes were determined

after reconstitution of model bile and used to quantitate the moles of PC or Ch bound to mucin.

Rapid filtration binding assay

To quantitate the binding of PC and Ch to gallbladder mucin in model bile, a rapid filtration binding assay was developed which used cellulose nitrate filters with a pore size of 0.1 μm (Sartorius Filters Inc., Hayward, CA). Validation experiments demonstrated that these filters retained 88 ± 3.9% (mean ± SEM) of native gallbladder mucin and 75 ± 4.2% of protease-digested mucin. Highly purified, lyophylized HGM was reconstituted at a concentration of 4 mg/ml in a 0.1 M sodium phosphate, 0.15 M NaCl, 0.03% sodium azide, pH 7.0 buffer containing 5 mM NaTC (PBS-TC). Aliquots of dual isotopically labeled model bile (^{3}H-PC and ^{14}C-Ch) and HGM were mixed and the final volume of the mucin-model bile mixtures was brought to 250 μl with the PBS-TC buffer. After incubation at 37°C for 2 hr, 200 μl of the original 250 μl mucin-model bile mixture was vacuum-filtered over the cellulose nitrate filter. A 2-ml wash of the PBS-TC buffer was immediately vacuum-filtered in an identical manner and radioactivity remaining on the filter was determined using a liquid scintillation counter (Prias model, Packard Instrument Co., Downers Grove, IL) to quantitate the ^{3}H-PC and ^{14}C-Ch bound to mucin. The radioactivity in an aliquot of the remaining 50 μl of the mucin-model bile mixture was determined to calculate the total radioactivity applied to the filter. The nonspecific binding of ^{3}H-PC and ^{14}C-Ch to the filter was determined for each data point by filtering an identical sample of model bile containing the PBS-TC buffer without HGM. The amount of PC and Ch bound to mucin was calculated from the radioactivity remaining on the filters in the presence of mucin minus the radioactivity on the filters in the absence of mucin. Nonspecific binding of PC and Ch to the filters in the absence of mucin accounted for approximately 30–40% of the total radioactivity remaining on the filters in the presence of mucin. On the other hand, only 0.08% of radiolabeled glucose remained on the filters when filtered in an identical manner with the same concentration of mucin, indicating that mucin had not plugged pores in the filter. Recovery of radioactivity applied to the filters ranged from 92–97% when counts remaining on the filter and in the eluate were compared to the radioactivity applied to the filter. Variation between the triplicate samples used to generate each data point averaged 5.2 ± 1.4%.

In this binding assay either the concentration of HGM or model bile could be varied. The specific activities of ^{3}H-PC and ^{14}C-Ch were used to quantitate the moles of bound lipid and an estimated molecular weight of 2×10^6 was used to calculate the moles of HGM in the assay. The buffer in these experiments contained 5 mM NaTC to maintain the critical micellar concentration of NaTC and prevent disruption of mixed micelles during dilution of model bile.

Sucrose density gradient ultracentrifugation

Sucrose density gradient ultracentrifugation was used to confirm the binding of PC and Ch in model bile to HGM. Step gradients (9 ml) of 30% (w/v) sucrose in 0.1 M sodium phosphate, 0.15 M NaCl, 0.03% sodium azide, pH 7.0, containing 10 mM NaTC were used to separate lipid bound to HGM from unbound lipid. Model bile containing ^{3}H-PC and ^{14}C-Ch with a total lipid concentration of 10 g/dl and a CSI of 1.4 was mixed with HGM with a final ratio of model bile–HGM of 1:5 (w/w). The mucin-model bile mixture was incubated for 4 hr and a 450-μl aliquot was layered on top of the gradient. The gradient was centrifuged at 20,000 *g* for 16 hr in a Ti 70.1 fixed-angle rotor on a Beckman L8-70 ultracentrifuge. Gradients were fractionated by aspiration into eight equal fractions of approximately 1.2 ml. ^{3}H-PC and ^{14}C-Ch were quantitated in each fraction in a liquid scintillation counter. Because of the differences in the ratio of mucin-model bile (w/w) used in centrifugation and rapid filtration binding experiments, the results of these experiments were not quantitatively comparable.

Nucleation experiments

Model bile (CSI = 1.4; total lipid = 10 g/dl) was incubated without protein addition (control, n = 8) or with albumin (Alb), purified bovine gallbladder mucin (BGM), trypsin-digested bovine gallbladder mucin (BGM-T), purified human gallbladder mucin (HGM), or trypsin-digested human gallbladder mucin (HGM-T). Each protein addition experiment was performed at three protein concentrations (2, 4, 8 mg/ml) and contained four samples at each concentration. Proteins were dissolved in deionized water, added to sterile incubations vials in the appropriate concentrations, lyophilized, and reconstituted in 250 μl of model bile. Proteins were dissolved by vortexing incubation vials for 30 sec, and the samples were incubated without agitation at 37°C. Cholesterol monohydrate crystals (ChMC) were identified by polarizing light microscopy as birefringent, notched rhomboidal plates. ChMC in each sample were counted in a hemocytometer chamber after 3, 6, and 9 days of incubation. ChMC count variability was less than 2% at low crystal numbers, but was as high a 10–12% at ChMC counts in excess of 2000/mm^3. The effect of mucins on the nucleation of ChMC was analyzed by comparing the incidence of nucleation in each protein addition group on day 3 with Fisher's exact test. In addition, the influence of protein type and concentration on nucleation was examined by comparing the number of ChMC on days 3, 6, and 9 by two-way analysis of variance (ANOVA) and Duncan's test for multiple comparisons.

RESULTS

Binding of phosphatidylcholine (PC) and cholesterol (Ch) in model bile to human gallbladder mucin

Binding of PC and Ch to highly purified HGM was demonstrated by the rapid filtration assay in model bile with a composition reflecting average values of lithogenic human gallbladder bile (total lipid concentration = 10 g/dl; CSI = 1.4) (**Fig. 1**). Binding for both PC and Ch was found to be linear in the concentration range of HGM used. Kinetic analysis of PC and Ch binding to HGM using a double reciprocal plot revealed numerous, low affinity binding sites on HGM for both PC and Ch. For PC, the dissociation constant (K_d) was 2.63×10^{-3} with the estimated number of binding sites, n, being 212. For Ch, the K_d was 2.49×10^{-3} with n = 119. The total molar concentrations of PC and Ch were used to calculate these binding parameters, rather than estimates of their monomeric concentrations. Since PC and Ch are present primarily in lipid aggregates in model bile (micelles, vesicles), these values need to be interpreted with caution and are not comparable to previous estimates of hydrophobic domains on mucin using monomeric solutions of l-anilino-8-naphthalene sulfonate (14). Also, since an exact molecular weight for HGM is not available, an estimated value of 2×10^6 was used. This value was based on previous study of bovine gallbladder mucin (14). The kinetic data should, therefore, be considered as approximate values.

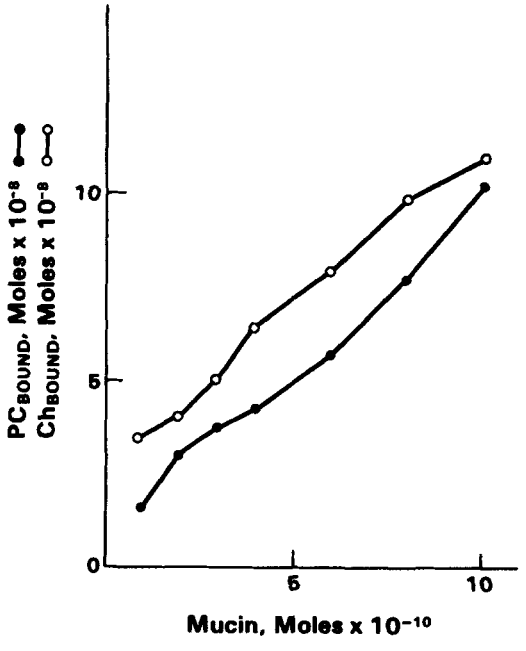

Fig. 1. Binding of PC and Ch to HGM in model bile. Lyophilized HGM was reconstituted in deionized water at a concentration of 1 mg/ml and 50–500 μg was placed in sterile incubation vials and relyophilized. HGM in incubation vials was then reconstituted in 250 μl of dual isotopically labeled model bile (^{3}H-PC and ^{14}C-Ch) with a CSI = 1.4 and total lipid = 10 g/dl, and the mixture was incubated for 4 hr at 37°C. Two hundred μl of the HGM-model bile mixture was then vacuum-filtered over a cellulose nitrate filter that had a pore size of 0.1 μm. Two ml of a PBS buffer containing 5 mM NaTC was then vacuum-filtered over the same filter. Radioactivity remaining on the filter was determined in a scintillation counter to quantitate the amount of PC and Ch bound to HGM. Background binding to the filter was determined by filtering model bile in the absence of HGM. Results are expressed as the moles of PC and Ch bound versus the moles of HGM present in the HGM-model bile mixture.

Effect of CSI on the binding of PC and Ch to HGM in model bile

PC and Ch binding to HGM was found to be dependent on the degree of cholesterol saturation in model bile (**Fig. 2**). In model bile that was unsaturated (CSI = 0.6) or just saturated (CSI = 1.0), there was demonstrable binding of PC to HGM, but negligible binding of Ch to HGM. In supersaturated model bile with a CSI of 1.4, however, the binding of both Ch and PC to HGM was markedly increased and did not exhibit saturation at the concentration of HGM used. The disproportionate increase in the binding of Ch to HGM in supersaturated bile as compared to PC suggests that in supersaturated model bile a cholesterol-rich particle, such as the cholesterol-lecithin microprecipitate described by Mazer and Carey (19), may be binding to HGM.

Sucrose density gradient ultracentrifugation

Sucrose density gradient ultracentrifugation confirmed the binding of PC and Ch to HGM in model bile. Because of their low buoyant density, lipids are recovered in the low density fractions at the top of a 30% (w/v) sucrose gradient, while HGM, because of its high buoyant density is recovered in the high density fractions at the bottom of the sucrose gradient (20). In **Fig. 3**, the elution profiles of ^{3}H-PC and ^{14}C-Ch in model bile on a 30% sucrose (w/v) step gradient are compared in the presence or absence of HGM. In the absence of HGM, both ^{3}H-PC and ^{14}C-Ch eluted as a single peak in the low density fractions at the top of the gradient as expected. However, in the presence of HGM, 11% of the ^{3}H-PC and 13% of the ^{14}C-Ch coeluted with HGM in the high density fractions at the bottom of the gradient, thereby confirming their binding.

Effect of proteolysis of HGM on binding of PC and Ch in model bile

Figure 4 shows the Sepharose 2B column chromatography of native purified HGM, pronase-digested and trypsin-digested HGM. Both pronase and trypsin digestion resulted in a shift in the elution profile of HGM from the void volume to the included volume. As indicated in Fig. 4, peak glycoprotein fractions in the included volume were pooled, dialyzed against deionized water at 4°C for 72 hr, lyophilized, and stored at −20°C prior to use in binding or nucleation experiments.

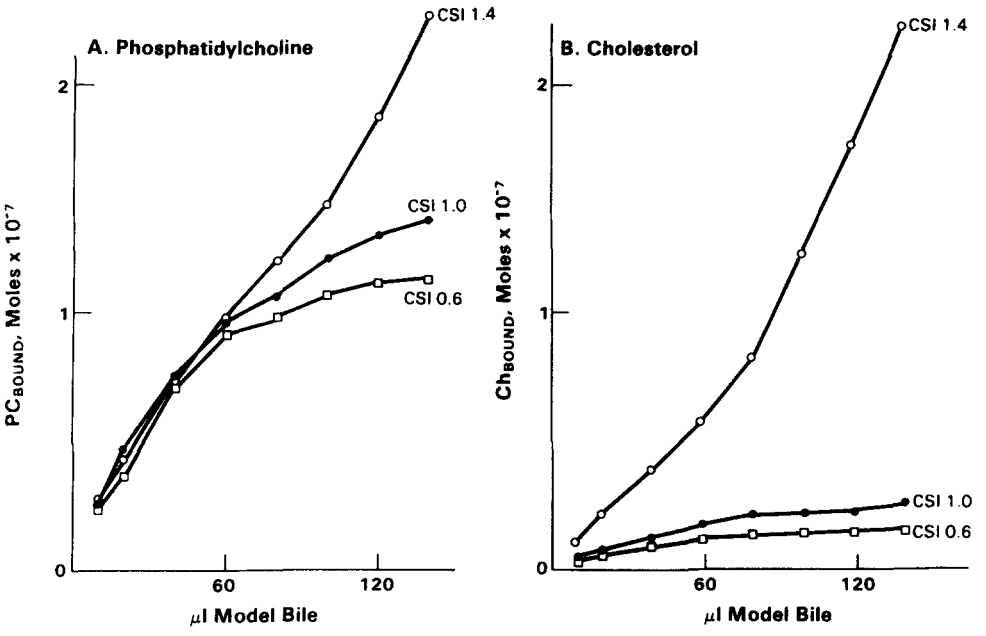

Fig. 2. Effect of cholesterol saturation index on the binding of PC and Ch to HGM in model bile. Lyophilized HGM was reconstituted in a PBS buffer containing 5 mM NaTC and 50 μl (200 μg) was mixed with increasing amounts of dual isotopically labeled model bile (10–140 μl). Model bile had a total lipid = 10 g/dl and CSI of 0.6, 1.0, or 1.4. All samples were brought to a final volume of 250 μl with a PBS buffer containing 5 mM NaTC. Binding of PC and Ch to HGM was determined as in Fig. 1. Panel A shows the moles of PC and panel B the moles of Ch bound to HGM versus the quantity of model bile added.

Pronase digestion of HGM caused a nearly complete elimination of the binding of the hydrophobic fluorescent probe 8-anilino-1-naphthalene sulfonic acid (ANS) to HGM (**Fig. 5**). The binding of PC and Ch to native HGM and pronase-digested HGM was compared using the same concentration for each protein (**Fig. 6**). Pronase digestion decreased the binding of PC and Ch to HGM by 91% and 78%, respectively.

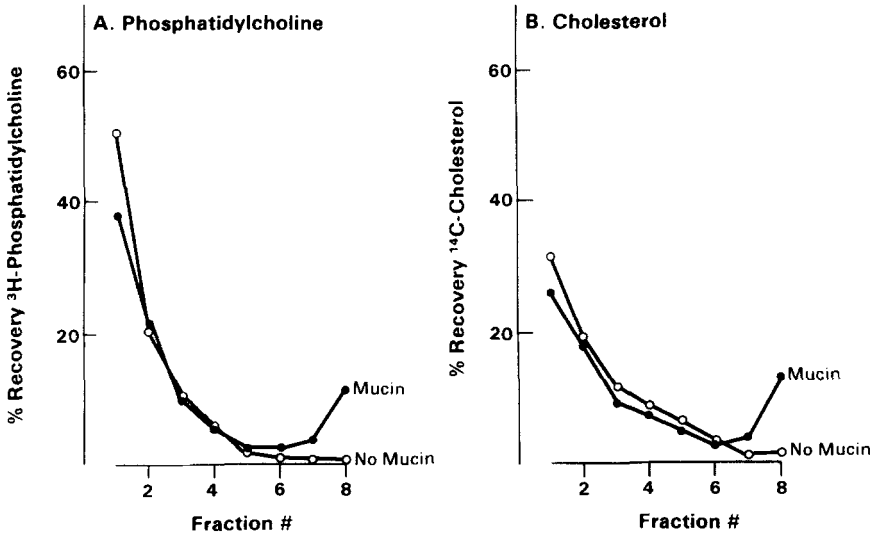

Fig. 3. Sedimentation of PC and Ch with HGM during sucrose density gradient ultracentrifugation. HGM (5 mg) was mixed with 1 mg of model bile (CSI = 1.4; total lipid = 10 g/dl) in a total volume of 450 μl which was then layered on a 30% (w/v) sucrose gradient (9 ml) in a PBS buffer containing 10 mM NaTC. The gradient was centrifuged in a Ti 70 fixed-angle rotor for 16 hr at 20,000 *g*. The gradient was fractionated into eight fractions of 1.2 ml and radioactivity was determined in a scintillation counter. Panel A shows the sedimentation profile of PC and panel B that of Ch in the presence (closed circles) or absence (open circles) of HGM. HGM was recovered in fractions 7 and 8 (not shown).

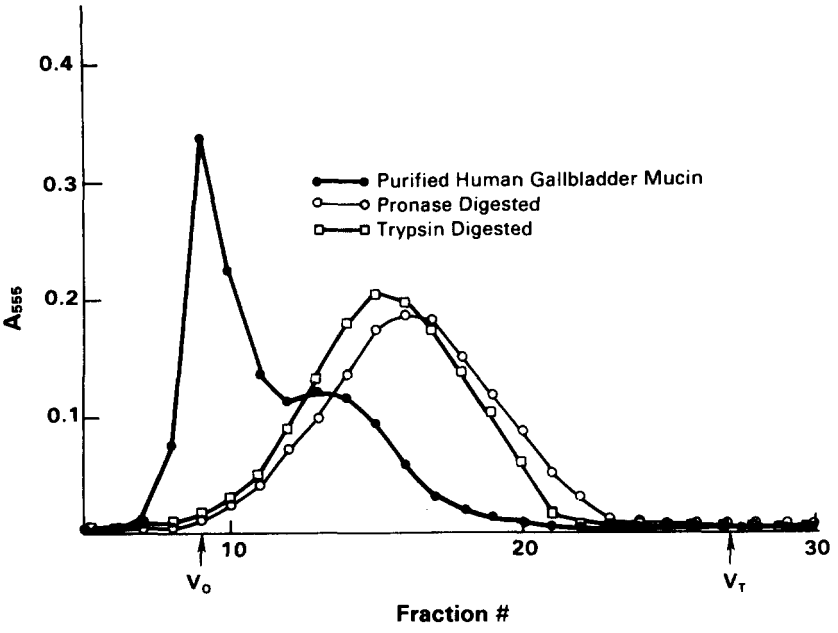

Fig. 4. Sepharose 2B column chromatography of proteolytically digested HGM. Purified human gallbladder mucin was digested with either pronase or trypsin. Pronase digestion was performed in a 0.1 M sodium phosphate, 0.15 M NaCl, 0.03% sodium azide, pH 7.0 buffer. Trypsin digestion was performed in a 0.46 M Tris, 0.0115 M CaCl, 0.03% sodium azide, pH 8.0 buffer. Highly purified, lyophilized gallbladder mucin was reconstituted in the appropriate buffer at a concentration of 4 mg/ml and enzyme was added at a mucin-enzyme ratio of 1:20 (w/w) at 0, 24, and 48 hr. Mucin-enzyme mixtures were incubated at 37°C for 72 hr and then dialyzed against 0.2 M NaCl, 0.03% sodium azide for 24 hr at 4°C prior to column chromatography. After dialysis, mucin-enzyme mixtures were chromatographed over a Sepharose 2B column, 87 cm x 2.6 cm, equilibrated in 0.2 M NaCl, 0.03% sodium azide. Fractions (15 ml) were monitored for glycoprotein at A_{555} using the periodic acid/Schiff assay. Glycoprotein in fractions 11 through 20 was pooled, dialyzed against deionized water at 4°C for 72 hr, lyophilized, and stored at −20°C until used.

Nucleation experiments

Two parameters of cholesterol monohydrate crystal (ChMC) nucleation in model bile were examined: the incidence of nucleation at day 3 and the quantity of ChMC nucleated at days 3, 6, and 9. Differences in the incidence of nucleation at day 3 between different protein-addition groups was compared with Fisher's exact test using a chi square analysis (**Table 1**). HGM differed from control and HGM-T with $P < 0.001$ and $P < 0.002$, respectively. BGM differed from control and BGM-T with $P < 0.02$ and $P < 0.001$, respectively. Neither HGM-T nor BGM-T differed significantly from control.

The number of ChMC nucleated with the different protein additions is shown in **Table 2**. Analysis of variance demonstrated that the number of ChMC nucleated at 3, 6, and 9 days was significantly greater with HGM than with control, albumin, or HGM-T ($P < 0.0001$ for each). Moreover, the concentration of HGM had a significant effect on the number of ChMC nucleated ($P < 0.0001$) confirming the concentration-dependent effect of HGM on nucleation. The number of ChMC at each time point and concentration was also significantly increased with BGM when compared to control, albumin, or BGM-T ($P < 0.0001$ for each).

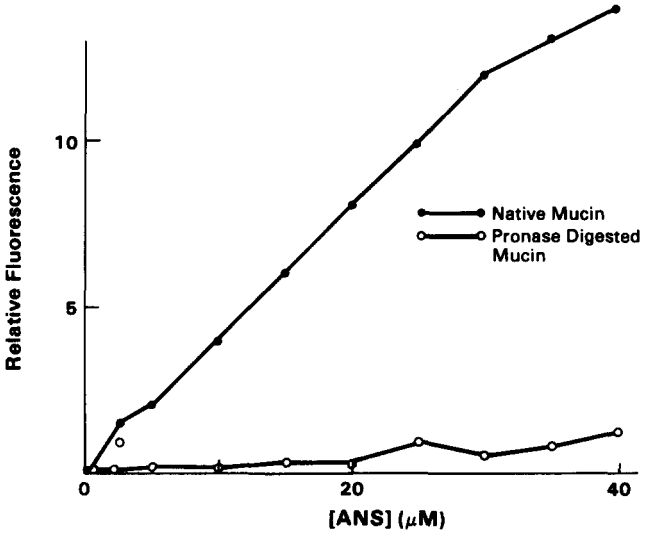

Fig. 5. Effect of proteolysis on ANS binding by HGM. ANS was mixed with either native HGM (closed circles) or pronase-treated HGM (open circles, see Methods). A fixed amount of mucin (200 μg/ml) was mixed with increasing concentrations of ANS (2–40 μM). Fluorescence was measured with an excitation wavelength of 365 nm and an emission wavelength of 475 nm. Results are expressed as relative fluorescence versus ANS concentration.

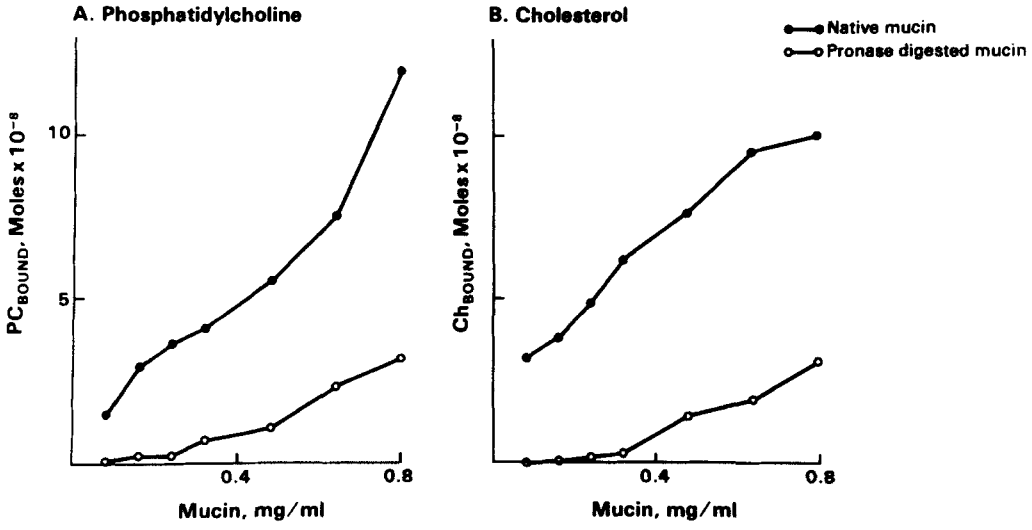

Fig. 6. Effect of proteolysis on PC and Ch binding to HGM in model bile. Two hundred μl of model bile (CSI = 1.4; total lipid = 10 g/dl) was mixed with increasing quantities of mucin (20–200 μg) and the binding of PC and Ch was determined as in Fig. 1. Results are expressed as the moles of PC (panel A) or Ch (panel B) bound versus the amount of native mucin (closed circles) or pronase-digested mucin (open circles) added.

DISCUSSION

Gallbladder mucin, a high molecular weight glycoprotein, is the major secretory product of the gallbladder epithelium, and the principal organic constituent of gallbladder mucus. Compositional analysis of bovine gallbladder mucin (14) has shown that 75% of mucin weight is contributed by branching oligosaccharide side chains which are primarily responsible for the distinctive visco-elastic and gel-forming properties of mucin (21). The mucin peptide core contributes approximately 16% of mucin weight and appears to have at least two functionally separate domains. One domain is resistant to proteolytic digestion and contains the majority of covalently linked carbohydrate. Steric hinderance caused by tight spatial packing of the oligosaccharide side chains may be responsible for protease resistance (22). Another domain on the peptide core is sensitive to proteases, and has been shown to contain hydrophobic binding domains for the fluorescent probes 8-anilino-l-naphthalene sulfonic acid and N-phenylnaphthylamine (14). Compositional analysis of protease-digested bovine gallbladder mucin demonstrated a reduction in the contribution of protein to mucin weight from 16.35% to 9.1% without demonstrable

TABLE 1. Incidence of nucleation on day 3

	Protein Concentration (mg/ml)															
	Control	Albumin			BGM			BGM-T			HGM			HGM-T		
	0	2	4	8	2	4	8	2	4	8	2	4	8	2	4	8
Incidence of nucleation	1/8	0/4	0/4	1/4	2/4	3/4	3/4	0/4	0/4	0/4	4/4	4/4	4/4	0/4	1/4	4/4
Total	1/8	1/12			8/12[a,b]			0/12[c]			12/12[d,e]			5/12[e]		

Model bile (CSI = 1.4, total lipid = 10 g/dl) was incubated with either no protein (control, n = 8), albumin, native mucins (BGM, HGM), or trypsin-digested mucins (BGM-T, HGM-T). Proteins were added at concentrations of 2, 4, and 8 mg/ml (n = 4 at each concentration). Nucleation, defined as the presence of cholesterol monohydrate crystals, was determined by polarizing microscopy after incubation at 37°C for 3 days. The numerator in each ratio indicates the number of samples in each group that contained crystals and the denominator indicates the number of samples in each group. Differences in the incidence of nucleation between different groups was determined with

[a] $P < 0.02$ versus control.
[b] $P < 0.001$ versus BGM-T.
[c] Not significantly different from control.
[d] $P < 0.001$ versus HGM-T.
[e] $P < 0.002$ versus control.

TABLE 2. Cholesterol monohydrate crystal nucleation in model bile

	Protein Concentration (mg/ml)															
	Control	Albumin			BGM			BGM-T			HGM			HGM-T		
	0	2	4	8	2	4	8	2	4	8	2	4	8	2	4	8
	ChMC/mm³															
Day 3	1	0	0	3	10	13	10	0	0	0	18	10	48	0	3	18
Day 6	0	20	43	20	178	383	663	23	32	58	260	475	635	33	75	158
Day 9	191	158	150	138	883	2248	3085	200	212	245	2160	3130	5340	1082	368	363

Model bile (CSI = 1.4, total lipid = 10 g/dl) was incubated with either no protein (control, n = 8), albumin, native mucin (BGM, HGM), or trypsin-digested mucin (BGM-T, HGM-T). Proteins were added at concentrations of 2, 4, and 8 mg/ml (n = 4 at each concentration). The number of cholesterol monohydrate crystals (ChMC/mm³) was determined by means of polarizing light microscopy after incubation at 37°C for 3, 6, and 9 days. Values are means for each group. Results were analyzed with two-way ANOVA for the type and concentration of protein and Duncan's test for multiple comparisons. HGM was significantly different from control, albumin, and HGM-T at days 3, 6, and 9 ($P < 0.0001$ for each). BGM was significantly different from control, albumin, and BGM-T at days 3, 6, and 9 ($P < 0.0001$ for each).

loss of carbohydrate (14). Hydrophobic domains have also been identified on the peptide core of bovine cervical mucin (23). During purification of mucin from gallbladder mucosal homogenates, PC, Ch, and other lipids coelute with macromolecular mucin during molecular sieve chromatography (14), suggesting that mucin may bind these lipids. Lipid binding has been reported for both respiratory (24) and gastric (25) mucin, and may represent a generalized phenomenon among epithelial mucins.

The mechanism by which mucin promotes ChMC nucleation is unknown. Several studies in both man and experimental animals have observed that ChMC nucleation occurs in a mucus gel layer adherent to the gallbladder epithelium, rather than in the bulk aqueous phase of gallbladder bile (6, 7, 9, 10). This suggests that the mucus gel may provide a favorable environment for the heterogeneous nucleation of ChMC from supersaturated bile. Synthetic gels are commonly used to enhance crystal nucleation during the industrial purification of inorganic salts (26), and a similar nucleation-promoting effect may be caused by the physical chemical interaction of mucus gel and supersaturated gallbladder bile. Physical chemical interactions of lipids and gastric mucin have been reported to increase the viscosity of gastric mucus in vitro (27). Alternatively, components of aqueous gallbladder bile may be selectively sequestered in the mucus gel, and nucleation of ChMC may be promoted by this segregation phenomenon. Several observations indicate that the more hydrophobic components of aqueous gallbladder bile are concentrated in gallbladder mucus gel. Allen et al. (28) demonstrated that the hydrophobic bile pigments, bilirubin and bilirubin monoglucuronide, were present in large quantities in the gel phase of gallbladder bile while only trace amounts of the hydrophilic bilirubin diglucuronide were present. The ability of mucin to hydrophobically bind bilirubin may be responsible for this phenomenon (20). In addition, Womack (7) demonstrated a two- to threefold increase in the cholesterol content of the mucus gel phase of gallbladder bile as compared to the bulk aqueous phase of gallbladder bile. The present report offers a potential explanation for the apparent association of gallbladder mucus and biliary lipids.

The current study demonstrates binding of PC and Ch to HGM using physical separation techniques based on molecular size (filtration binding assay) and buoyant density (sucrose density gradient ultracentrifugation). The rapid filtration binding assay demonstrated binding of PC and Ch to HGM in supersaturated model gallbladder bile which was linear over the concentrations of HGM used (Fig.1). Kinetic analysis using a double-reciprocal plot demonstrated multiple low affinity binding sites on HGM for PC and Ch. The low binding affinities may reflect competition between mucin and NaTC-containing mixed micelles for the binding of PC and Ch in model bile. The binding of PC and Ch to HGM was confirmed with sucrose density gradient ultracentrifugation (Fig. 3). This technique has been previously used to demonstrate the binding of bilirubin to BGM in vitro (20).

The disproportionate increase of Ch binding to HGM seen with supersaturated model bile (CSI = 1.4) suggests that the physical state of Ch in model bile has a major effect on mucin-Ch binding. In model bile with a CSI = 1.4, Ch is solubilized in mixed micelles as well as lipid vesicles with a Ch/PC molar ratio of approximately 2:1 (19, 29). Our data indicate that nonmicellar Ch may bind preferentially to HGM (Fig. 2).

Highly purified HGM digested with either pronase or trypsin eluted in the included volume of a Sepharose 2B column rather than in the void volume, indicating a disaggregation of the native mucin polymer (Fig. 4). Moreover, proteolysis effectively removed hydrophobic domains on the nonglycosylated portion of the mucin peptide core as measured by ANS binding (Fig. 5). This finding is in good agreement with previous observations of the polymer structure and hydrophobic binding properties of BGM, which suggest that the gallbladder mucin polymer may be formed by noncovalent aggregation of

mucin subunits (14). Currently, it is not possible to remove the hydrophobic domains on the mucin peptide core without disaggregating the mucin polymer. Proteolytic digestion of HGM also resulted in significantly reduced binding of PC and Ch to HGM (91% and 78%, respectively) (Fig. 6). This finding suggests that PC and Ch bind to hydrophobic domains on the mucin peptide core in a fashion analogous to the previously demonstrated binding of ANS. Because of the limited information available regarding the structure of the mucin peptide core, one cannot conclude that the ANS and PC and Ch binding sites are identical. However, the protease sensitivity of binding suggests that both these molecules bind to the mucin peptide core rather than to the oligosaccharide side chains of mucin.

It is unlikely that hydrophobic binding sites on HGM are specific for PC or Ch. Hydrophobic domains are present on several epithelial mucins (23–25), and may be important for the protection of epithelial cells by mucus. The nonspecific hydrophobic binding properties of mucus may be of particular importance in the gallbladder whose epithelium is exposed to the detergent properties of concentrated bile. Other proteins in gallbladder bile may also interact with biliary lipids (4, 5). Recent studies have reported that apolipoproteins are present in bile (30), and have demonstrated that apolipoproteins A-I and A-II inhibit ChMC nucleation in model bile (31). These observations do not preclude the apparent importance of gallbladder mucus in promoting gallstone formation (6–10), but rather indicate the complexity of factors that modulate ChMC nucleation in vivo.

Structural integrity of the mucin peptide core appears to be essential for the pro-nucleating properties of mucin. Two mucins (HGM and BGM), which promote ChMC nucleation in model bile (12), were digested with trypsin to remove the hydrophobic domains on the peptide core. The trypsin-digested mucins (BMG-T and HGM-T) were then compared with native HGM and BGM with regard to their ability to promote ChMC nucleation in supersaturated model bile. Both native mucins demonstrated a highly significant concentration dependent increase in the number of ChMC nucleated at days 3, 6, and 9 (Table 2). Trypsin-digested mucins, however, did not increase the nucleation of ChMC above control samples nor was a concentration dependence of nucleation present. This finding extends previous observations on the pro-nucleating properties of mucin by demonstrating that structural integrity of the mucin peptide core is essential for mucin to promote ChMC nucleation in supersaturated model bile.

Previous in vitro studies of ChMC nucleation have measured a single parameter of nucleation, the nucleation time, which has been defined as the time required during in vitro incubation for the initial appearance of ChMC (13, 29, 32). This study has employed a more quantitative analysis of the effect of mucin on ChMC nucleation. This study has attempted to determine whether mucin accelerates nucleation of ChMC or merely serves to support the growth of spontaneously nucleated crystals (33). The significantly increased incidence of ChMC nucleation in mucin-containing samples (Table 1) at day 3 strongly suggests that mucin is responsible for the initiation of ChMC nucleation rather than supporting the growth of spontaneously nucleated crystals. This finding, coupled with the significantly increased numbers of ChMC present at each time point (Table 2), indicates that HGM is a potent nucleator of ChMC in model bile. The ability of mucin from normal and bovine gallbladders to nucleate ChMC in supersaturated model bile suggests that the nucleation-promoting properties of mucin do not require the synthesis and secretion of an abnormal mucin by the gallbladder epithelium of lithogenic individuals. Recent studies do suggest, however, that abnormal quantities of mucin are secreted and retained as a viscous gel within the gallbladder lumen prior to gallstone formation in experimental animals and humans (6–10, 12).

The concentration range of mucin in this nucleation study (2–8 mg/ml) is lower than concentration of mucin in biliary sludge (30–37 mg/ml), but higher than that in the aqueous phase of gallbladder bile (34). Studies in our laboratory indicate that a significant increase in mucin viscosity occurs at concentrations greater than 10 mg/ml (Smith, B. F., unpublished observations). In order to study the influence of mucin on nucleation of ChMC independent of any effects which viscosity may have on the nucleation process, the concentration of mucin was kept below 10 mg/ml.

In summary, this study presents two findings that may have direct relevance in understanding the role of gallbladder mucin in the pathogenesis of cholesterol cholelithiasis in humans. First, highly purified HGM binds PC and Ch in model bile with the composition of lithogenic human gallbladder bile. Second, proteolytic digestion significantly reduces the ability of mucin to bind PC and Ch and to promote the nucleation of ChMC in supersaturated model bile. This finding suggests that structural integrity of the hydrophobic domains on the mucin peptide core is necessary for mucin to promote ChMC nucleation. This study provides experimental evidence at the molecular level which supports the findings of previous studies using whole animals (8, 9) that indicate that gallbladder mucus is a pronucleating agent for the formation of ChMC in cholesterol-supersaturated bile. We speculate, based on these results, that the hydrophobic binding properties of mucin are related to its ability to promote cholesterol crystal nucleation in model bile.

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