Epitope mapping of the human biliary amphipathic, anionic polypeptide: similarity with a calcium-binding protein isolated from gallstones and bile, and immunologic cross-reactivity with apolipoprotein A-I

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Abstract Biliary amphipathic anionic polypeptide (APF) the major protein of the pigment-lipoprotein complex in bile, and calcium-binding protein (CBP) from gallstones are both small (<10 kDa), highly acidic, amphipathic proteins present in bile and closely associated also with pigmented areas in human gallstones. Polyclonal antibodies against APF have shown cross-reactivity with plasma high density lipoproteins (HDL). This study examines the hypothesis that APF and CBP might be closely related or even identical, and might also share common epitopes with the larger apoA-I (23 kDa). To assess this, immunoreactivity of the three delipidated, highly purified proteins was determined against a panel of 12 monoclonal antibodies (MAbs) prepared against APF and a panel of 4 MAbs against apoA-I. APF was isolated from bile by zonal ultracentrifugation. CBP was isolated from proteins precipitated from bile by $CaCl_2$, as well as from the calcium bilirubinate shells of cholesterol gallstones, by extraction successively with methyl-t-butyl ether, methanol, and Na_2EDTA, followed by Sephadex G-25 chromatography and two-stage preparative SDS-PAGE. ApoA-I was prepared by two types of chromatography: Sephacryl S200 chromatography and heparin-chromatographic immunoaffinity. Specific polyclonal antibodies to APF and apoA-I were prepared from immunized rabbits. MAbs to APF and apoA-I were prepared by immunization of mice, using standard hybridoma technique. Western blotting of APF and CBP in 15% SDS-PAGE yielded one band with an apparent molecular weight of 6.5 kDa, which, along with apoA-I, was immunostained by polyclonal antibodies to APF and apoA-I. Using 12 MAbs against APF with three types of ELISA (direct antigen binding, competitive antigen displacement, and epitope competition between antibodies), it was shown that APF and delipidated apoA-I shared six epitopes, three of which were detected also on the surface of intact HDL particles. Six other epitopes were present in APF but not apoA-I, four of which were exposed on the surface of HDL. Four MAbs against apoA-I reacted with APF and CBP. Amino acid analyses of APF and CBP were similar with 20–23% acidic and 7–11% basic amino acids and low contents of cysteine, methionine, and tyrosine; both differed from apoA-I in containing isoleucine and cysteine. Using ELISA and one MAb (no. 32) against APF, this polypeptide was detected in human plasma HDL, the pigment-lipoprotein complex in the bile of humans, dogs, and rats, and in both pigment and cholesterol gallstones. Like CBP, APF contained tightly bound bile pigments and arrested the precipitation of calcium carbonate from a supersaturated solution in vitro. These common properties and immunological cross-reactivity between APF and CBP suggest that the two proteins may be identical, and likely play a role in both transport of cholesterol and precipitation of calcium salts in bile, and therefore in the formation of both cholesterol and calcium/pigment-containing gallstones. APF/CBP also shares some epitopes with apoA-I and plasma HDL. The presence of amino acids in APF/CBP not found in apoA-I, however, renders it probable that APF is a true minor apolipoprotein of HDL, distinct from apoA-I, that binds tightly to the surface of HDL.—**Domingo, N., J. Grosclaude, E. D. Bekaert, D. Mège, M. J. Chapman, S. Shimizu, M. Ayrault-Jarrier, J. D. Ostrow, and H. Lafont.** Epitope mapping of the human biliary amphipathic, anionic polypeptide: similarity with a calcium-binding protein isolated from gallstones and bile, and immunologic cross-reactivity with apolipoprotein A-I. *J. Lipid Res.* 1992. **33:** 1419–1430.

Supplementary key words monoclonal antibodies • polyclonal antibodies • high density lipoproteins • bile pigment-lipoprotein complex

Abbreviations: VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; apo, apolipoproteins; ELISA, enzyme-linked immunosorbent assay; MAb, monoclonal antibody; PAb, polyclonal antibody; BLC, bile lipoprotein complex; APF, anionic polypeptide fraction; CBP, calcium-binding protein; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline.

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Several particles may be involved in cholesterol transport in bile, including mixed micelles, large vesicles (1), and/or the pigment-lipoprotein complex (2). This last particle, isolated by zonal centrifugation, consists mainly of biliary lipids (phosphatidylcholines, free cholesterol, and bile salts) and an anionic polypeptide fraction (APF) (3). This biliary polypeptide has been shown more recently to consist almost entirely of a single, amphipathic, highly acidic, small (< 10 kDa) polypeptide (APF), which is the third most abundant protein in human bile from normal subjects and patients with gallstones (4). Using specific polyclonal antibodies against APF (4), it was shown that APF was synthesized by isolated rat hepatocytes (4), whose plasma membranes contain saturable and specific binding sites for APF (5). APF was detected in plasma HDL particles, whose major apolipoprotein is apoA-I (6).

APF has been found in mixed cholesterol gallstones, where immunostaining reveals it is concentrated at the interface between pigment and cholesterol zones (7). Shimizu and his colleagues (8) have recently described a calcium-binding protein (CBP) from human cholesterol (8) and black pigment (9) gallstones which is, like APF small (< 10 kDa), amphipathic, highly acidic, and contains tightly bound bile pigment. CBP strongly inhibits the precipitation of calcium carbonate from supersaturated solutions in vitro (8, 9), suggesting it might play an important role in regulating the precipitation of calcium salts from bile and thus the formation of calcium-containing (usually pigmented) gallstones. As APF has an obvious role in cholesterol transport in bile (2), it would be of great interest if CBP and APF were a single, identical biliary protein that could play a role in regulating the transport and precipitation of the two major crystalline components of gallstones.

The present work examines, primarily by immunologic methods, the hypothesis that APF and CBP might be closely related or even identical, and might share common epitopes with the larger protein, apoA-I (23 kDa). Highly purified, delipidated APF, CBP, and apoA-I were probed with a panel of 12 monoclonal antibodies (MAbs) prepared against APF and 4 MAbs prepared against apoA-I, using Western blotting and three types of ELISA assays. The similarity of APF and CBP was demonstrated by commonality of multiple epitopes and by comparable amino acid analyses. In addition, the study demonstrated the presence of APF in normal bile and quantitated the APF content of human and animal biles and human plasma HDL. Shared epitopes between APF and apoA-I were demonstrated that clarified relationships between these two proteins.

MATERIALS AND METHODS

Biological samples

Bile samples and purification of APF. Gallbladder bile was obtained by peroperative puncture of the gallbladder from normal calves and dogs, as well as humans with cholesterol cholelithiasis. Human hepatic bile was obtained from T-tube drainage of patients operated upon for cholesterol choledocholithiasis. Biles were placed immediately on ice for up to 4 h prior to study, with sodium azide added to a final concentration of 0.02%. Biles were discarded if thin-layer chromatography revealed appreciable quantities of lysophosphatidylcholines. Biles were dialyzed for 24 h against running water (5°C) using Visking tube (cut-off 6,000 mol wt), and then centrifuged at 15,000 *g* for 1 h to remove mucoproteins. Human bile-lipoprotein complex was then isolated by preparative zonal ultracentrifugation (10), and contaminating proteins, i.e., albumin, IgA fragments, apoA-I, and other proteins with molecular weights higher than 10,000 were removed by ultrafiltration through a YM-10 membrane (Amicon, Danvers, MA). SDS-PAGE in a 15% acrylamide Laemmli system in the presence of β-mercaptoethanol (10) showed a single pigmented band (APF) with an approximate molecular mass of 6.5 KDa.

Purification of CBP from bile and the pigmented shells of cholesterol gallstones. "Normal" T-tube bile was collected from patients who had undergone orthotopic liver transplantation at least 1 week earlier. Bile was drained by gravity into plastic bags, wrapped in aluminum foil and kept on ice, that contained protease inhibitors (8), and was frozen every 24 h. After thawing, bile was centrifuged for 1 h at 4°C and 13,000 *g* to remove insoluble mucins and particulate material, then mixed with one-fourth volume of 50 mM Tris buffer; the pH was adjusted to 8.0, and calcium chloride was added to a final concentration of 0.25 M. The mixture was stirred for 4 days in the dark at 4°C; the resulting precipitate, collected by centrifugation, was washed twice with 0.25 M $CaCl_2$ in Tris buffer and then dried by washing with acetone.

Cholesterol gallstones, 1.0 to 2.5 cm in diameter, with brownish-black outer shells 2 to 5 mm thick, were collected at uncomplicated cholecystectomy from nine patients, washed with distilled water, blotted dry, and stored in the dark at −20°C until analyzed. The pigmented shells were removed with a scalpel from the body of each stone and the pooled fragments were desiccated in vacuo over anhydrous $CaSO_4$ for 1 week, then pulverized with an agate mortar and pestle.

The dried powders from the bile precipitates and pigmented gallstone shells were then desiccated for another

2 to 6 weeks to a stable dry weight. As described previously (8), each powder was then extracted successively for 4 days each with methyl-t-butyl ether (in a Soxhlet apparatus, to remove cholesterol and lipids), and three times with cold methanol (to remove bile acids, lipids, and some pigments) and then demineralized for 4 days at 4°C with 50 mM Tris buffer containing 0.5 M EDTA, pH 8.0, and protease inhibitors. After centrifugation at 20,000 *g* for 20 min, the EDTA in the extracts was removed by dialysis and the proteins were concentrated by repeated ultrafiltration and redilution with water at 4°C (YM5 and then YM2 membranes, Amicon, Danvers, MA). Three ml-concentrated retentate was then applied to a 2.2 × 27 cm column of Sephadex G-25 (Pharmacia, Piscataway, NJ) that was pre-equilibrated with 0.15 M NaCl and then eluted at 2.7 ml/min with 0.15 M NaCl.

The proteins in the intensely brown fraction at the void volume were concentrated by ultrafiltration at 4°C as above, and then subjected to a two-stage purification by preparative SDS-PAGE. Stage 1 utilized the 12.0T, 2.67C Laemmli system as described previously (8), but in an LKB electrophoresis apparatus with a hydraulic cooling core and 3.0 mm thick × 14 cm wide × 10 cm high running gels, with an overlying 2-cm stacking gel. After 15 min at 50 V to allow the samples to penetrate the stack, electrophoresis was continued at constant 200 V for another 1.5 h until the front had migrated 4.0 cm into the running gel. The narrow yellow band just behind the front was excised and electroeluted with 50 mM Tris–50 mM glycine buffer, pH 8.9, using the LKB 2014 Extraphor electrophoretic concentrator with 1 M NaCl in Tris-glycine buffer as the high-salt bridge. After concentration of the protein and dilution of the NaCl to 10 mM by repeated dilution and ultrafiltration in a Centricon-3 (Amicon), Stage 2 SDS-PAGE was performed, using the 16.5T, 3C system of Schägger and von Jagow (11) which contains tricin in the cathode buffer. Gels, 1.5 mm thick × 8.0 cm wide × 5.5 cm high, with a 1.0-cm stacking gel, were run in the LKB 2050 Midget apparatus at 50 V for 15 min and then at 135 mA until the front had migrated 5.0 cm. Monitor lanes, including very low molecular mass standards (16.9–2.8 kD) from LKB, were silver-stained (12), and the darker yellow band at about 11.5 kDa that ran at the upper edge of the pigment smear was excised, electroeluted, concentrated, and desalted by ultrafiltration as above. The yield of pure protein was 1.04 mg per g dry stone powder. This material was used for the analyses of epitopes, for analytical SDS-PAGE in the 12.0T, 3C system of Schägger and von Jagow (11), and for its effect on precipitation of calcium carbonate in vitro by the method of Dawes et al. (13).

Human plasma lipoproteins. Human lipoprotein density classes were isolated from normal human plasma by sequential ultracentrifugation, using a Type 40.3 rotor in a Beckman ultracentrifuge, according to Havel, Eder, and Bragdon (14) as follows: very low density lipoproteins (VLDL, d 1.006 g/ml), low density lipoproteins (LDL, d 1.006–1.063 g/ml), high density lipoproteins (HDL_2, d 1.063–1.125 g/ml and HDL_3, d 1.125–1.210 g/ml). Immature high density lipoproteins (d 1.075–1.175 g/ml) were isolated from perfusates of pig liver by a similar procedure (14). The composition of the perfusion medium was based on that described by Henry et al. (15) and supplemented with red blood cells, glucagon, insulin, and albumin. The perfusion rate was 500 ml/min. All lipoprotein fractions were washed at their highest limiting density by means of an additional centrifugation using two to three times their volume of the appropriate NaCl/KBr solution. Each density class fraction was then extensively dialyzed against a solution containing 5 mM NH_4HCO_3 buffer, pH 7.4, containing 50 mM NaCl, 0.04% EDTA, 0.01% NaN_3, and 0.001% sodium merthiolate. Delipidated purified apoA-I, prepared by Sephacryl S200 chromatography and heparin immunoaffinity chromatography (16), was a generous gift from Dr. A. Girard-Globa (INSERM U-286, Hôpital Bichat, Paris).

Immunological Reagents

Polyclonal antiserum (PAb) to APF. After lipid extraction using centrifugation, the protein was dissolved in 0.05 M Tris buffer, pH 9.6, containing 1.5 mM sodium glycodeoxycholate. Three male rabbits (New Zealand White), weighing 2.5 kg, were immunized with APF emulsified in complete Freund's adjuvant according to the method of Henry, Bernard, and Depieds (17). Each animal received three injections totaling 0.8 mg APF. The specificity of the polyclonal antiserum raised to APF was tested by double diffusion according to Ouchterlony (18) using albumin (Sigma, France), IgA (Institut Pasteur, France), and APF purified in our laboratory.

Monoclonal antibodies (MAbs) to APF. These were produced in Balb/C mice, immunized intraperitoneally with three doses of 20 μg of APF as described previously (19). Cell fusions were performed as described by Nowinsky et al. (20), using SP2-0 Ag14 myeloma cells (21); the selection medium contained azaserine and hypoxanthine (22).

Positive clones were detected by ELISA (test 1, see below), and asserted to be monoclonal cell populations by the limiting dilution technique; ascitic reagents were produced for each monoclonal hybridoma in Balb/C mice. Antibody isotypes were determined by ELISA (test 1, see below) with anti-mouse IgG subclass rabbit antisera (Amersham, Les Ullis, France) followed by anti-murine IgG goat antisera conjugated to alkaline phosphatase (Biosys Company, Compiègne, France).

Monoclonal and polyclonal antibodies to apoA-I. Most of the monoclonal antibodies to apoA-I used in the present study had been well characterized in earlier reports (23, 24). MAb F28 G11, 1F3 was shown to react with the CNBr-1 NH_2-terminal fragment of apoA-I (AA 1-85), whereas MAb F59 4A12 2F4 reacted with the CNBr-4, COOH terminus of apoA-I. The epitope recognized by MAb F59 4B11, 1F4 appeared to be highly conformation-dependent (23). MAb A11, a gift from Pr. J. C. Fruchart (SERLIA, Institut Pasteur, Lille), was shown to react with the CNBr-2 fragment (AA 87-11) in a region known to be crucial for activation of lecithin:cholesterol acyltransferase (25). Polyclonal antiserum to apoA-I was obtained as reported previously (23).

Immunological titrations

Detection of APF or antibodies by direct ELISA (Test 1). Microtiter plates (Linbro E.I.A., microtitration plate, Flow Laboratories, Puteaux, France) were coated with diluted samples of purified APF (0.5–20 μg protein per well) in 0.1 M sodium carbonate buffer, pH 9.6. Plates were then washed three times with phosphate-buffered saline (PBS) to remove the unbound components. After addition of polyclonal and ascitic monoclonal antibodies at dilutions of 1/200 and 1/1.000, respectively, in PBS containing 0.05% polyoxyethylene sorbitan monolaurate (Tween 20), the plates were incubated for 90 min at 37°C. After washing, bound antibodies were detected using alkaline phosphatase-conjugated anti-rabbit or anti-murine immunoglobulin goat antiserum (Sigma) at a dilution of 1/2.000. Alkaline phosphatase activity was measured with *p*-nitrophenyl phosphate (PnPP, 1 mg/ml) as the substrate, incubated for 30 min at 37°C in the dark. Absorbance in each well was measured at 405 nm using a Multiskan Titertek MC (Flow Laboratories, Puteaux, France).

For detection of antibodies in growing clones, supernatants were diluted with an equal volume of PBS containing 0.1% Tween 20, then ELISA was performed as above, but with APF 0.5 μg/ml and alkaline phosphatase conjugated anti-mouse immunoglobulin goat antiserum (Biosys). Clones were considered as positive when the ELISA signal was twice the background.

Antigen competition (Test 2). Each monoclonal antibody was tested for recognition of coated APF, when competing with delipidated APF in solution or with other compounds in solution suspected of containing APF-like epitopes. Defined amounts of MAbs, preincubated with increasing concentrations of competitive antigens, were added into the wells coated with APF (0.5 μg per well) at pH 9.6, and incubated for 90 min at 37°C. The binding capacity that remained in each well was revealed using alkaline phosphatase-conjugated anti-murine immunoglobulin rabbit antiserum.

Epitope competition between monoclonal antibodies to APF (Test 3). Competition experiments were performed according to Triplett et al. (26). Glutaraldehyde-treated plates were first coated with one monoclonal antibody (0.5 μg/well). Constant amounts of APF preincubated with increasing amounts of a second homologous competitive antibody were then added to the plates. This second antibody could be identical or not to the coated one. The APF retained by the coated MAb was revealed by adding polyclonal antibodies to APF, followed by conjugated anti-rabbit immunoglobulin goat antibodies. All steps were performed in PBS containing 0.25% gelatin and 0.05% Tween 20.

Western blot. The proteins were separated on a fast-gel apparatus (Phast-gel system, Pharmacia, Uppsala, Sweden), using a 15%T, 2.67%C Laemmli gel transferred to Immobilon membrane (27) (Millipore-Waters, St. Quentin en Yvelines, France) and revealed with polyclonal antibodies against APF or apoA-I at a dilution of 1/200. The antigen–antibody complexes were revealed by silver staining.

Quantitation of APF in different biological fluids. Bile or plasma samples were tested using two different types of ELISA. In test 1, quantitation was performed using a standard calibration curve obtained from different dilutions of purified, delipidated coated APF. It was assumed in this assay that the efficiency of binding of the protein fractions to the plastic surface was almost independent of

TABLE 1. Reactivity and characterization of monoclonal antibodies to APF

	Monoclonal Antibody No.											
	3	5	6	8	9	10	11	16	17	23	28	32
Reactivity to different forms of purified APF[a]												
Coated	+	+	+	+	+	+	+	+	+	+	+	+
In solution	+	–	+	+	+	–	+	+	+	+	+	+
Ig isotype[b]	G1	G1	G2b	G2b	G1	G2b	G3	G1	G1	G1	G1	G1
Affinity constants[c] ($\times 10^7$ M^{-1})	1.11	nd	2.83	nd	nd	1.11	1.79	2.32	nd	12.5	nd	3.80

[a] Assessed by ELISA: test 1 for "coated" APF; test 2 for APF in solution (see Materials and Methods and Fig. 1).
[b] Determined by ELISA test 1 using anti-murine isotype, specific rabbit antibodies.
[c] Affinity constants were determined by the method of B. Friguet et al. (45); nd, not determined.

the other biological components present in the sample. Using test 2, we investigated the capacity of a given dilution of crude biological fluid to compete with coated APF for the binding of a monoclonal antibody. Quantitation was based on the comparison of the displacement curves obtained with serial dilutions of a given sample with that obtained with purified APF in solution, under the same experimental conditions.

Protein and lipid analysis

Total protein concentrations in lipoproteins were quantified by the procedure of Lowry et al. (28), using bovine serum albumin (Fraction V, Sigma) as standard. In bile samples, quantitative determination of albumin was performed by radial immunodiffusion on LC Partigen albumin plates (Behring Laboratories, Marburg, Germany). Total biliary protein estimations were made using BCA protein assay reagent (29) (Pierce, Interchim, Montluçon, France).

Phosphatidylcholine was estimated using the semi-automated technique of Amic, Lairon, and Hauton (30), on dialyzed fractions obtained after ultracentrifugation of HDL and bile samples.

RESULTS

Epitopic mapping of APF

A panel of 12 murine MAbs was obtained from one fusion experiment. Isotypes and affinity constants for the MAbs (**Table 1**) exhibited the customary variations between antigen (APF) in solid-phase (coated) as compared with solution (Test 2). For example, MAbs no. 5 and 10 had very low affinity for APF in solution compared to that for coated APF. In contrast, MAb no. 32 exhibited a very high capacity for binding to solubilized APF (**Fig. 1**).

On the basis of competition experiments using test 3 (**Fig. 2**), epitopes recognized by MAbs no. 5 and 16 were found to be independent whereas epitopes to MAbs no. 9 and 3 overlapped. Epitope to MAb no. 9, however, remained distinct from that recognized by MAb no. 3, since only the latter epitope overlapped with epitope recognized by MAb no. 11.

Results of the full panel of competitive assays allowed formulation of an epitope map of APF (**Fig. 3**), which defined three domains within the APF molecule. Two isolated domains corresponded to single antigenic markers, reacting with MAbs no. 6 and 10, respectively. The principal domain consisted of a cluster of the remaining ten distinct epitopes, each of which overlapped with at least one other epitope in the cluster.

Comparisons of APF and CBP

On second stage SDS-PAGE under reducing conditions in the Schägger-von Jagow 16.5T, 3C system (11), CBP

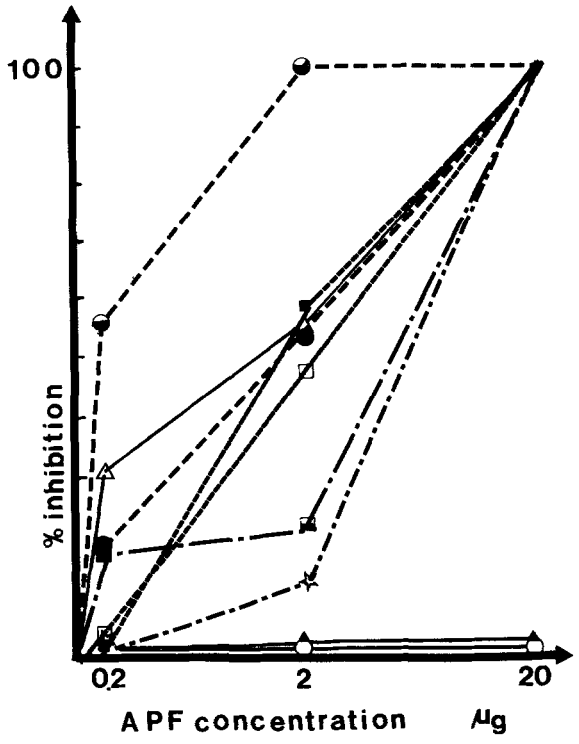

Fig. 1. Inhibition of the binding of monoclonal antibodies to coated APF by APF in solution according to test 2. Three different concentrations of APF in solution were used. Corresponding curves as follows: MAb no. 32 (◒—◒); MAb no. 17 (♦—♦); MAb no. 3 (△—△); MAb no. 8 (●—●); MAb no. 16 (◇—◇); MAb no. 23 (⬓—⬓); MAb no. 6 (*—*); MAbs no. 5 and 10 (▲—▲, ○—○).

prepared from the body of cholesterol gallstones (8), the demineralized pigment shells of cholesterol gallstones, and the calcium-precipitated proteins of human bile, as well as APF prepared from bile by a different method (10), all migrated primarily as a yellow band with an apparent molecular mass of 11.5 kDa. A weaker yellow band at 6.5 kDa was present also. On repeat SDS-PAGE of the eluted bands, each yielded both the 6.5 and 11.5 kDa bands (unpublished data). In a 16.5T, 6C Schägger-von Jagow system, the 6.5 kDa band was more prominent than the 11.5 kDa band especially when smaller quantities of protein were applied. On Western immunoblots in a 15% Laemmli gel, both the 11.5 kDa preparations of CBP and the 6.5 kDa preparation of APF yielded only a 6.5 kDa band, each of which immunostained identically with each antibody tested (**Fig. 4**) (**Table 2**).

The immunologic analogy between APF and CBP was supported further by ELISA test 1, using plates coated either with APF or CBP and MAbs against APF and apoA-I (Table 2). CBP was recognized by seven MAbs that were tested, including three MAbs against APF and four MAbs against apoA-I. The ELISA absorbance obtained with CBP, however, was usually less than that ob-

served with APF at similar protein concentrations.

At a final concentration of 8 μg/ml, both the pigment shell and bile CBP, as well as APF from bile, arrested precipitation of calcium carbonate in vitro, as had been observed with CBP from both pure and mixed cholesterol gallstones (8) and black pigment gallstones (9). Amino acid analyses of the 11.5 kDa CBP bands from cholesterol gallstones (8) and bile gave patterns similar to those of the 6.5 kDa APF preparations isolated from cholesterol gallstones (7) and bile (10) (**Table 3**).

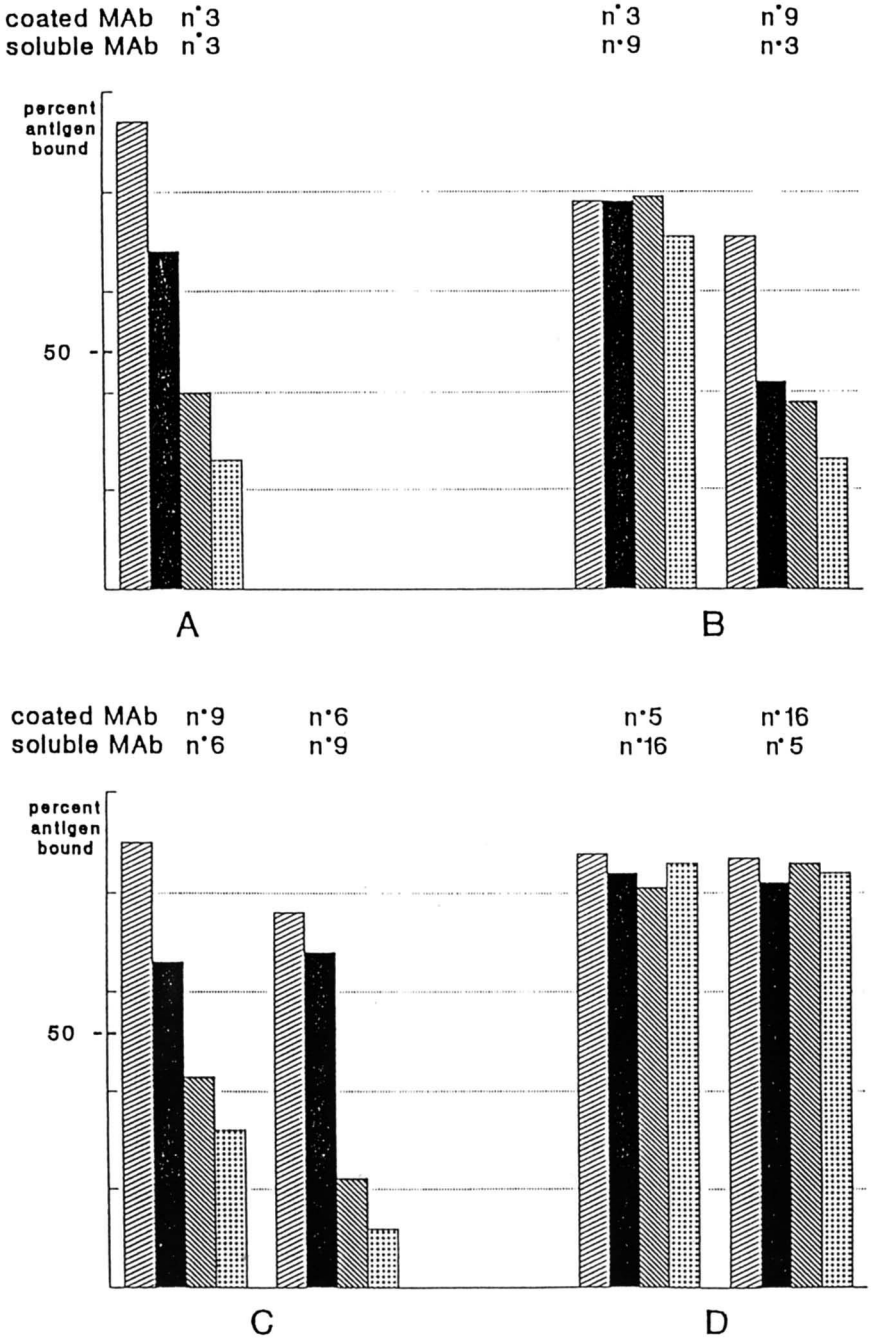

Fig. 2. Competition for epitopes binding between two monoclonal antibodies to APF according to test 4. The first MAb was coated to the solid phase and the second (at serial dilutions) was subsequently added after preincubation with a constant amount of APF. APF bound to the plates was then revealed using a polyclonal antibody. A: Homologous competition in which MAb no. 3 was used both in solid phase and in solution. B: Reciprocal, nonhomologous competition between MAbs no. 3 and 9. C: Nonreciprocal nonhomologous competition between MAbs no. 6 and 9. D: No competitive interaction between MAbs no. 5 and 16. On each panel, the concentrations of the soluble MAb are (▨) 0 μg; (■) 10 μg; (▩) 50 μg; (⊞) 100 μg/ml.

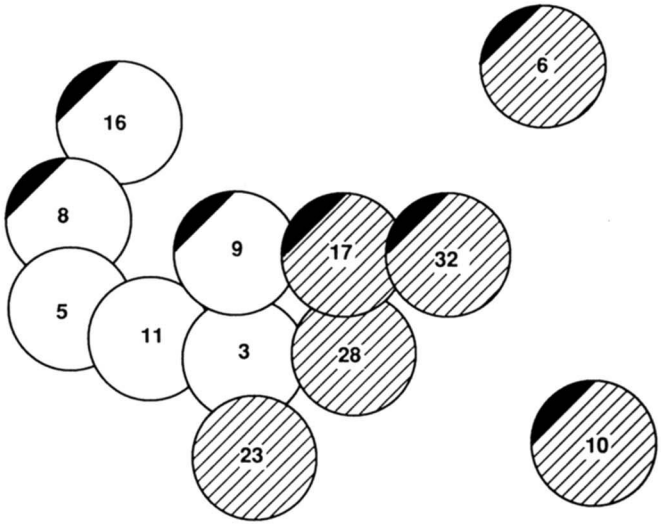

Fig. 3. Epitopic map of delipidated APF. Each circle denotes a specific epitope and the corresponding antibody number. Hatched circles (6, 10, 17, 23, 28, 32) represent those epitopes specific to APF. Open circles (3, 5, 8, 9, 11, 16) represent those epitopes reacting to both APF and apoA-I. Thickened contours indicate epitopes that are exposed on HDL_3 particles.

Antigenic homologies of APF with apoA-I and HDL_3

Using 12 MAbs against APF to probe purified delipidated apoA-I, direct-binding studies (ELISA Test 1) and antigen competition studies (ELISA Test 2) revealed many cross-reactivities (Table 2 and Fig. 3). Six anti-APF MAbs (nos. 16, 8, 9, 5, 11, and 3) recognized delipidated apoA-I; only the first three of these also reacted with intact HDL particles. The common APF-apoA-I epitopes recognized by MAbs no. 5, 11, and 3 were not detected on HDL and were presumably buried below the surface of this lipoprotein particle. Four other APF-reactive MAbs (no. 17, 32, 6, and 10), which did not react with apoA-I, did however react with epitopes expressed on the surface of intact HDL particles, including the two isolated epitopes (no. 6 and 10) on delipidated APF. Finally, the two epitopes recognized by MAbs no. 23 and 28, which did not react with apoA-I or HDL, were detected in the bile pigment-lipoprotein complex.

These studies were complemented with direct and competitive ELISA assays using four MAbs prepared against apoA-I (Table 2). MAb no. 2G11 (recognizing the NH_2-terminal fragment of apoA-I) and MAb no. 4A12 (recognizing the COOH-terminus) cross-reacted with APF. In contrast, anti apoA-I MAbs no. 4B11 and A11 did not react with delipidated APF.

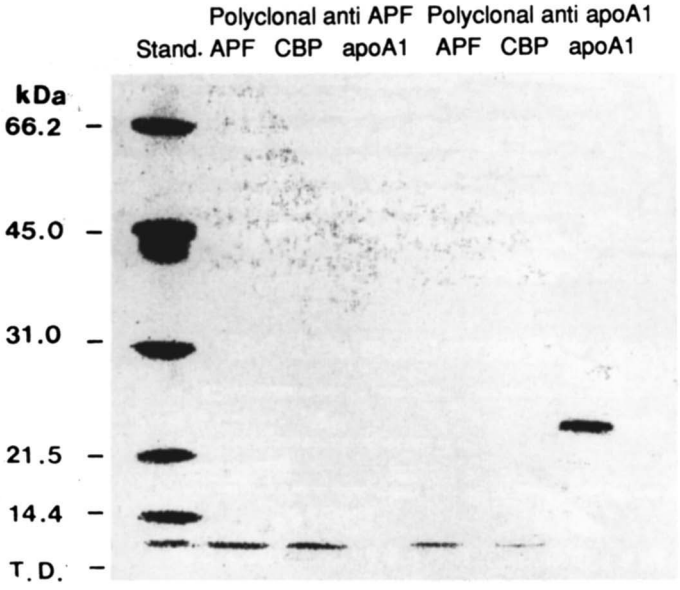

Fig. 4. Western blot of APF-CBP, apoA-I. Immunoreactivity of rabbit anti-human APF and apoA-I against APF, CBP, apoA-I. Line 1: protein standards separated by gel electrophoresis. Line 2: APF tested against rabbit polyclonal antibodies against purified APF. Line 3: CBP tested against rabbit polyclonal antibodies against purified APF. Line 4: apoA-I tested against rabbit polyclonal antibodies against purified APF. Line 5: APF tested against rabbit polyclonal antibodies reacting against purified apoA-I. Line 6: CBP tested against rabbit polyclonal antibodies reacting against purified apoA-I. Line 7: apoA-I tested against rabbit polyclonal antibodies reacting against purified apoA-I.

Antigen detection and quantitation of APF in biological samples

Various biological samples were probed with MAb no. 32, which had high binding capacity and high affinity for APF. The data in **Table 4** indicate that APF constituted at least one-third of the total protein in both gallbladder and hepatic biles from patients with cholelithiasis, and approximately half the proteins in normal gallbladder bile from calves and dogs. The weight ratios of APF/protein and APF/phospholipids showed little variation among bile samples within each individual group. The weight ratios of APF to phospholipid in human gallbladder and hepatic biles were similar and were significantly higher than those in gallbladder biles from dogs ($P < 0.5$) and calves

TABLE 2. Cross-reactivity of polyclonal (PAb) and monoclonal (MAb) antibodies to APF/CBP/apoA-I

Method	Antibody	APF	CBP	ApoA-I
Elisa				
	Mono-anti-apoA-I A11	0	0	+ +
	Mono-anti-apoA-I 4A12	+ +	+	+ +
	Mono-anti-apoA-I 4B11	0	0	+
	Mono-anti-apoA-I 2G11	+	+	+
	Mono-anti-APF#5,11,3,9	+	+	+
	Mono-anti-APF#28,32,6	+	+	0
Western				
	Mono-anti-apoA-I A11	0	0	+ +
	Mono-anti-apoA-I 4A12	+	+	+ +
	Mono-anti-apoA-I 4B11	0	0	+
	Mono-anti-apoA-I 2G11	+	+	+
	Mono-anti-APF#6	+	+	0
	Poly-anti-apoA-I	+	+	+
	Poly-anti-APF	+	+	0

TABLE 3. Amino acid composition of APF and CBP

	APF (Ref. 10)		CBP (Ref. 8)	
	Bile	Stones	Bile	Stones
Asp	10.90	10.20	9.34	12.85
Thr	5.20	4.70	3.31	4.75
Ser	4.80	5.90	6.28	6.05
Glu	12.00	10.70	12.60	11.55
Pro	4.10	4.00	4.05	7.50
Gly	8.50	8.50	27.43	22.10[a]
Ala	11.10	11.00	6.04	10.45
1/2 Cys	0.70	7.40	0.42	0.65
Val	9.80	6.40	4.23	6.45
Met	2.30	2.00	0.19	0.48
Ile	5.50	3.60	2.90	1.50
Leu	10.00	11.40	5.77	5.60
Tyr	2.90	3.00	3.26	3.01
Phe	3.50	4.70	3.26	2.10
His	1.90	1.00	2.57	1.60
Lys	5.40	2.80	3.76	3.55
Arg	4.00	3.40	1.32	1.75
Tau	2.58	0.37	3.28	1.60

All values are expressed in molar percentage (ref. 7).
[a]Value reflects contamination with conjugated bile salts as revealed by taurine detected in the amino acid analyses.

($P < 0.5$). Human cholesterol gallstones, dissolved in 0.5 M Tris buffer containing 10% sodium deoxycholate and 1% (w/w) SDS, contained a mean APF concentration of 1.05 ± 0.09 mg/g dry weight of stone (7).

The weight ratios of APF to proteins and to phospholipids in human plasma HDL subfractions are presented in **Table 5.** The detection and the quantification of APF both by PAb and MAb gave a good correlation: APF appeared as a minor protein constituent of HDL_2 and HDL_3. By the same ELISA, HDL_3 contained three times more APF than HDL_2, but APF was not detected in the apolipoproteins of human plasma VLDL or LDL (data not shown). APF was, however, detected in immature HDL at a concentration of 0.1 mg/dl.

DISCUSSION

In the present study, we used a panel of 12 murine MAbs prepared against delipidated APF, and 4 prepared against delipidated apoA-I, to probe, by ELISA assays, the epitopic map of APF and its homologies with CBP and apoA-I. The major findings were demonstration of the close similarity, if not identity, of APF and CBP, and their partial immunologic cross-reactivity with apoA-I, the major apolipoprotein of plasma HDL.

In initial reports, APF was isolated by zonal centrifugation from the pigment-lipoprotein complex of bile from patients with cholesterol gallstones (10), whereas CBP was isolated from cholesterol and mixed gallstones by serial solvent extraction, followed by demineralization with Na_2EDTA and then Sephadex gel chromatography and preparative SDS-PAGE (8). Subsequently, APF (7) and CBP (9) were detected also in black pigment gallstones. The present work demonstrates that CBP can be isolated from the calcium bilirubinate shells of cholesterol gallstones, as well as from the material precipitated from normal human bile by addition of calcium. Both APF and CBP are thus present in normal bile and in the bile and calculi of patients with cholesterol or pigment gallstones, suggesting a role in formation of all gallstones.

Purity and similarity/identity of APF and CBP

APF and CBP are both small (about 7 kDa), highly acidic, amphipathic proteins that contain tightly (possibly covalently) bound bile pigment and arrest the precipitation of calcium carbonate from supersaturated solutions in vitro. Both proteins show the same variations in apparent molecular weights on different types of SDS-PAGE, and identical patterns on Western blots immunostained with a variety of PAbs and MAbs against APF and apoA-I. The reactivity of CBP on ELISA with seven MAbs prepared against pure APF is even stronger evidence for their probable identity.

TABLE 4. Concentrations of proteins and phospholipids in bile and percentage ratios by weight of APF to proteins and phospholipids

Bile Samples	Proteins[a]	Phospholipids	APF[b]	
			Proteins	Phospholipids
	mg/ml		*mg/100 mg*	
Human gallbladder bile[c] (16)	4.68 ± 0.04	32.4 ± 0.04	41.0 ± 4.1	5.94 ± 0.10
Human hepatic bile[c] (7)	1.12 ± 0.01	6.6 ± 0.01	35.0 ± 1.1	5.95 ± 0.13
Normal calf gallbladder bile (7)	2.43 ± 0.02	28.7 ± 0.09	54.0 ± 4.1	4.78 ± 0.18
Normal dog gallbladder bile	2.89 ± 0.04	24.2 ± 0.08	46.0 ± 1.5	5.40 ± 0.16

Number of separate preparations in parentheses. Values given as mean ± SD.
[a]Albumin and mucoproteins were discarded.
[b]APF was quantitated by ELISA using MAb no. 32.
[c]Biles from patients with gallstones.

TABLE 5. Absolute plasma concentrations of proteins and phospholipids in human plasma HDL subfractions and percentage weight ratios of APF to proteins and phospholipids

High Density Lipoproteins	Proteins	Phospholipids	APF[a]			
			Proteins		Phospholipids	
			MAb	PAb	MAb	PAb
	mg/dl		*mg/100 mg*			
Human HDL_2 (5)	43.1 ± 6.3	37.4 ± 3.0	0.19 ± 0.02	0.22 ± 0.02	0.22 ± 0.02	0.25 ± 0.02
Human HDL_3 (6)	55.0 ± 15.4	31.6 ± 2.9	0.60 ± 0.07	0.79 ± 0.09	1.10 ± 0.10	1.50 ± 0.20

Number of preparations in parentheses. All values are means ± SD.
[a]APF was quantitated by ELISA using either monoclonal antibody no. 32 (MAb) or a polyclonal antiserum (PAb).

The occurrence of a major cluster of 10 epitopes in APF is indicative of a high degree of structural homogeneity of the preparation, including the APF used for immunization. It is unclear, however, whether the one independent epitope (to MAbs no. 6) is small, tightly associated polypeptide or is an integral peptide of the same protein; this presence in both APF and CBP, despite different methods of preparation, favors the interpretation that CBP and APF are probably free from contamination with other proteins. There is also little or no contamination with lipids, which were readily extracted by the standard delipidation techniques that were used and were thus probably bound by hydrophobic interactions. There is, however, contamination with bile pigments and bile salts, which bind tightly to the highly amphipathic APF and CBP. Some pigment is so intimately bound that it is not fully removed by molecular sieve chromatography, dialysis, or PAGE in the presence of SDS (8, 9), or by solvent partition using lipid solvents and chaotropic solutes. As it remains associated even to small fragments of the degraded protein, the pigment is likely covalently bound (Ostrow, J. D., unpublished observations). The taurine detected in the amino acid analyses must be derived from tauroconjugated bile salts; similarly bound glycine-conjugated bile salts probably account for much of the variability in glycine content found among amino acids analyses of various preparations of APF and CBP. With this exception, the amino acid compositions of the two proteins from both gallstones and bile were remarkably similar and characteristic of small, amphipathic, anionic proteins that regulate calcium precipitation in a variety of normal and pathological biomineralization systems throughout nature (8, 31, 32).

On some SDS-PAGE gels, APF and CBP yielded only a 6.5 kDa band, on others only an 11.5 kDa band; on yet other gels, both bands were present. It is likely that the 11.5 kDa band is the dimer of the 6.5 kDa band, since *a)* both bands could be obtained from repeat SDS-PAGE of either band; *b)* both 11.5 kDa CBP and 6.5 kDa APF revealed identical immunological reactions with a variety of PAbs and MAbs on both direct ELISA and Western blotting; and *c)* both bands revealed closely similar amino acid analyses. Analogous amphipathic, highly acidic, low molecular weight proteins, isolated from other biomineralized tissues, likewise exhibit a high tendency to aggregate (31), even in the presence of SDS.

Relationships of APF/CBP to apoA-I

Cross-reactivity of many of the epitopes of APF with apoA-I suggests structural relationships between these two proteins. With the 12 MAbs against APF, 6 epitopes were common to APF and apoA-I, whereas the other 6 were monospecific for APF. Since APF (< 7 kDa) is a much smaller protein than apoA-I (23 kDa), the possibility must be considered that the former is a fragment of the latter. This is unlikely, however, in view of the presence in APF (as well as the likely identical CBP) of isoleucine, leucine, and cysteine, which are not found in apoA-I (33). Of the 4 MAbs prepared against apoA-I fragments, 2 reacted with APF, one each was derived by immunization with the -COOH- or $-NH_2$-terminal fragment of apoA-I. By contrast, 2 MAbs derived from the middle segments of apoA-I (no. 4B11 and A11) did not bind to delipidated APF nor to a CNBR-2 fragment of apoA-I, which was not present in APF. These immunological analogies between APF and apoA-I are confirmed by the use of 5 other MAbs against apoA-I prepared by Dr. Y. Marcel (34). As a single polypeptide (i.e., APF) cannot be derived from cleavage of the two distant termini of a single larger protein (i.e., apoA-I), these results confirm that APF is an entity different from apoA-I. The partial homology between APF and apoA-I might be best explained, therefore, by independent transcription or alternative splicing of a respective structural gene from a common ancestor, akin to the situation with the microsomal glucuronyl transferases (35).

Relationships of APF/CBP to HDL_3

APF was detected by ELISA of plasma HDL and, as noted above, showed considerable immunologic cross-reactivity with apoA-I, the major apolipoprotein of HDL.

Of the six epitopes common to APF and apoA-I, however, only three (no. 16, 8, and 9) were detected on HDL particles (Fig. 3). By contrast, three other APF epitopes (no. 17, 32, and 6) that were detected on HDL did not react with apoA-I. These findings confirm that APF is a minor structural entity in HDL that is distinct from apoA-I, although they exhibit significant, partial structural homology. These differences in cross-reactivity of anti-APF MAbs with HDL as compared with apoA-I suggest that the MAbs that react with HDL but not apoA-I are reacting with APF on the surface of HDL, whereas the MAbs that react with apoA-I but not HDL presumably recognize epitopes on apoA-I that are buried within the lipid core of the HDL particle.

The epitopes recognized by anti-APF MAbs no. 23 and 28, which did not react with apoA-I, were not recognized in plasma HDL, but have been detected in the bile pigment-lipoprotein complex (36). This suggests that the tertiary structure of APF differs in the particles of plasma HDL and the bile pigment-lipoprotein complex.

Significance of APF/CBP in biological fluids and gallstones

APF/CBP was detected by ELISA in both the bile and plasma compartments: in bile as the major protein of the bile pigment-lipoprotein complex and in plasma as a minor apolipoprotein of plasma HDL. CBP from cholesterol or pigment gallstones, and both CBP and APF from bile, have been shown to arrest the precipitation of calcium carbonate in vitro, and both CBP and APF contain tightly bound bile pigment and are associated most closely with the pigment/calcium zones in gallstones (7–9). These results suggest that APF/CBP may be involved in the regulation of cholesterol as well as calcium and pigment precipitation from bile, and thus a key factor in the formation of all types of gallstones. They suggest also that APF/CBP may be involved in the transport of cholesterol and bile pigments from the hepatocyte into bile and of cholesterol from plasma into the bile (37). The high concentrations of APF in bile and its association with the bile pigment-lipoprotein complex suggest it is the major apolipoprotein in bile. Other apolipoproteins, present in bile in much lower concentrations, have been shown to inhibit cholesterol precipitation from model bile systems (38), although a variety of glycoproteins have been isolated from bile that are apparently more potent in promoting or inhibiting cholesterol precipitation (39–41). All these findings only underscore the importance of including biliary proteins in model systems designed to study physicochemical relationships between bile salts and biliary lipids (42). Most models reported to date (38, 42, 43) have not truly simulated the physiological situation, since they did not include bile proteins, bile pigments, or calcium.

Future directions

Unequivocal confirmation of the probable identity of APF and CBP, and explanation of their partial structural homologies with apoA-I, will require amino acid sequencing of the proteins and DNA sequencing of the relevant genes. Attempts in both laboratories (H. Lafont et al. and J. D. Ostrow et al.) to obtain amino acid sequences of APF/CBP have been hindered by an apparently blocked N-terminal amino acid. Based on the evidence presented in this paper and elsewhere (7–10, 36) it seems highly likely that they are closely related, if not identical.

Our hypothesis that APF may be involved in regulating the precipitation of both calcium salts (including bilirubinates) and cholesterol from bile, and thus play a key role in the pathogenesis of all types of gallstones, needs to be tested directly in model systems containing APF and other bile proteins. This will require large quantities of pure APF, isolation of which will be facilitated by immunoaffinity chromatography using the specific MAbs reported in this paper, and ultimately by synthesis of APF programmed by the cloned gene in expression vectors. The MAbs and cDNA probes will also permit determination of whether the concentrations and/or structure of APF in bile is abnormal in patients with various types of gallstones. The MAbs can be used to study the appearance and progressive association of APF with lipids within the hepatocyte as well as in bile and to determine the correspondence between the bile pigment-lipoprotein complex and biliary lipid particles obtained by other techniques (44). Finally, the MAbs can be used to determine the portions of the APF molecule that interact with lipids in plasma HDL and in the bile pigment-lipoprotein complex. These MAbs will also allow us to study the appearance and progressive association of APF with lipids in endothelial cells and other tissues and to investigate the role of this minor apoprotein in the HDL fraction in the reverse transport of cholesterol.

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