ORIGINAL ARTICLES

Purification and characterization of a novel human 15 kd cholesterol crystallization inhibitor protein in bile

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Crystallization-inhibiting proteins can explain longer nucleation times associated with bile from gallstone-free subjects as compared with bile from patients with cholesterol gallstones. We partially characterized and examined the crystallization inhibitory potency of a newly purified 15 kd human biliary protein. Gallbladder bile was passed through an anti-apolipoprotein A-I (apo A-I) immunoaffinity column to extract lipid-associated proteins. The bound fraction was separated by 30 kd ultrafiltration. Sodium dodecyl sulfate-polyacrylamide gel electrophesis (SDS-PAGE) was performed under nonreducing and reducing conditions. Cholesterol crystallization activity was tested in a photometric cholesterol crystal growth assay. Isoelectric focusing was performed by using a standard gel. The purified 15 kd protein was subjected to N-terminal amino acid sequencing. Although the whole apo A-I–bound fraction contained a variety of proteins and lipids, its 30 kd filtrate yielded a nearly pure 15 kd protein with only minor contamination from apo A-I. Amino acid sequencing showed that the protein was unique. Enzymatic deglycosylation revealed no evidence for glycosylation. At a protein concentration of 10 μg/ml, crystallization time was delayed as compared with control and apo A-I, and final crystal mass was reduced to 75% of control. Its isoelectric point was 6.1 without isoforms. Under nonreducing conditions, the protein formed a 30 kd dimer and a 60 kd tetramer. We conclude that this protein is a novel potent biliary crystallization inhibitor protein. (J Lab Clin Med 1996;127:169-78)

Abbreviations: apo A-I = apolipoprotein A-I; BCIP = 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt; IEF = isoelectric focusing; M_r = relative molecular mass; NBT = nitroblue tetrazolium chloride; PBS = phosphate-buffered saline solution; pI = isoelectric point; SDS-PAGE = sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TBS = tris-buffered saline solution

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Supported by Research Grant DK-17562 from the National Institutes of Health. Dr. Secknus was the recipient of a fellowship from the Feodor Lynen Program of the Alexander von Humboldt Foundation of Germany. Drs. Secknus and Yamashita were supported in part by funds from the Research Institute, The Cleveland Clinic Foundation. Dr. Ginanni Corradini was the recipient of a National Institutes of Health Fogarty International Research Fellowship.

Presented in preliminary form at the annual meeting of the American Gastroenterological Association, May 15, 1994, New Orleans, La., and published in abstract form (Gastroenterology 1994; 106:979A).

Submitted for publication May 31, 1995; revision submitted Sept. 12, 1995; accepted Sept. 18, 1995.

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0022-2143/96 $5.00 + 0 **5/1/69457**

Biliary cholesterol supersaturation is frequently seen in normal individuals as well as in patients with gallstones.[1] A rapid cholesterol nucleation time and an increased cholesterol crystal growth rate are two of the most important indexes that influence gallstone formation and that are affected by cholesterol supersaturation.[2] A variety of biliary proteins can influence these two factors.[3-6] It has been suggested that this influence may be the result of a nonspecific interaction of a given protein with the process of nucleation and stone formation that is based on the physicochemical properties of the protein rather than reflecting a site- and protein-specific effect.[7] Proteins that share common molecular properties (e.g., the degree of glycosylation or lipophilicity) may influence cholesterol nucleation in a similar manner.

In keeping with this concept, many proteins that bind to the lectin concanavalin A, such as α_1-acid glycoprotein,[7,8] haptoglobin,[9] and aminopeptidase N,[10] have been shown to promote cholesterol nucleation and crystal growth. Others, binding to the *Helix pomatia* lectin (e.g., a 120 kd dimer[11] and a recently introduced family of cholesterol crystal-associated proteins[12]), appear to act as crystallization inhibitors. However, with the sole exception of apolipoproteins, no inhibitor protein so far has been identified, fully characterized, or quantified. Quantitation of apo A-I has suggested that its physiologic concentration is far lower than that shown to have a definite inhibitory effect in vitro. In both groups, proteins are thought to have a similar glycosylation pattern, because the lectins are specific for certain sugars: concanavalin A is specific for mannose, and *Helix pomatia* is specific for N-acetyl-α-D-galactosamine. Inhibitory lectin-binding proteins may interfere with cholesterol crystal formation by preventing cholesterol apposition to the crystal surface.[12,13]

In contrast to the assumed direct interaction of glycoproteins with the cholesterol crystal during its growth process, another biliary inhibitor protein, apo A-I, has been reported either to partially shift the distribution of cholesterol in bile from vesicles toward the more stable mixed micellar form[14] or to stabilize cholesterol carriers such as phospholipid lamellae.[15] This increase in stability appears to be based on its amphiphilic properties, allowing integration of apo A-I into lipid aggregates.

We accordingly tested the hypothesis that other proteins with properties similar to apo A-I are present in bile and act as cholesterol crystallization modifiers. In this study we describe a novel and potent 15 kd human crystallization inhibitor protein that we purified from the same biliary lipid compartments that contain apo A-I.

METHODS

Materials. SDS, acrylamide, bisacrylamide, ammonium persulfate, riboflavin-5′-phosphate, and N,N,N^1,N^1,-tetramethyl-ethylenediamine were purchased from Bio-Rad Laboratories, Richmond, Calif. Egg lecithin, grade I, was obtained from Lipid Products, South Nutfield, Surrey, England. Its purity was greater than 99% as measured by high-performance liquid chromatography.[16] Cholesterol purchased from Eastman Kodak Co., Rochester, N.Y., was 99.8% pure as measured by differential scanning calorimetry.[17] Sodium salts of taurocholic acid and taurodeoxycholic acid (more than 99% pure) were purchased from Calbiochem, San Diego, Calif. Stirring cells and ultrafiltration membranes (YM 10) and concentrators (Centriprep 30) with molecular weight cutoffs of 10 kd and 30 kd, respectively, were purchased from Amicon (Millipore Corp., Bedford, Mass.). NBT, BCIP, Tween 20, and streptavidin-alkaline phosphatase were purchased from GIBCO BRL, Gaithersburg, Md. Nitrocellulose sheets were purchased from Schleicher & Schüll, Keene, N.H. For SDS-PAGE and protein blotting studies, a Mini-Protean II electrophoresis cell and a semi-dry blot electrophoretic transfer system (Trans-Blot SD) were obtained from Bio-Rad. IEF was performed using an IEF-cell (Mini-IEF 111, Bio-Rad). Glutaraldehyde was purchased from EM Science, Gibbstown, N.J. Bovine serum albumin, human apo A-I, and biotinylated polyclonal rabbit anti-sheep antibodies were purchased from Sigma Chemical Co., St. Louis, Mo. Polyclonal sheep anti-human apo A-I antibodies were obtained from The Binding Site, San Diego, Calif. Immunoaffinity chromatography gel (AminoLink) was purchased from Pierce Chemical Co., Rockford, Ill.

Water was filtered, ion-exchanged, and glass-distilled (Corning Glass Works, Corning, N.Y.). Glassware was acid-washed and thoroughly rinsed with purified water before drying. Solutions were filtered through 0.22 mm micropore filters (Millipore Corp.) before they were used for model bile preparation and subsequent growth assay measurements. N-glycosidase F and endo-α-N-acetylgalactosaminidase were purchased from Boehringer Mannheim Corp., Indianapolis, Ind.

All other reagents were of the highest grade commercially available.

Methods

Bile and serum collection and preparation. On approval by the Cleveland Clinic Foundation's Research Projects and Institutional Review Committee regarding human studies, human bile samples were obtained from donors in liver or kidney transplant programs or at surgery for gastrointestinal diseases (e.g., pancreatic carcinoma) by needle aspiration of the gallbladder as previously described.[13] Patients with chronic or acute gallbladder-related diseases (e.g., gallstones or cholecystitis) were excluded. Bile samples found to be blood-free were

frozen at −80° C for up to 6 months. After thawing, they were ultracentrifuged for 90 minutes at 100,000 *g* (model L5- 50; Beckmann Instruments, Palo Alto, Calif.) to remove solids and high-molecular-weight proteins. The supernatant was then washed with PBS (200 mmol/L NaH_2PO_4/Na_2HPO_4, 150 mmol/L NaCl) on a stirring cell with YM10 membranes. The final volume was equal to the original bile sample volume.

Human serum from healthy donors was received from the Cleveland Clinic blood bank. After pooling and defibrination with $CaCl_2$, it was stored frozen at −20° C. For the following procedures, it was used without further preparation.

Immunoaffinity chromatography of Apo A-I. Apo A-I–containing lipid-protein complexes were purified from PBS- washed bile and from serum by using anti-apo A-I antibody affinity columns (1 × 5 cm) that were prepared by coupling 28 mg of anti-apo A-I antibody to 5 ml of AminoLink gel. Samples (2 ml) were applied to the columns, which had been equilibrated with 25 mmol/L Tris-HCl and 150 mmol/L NaCl, pH 7.4 (TBS). Each column was allowed to equilibrate overnight at 4° C to completely adsorb the antigen (apo A-I–containing lipid-protein complexes) from the samples. Each column was then washed with 20 vol TBS to remove unbound material. The column was then eluted with 0.1 mol/L glycine/HCl, pH 2.6, to collect the bound fraction, which was immediately adjusted to neutral pH with 1 mol/L Tris/HCl, pH 9.5. Bound fractions were then either concentrated directly or separated into protein subfractions of greater and less than 30 kd with the Centriprep 30 separator. Individual pools of the subfractions were obtained by combining the protein from several column runs by using different patient sample sources. These were washed with 25 mmol/L ammonium bicarbonate and adjusted to a final protein concentration of 0.1 μg/ml.

Lipid analysis. Lipid contents in the anti-apo A-I antibody-binding fraction were measured to establish the nature of the protein-lipid complexes. Phospholipid concentration was determined by a kit modification of the enzymatic method of Takayama (PL-Kit K; Nippon Shoji Kaisha Ltd., Osaka, Japan).[18,19] Cholesterol concentration was determined enzymatically with a commercially available assay kit (Boehringer Mannheim Corp.).[20]

SDS-PAGE. Both the complete (above and below 30 kd) apo A-I binding proteins and the filtration-separated fraction below 30 kd were run on SDS-PAGE (4% to 20% gradient) in the buffer system described by Laemmli[21] under reducing conditions with the following sample buffer: 60 mmol Tris, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.001% bromophenol blue. For nonreducing conditions, mercaptoethanol was omitted from this buffer system. Aliquots containing 1 μg of protein for the more- abundant fraction of complete apo A-I-binding proteins and 0.2 μg for the fraction less than 30 kd were solubilized with sample buffer and either boiled for 5 minutes (reducing conditions) or incubated at room temperature overnight (nonreducing conditions). On completion of the electrophoretic run, gels were prefixed in a 50% methanol, 10% acetic acid solution for 30 minutes followed by rinsing for 30 minutes in 5% methanol, 7% acetic acid. They were next fixed in a 10% glutaraldehyde solution for 30 minutes and then rinsed with water. The gels were stained with silver nitrate according to the method of Morrissey.[22]

Immunoblotting. Proteins were separated by SDS-PAGE and immediately transferred unstained to a nitrocellulose membrane in 48 mmol/L Tris, 39 mmol/L glycine, and 20% methanol (pH 9.2) with the Trans-Blot SD semi-dry blot electrophoretic transfer system. Electrophoretic transfer was completed in 25 minutes at a constant voltage of 18 V. Prestained molecular weight markers served to monitor transfer efficiency. After the transfer was completed and the membrane was blocked with 1% (wt/vol) bovine serum albumin in 10 mmol/L Tris, 150 mmol/L NaCl, and 0.01% Tween 20, the membrane was exposed to sheep polyclonal anti-human apo A-I antibodies as the primary antibody and anti-sheep antibodies as the secondary antibody. Reactivity was detected by coupling of alkaline phosphatase to the secondary antibody and developing this in the presence of NBT/BCIP as a substrate.

IEF. Isoelectric focusing was performed with the model 111 Mini-IEF cell at the 5% setting. The pH gradients were established with an ampholyte spanning the pH range of 4.8 to 8.3 (Bio-Lyte 5/8, Bio-Rad). Aliquots of 0.1 μg of protein in 25 mmol/L ammonium bicarbonate per lane were then loaded onto the IEF gel. The electrophoretic run was carried out under constant voltage conditions in a stepwise manner. The voltages applied were 100 V for 15 minutes, 200 V for 15 minutes, and 450 V for 60 minutes. On completion of the electrofocusing, as indicated by a decrease in the measured current through the gel from an initial 4 to 5 mA to less than 2 mA, gels were fixed and stained as described for SDS-PAGE. After the gels were developed, isoelectric points were determined by comparison with standards (apo A-I, Sigma; and Isoelectric Focusing Calibration Kit, Pharmacia Fine Chemicals, Piscataway, N.J.).

Cholesterol crystal growth assay. A recently described photometric assay was used to measure cholesterol crystal growth in model bile.[23] In brief, stock solutions of cholesterol, egg lecithin, and sodium taurocholic acid were mixed in appropriate quantities according to Carey's critical tables[24] to construct a model bile with a cholesterol saturation index = 1.4, a total lipid concentration of 12.5 gm/dl, and a bile acid/phospholipid molar ratio of 4.4. The mixture was evaporated to dryness under nitrogen, lyophilized, and then resolubilized with TBS, pH 7.4, at 55° C. After filtration (0.22 mm), aliquots (130 μl) of this model bile were mixed with either protein samples (in 20 μl of TBS) or control solutions (20 μl of TBS) and were incubated at 37° C with shaking. To measure the development of cholesterol crystals, aliquots of the mixtures were diluted with 10 mmol/L sodium taurodeoxycholic acid/TBS.

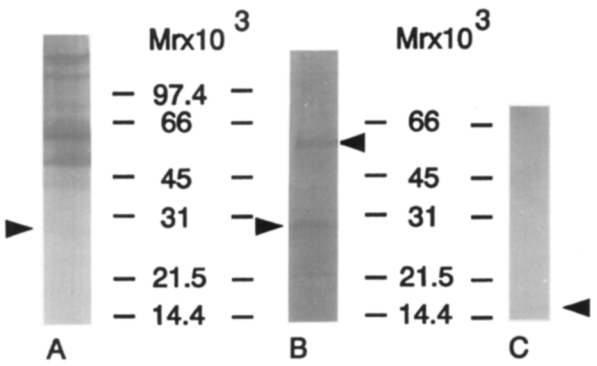

Fig. 1. A, SDS-PAGE of the anti-apo A-I antibody binding fraction of bile. A 1 μm sample of the bound fraction was applied to a 4% to 20% gradient gel under reducing conditions and stained with silver. Multiple bands are detectable, and only a small amount of the total protein mass is located at 30 kd (*arrowhead*). **B, C**, SDS-PAGE of the bile-derived 15 kd inhibitor protein. The bound fraction shown in **A** was ultrafiltered with a 30 kd cutoff, separated on a 4% to 20% gradient SDS gel (0.2 mg protein/lane), and stained with silver. Nonreducing conditions were applied in **B**, showing the 30 kd dimer and the 60 kd tetramer, and reducing conditions were applied in **C**, showing the 15 kd monomer. Protein bands are indicated by *arrowheads*. Different staining intensities of the protein bands are caused by different reactivity in the silver staining technique under reducing and nonreducing conditions.

After incubation for 20 minutes, absorbance was measured at 900 nm and mathematically corrected for the previous dilution. Cholesterol crystal growth curves were obtained from readings taken 24 to 240 hours after the start of the experiment; when the reading was taken depended on the growth rate of each reaction mixture. To compare the growth curves of the tested proteins, the time index (I_t) was used, which represented the onset time of crystal detection (onset time of the experimental group/onset time of the control group).

Amino acid sequencing. A 2 mmol sample of purified protein in 25 mmol/L ammonium bicarbonate was adsorbed to a polyvinylidine difluoride membrane with a sample preparation cartridge (ProSpin; Applied Biosystems Inc., Foster City, Calif.). The protein on the membrane was then subjected to N-terminal amino acid sequencing with an automated sequencer (model 477A; Protein Sequencer, Applied Biosystems) and analyzer (model 120A PTH; Applied Biosystems). The procedure is based on the Edman degradation method.[25] During the procedure the protein is held by a disk of porous glass-fiber filter in a heated, argon-purged reaction chamber. The filter was initially pretreated with Biobrene Plus (Applied Biosystems) and then cycled through repetitions of the Edman reaction with the protein loaded onto the filter. In brief, the steps of the Edman reaction consisted of phenylisothiocyanate coupling to the free N-terminal amino group of the peptide to form the phenylthiocarbamyl protein, followed by trifluoroacetic acid cleavage of the phenylisothiocyanate-coupled amino acid residue from the amino terminus of the protein and analysis of the residue on a PTC C-18 reverse-phase high-performance liquid chromatography column. The remaining protein is thus left with a new amino terminus for the next degradation cycle.

Enzymatic deglycosylation. Biliary and serum-derived 15 kd protein was enzymatically deglycosylated by treatment with N-glycosidase F according to the supplier's specifications, which are based on the work of Elder and Plummer et al.[26,27] The protein sample was boiled for 3 minutes in the presence of 0.1% (wt/vol) SDS, 1% (vol/vol) 2-mercaptoethanol, 1% (vol/vol) Triton X-100, and 25 mmol/L EDTA in 100 mmol/L sodium phosphate buffer, pH 7.4. Then N-glycosidase (1 U/mg protein) was added, and the reaction mixture was incubated for 18 hours at 37° C. The molecular weight of the deglycosylated protein backbone was then estimated by using SDS-PAGE.

Additionally, protein was exposed to endo-α-N-acetylgalactosaminidase enzyme. The protein sample was boiled for 3 minutes in the presence of 0.5% SDS and 0.1 mol β-mercaptoethanol and was then diluted with 10 mmol/L calcium acetate and 20 mmol/L sodium cacodylate buffer (pH 7.0) containing 1 U/ml neuraminidase. Endo-α-N-acetylgalactosaminidase was added, and this reaction mixture was incubated for 18 hours at 37° C. The effect of this enzyme on the molecular weight was then examined with SDS-PAGE.

Statistical analysis. The cholesterol crystal growth curves were analyzed by using analysis of variance at each time point to determine whether differences existed between different study groups. If the analysis of variance indicated statistical significance ($p < 0.05$), the unpaired t test was used to compare each of the study groups with the control and the other study groups.[28]

RESULTS

Characterization of the new biliary 15 kd inhibitor protein. Ultrafiltered, PBS-washed gallbladder bile was applied to an anti-apo A-I immunoaffinity chromatography column. In a representative pool of bound fractions, lipid-to-protein ratios were found to be 0.57 mg phospholipid per mg protein and 0.17 mg cholesterol per mg protein, thus demonstrating the mixed nature of these fractions. After elution, the bound fraction was analyzed by SDS-PAGE. As shown in Fig. 1, *A*, instead of a single band at the M_r of apo A-I (28 kd), multiple bands were detected with silver staining. The majority of these protein bands had an M_r greater than 50 kd, with dominant bands at 56 kd and 68 kd. A less dominant although clearly detectable band at 30 kd was also seen. After filtration of the bound fraction through a 30 kd cutoff membrane, SDS-PAGE revealed a 30 kd protein band as the major band (Fig. 1, *B*) under nonreducing conditions. Occasionally, however, under nonreducing conditions another protein band with

an apparent molecular weight of 60 kd was also found (Fig. 1, *B*). Which of both nonreduced forms was observed in a specific experiment appeared to be unpredictable and independent of experimental conditions. SDS-PAGE of the 30 kd filtered fraction under reducing conditions consistently showed a 15 kd band and the complete absence of protein bands at 30 or 60 kd (Fig. 1, *C*).

To identify apo A-I in the bound fractions, immunoblotting was performed on both the total-bound fraction and on the subfraction that had been filtered through a membrane with a 30 kd cutoff from serum and bile. In the total-bound fraction, some bands shown by silver staining also emitted a signal with immunoblotting. The 56 kd band yielded the strongest signal, thus indicating the probable presence of an apo A-I dimer. Other positive bands, however, were not simple polymers of apo A-I. Their molecular weights were not exact multiples of 28 kd, leading to the reasonable assumption that they represented protein-protein or protein-lipid complexes of biliary apo A-I (Fig. 2, *A*). The 30 kd filtered fraction, on the other hand, showed only minimal reactivity with anti-apo A-I antibody (Fig. 2, *B*), thus indicating that the 30 kd band was composed predominantly of an entirely different protein.

When IEF of the purified biliary 15 kd protein (Fig. 3) was performed under native conditions, the procedure yielded only a single band with a pI of 6.1. The lack of other bands indicated that isoforms with different isoelectric points were not present. Control experiments with apo A-I showed its primary pI to be at 5.6 and showed that its known isoforms have a pI ranging between 5.5 and 5.7, in accordance with published data.[29,30]

N- and O-enzymatic deglycosylation of the isolated 15 kd protein were performed to identify the length of its polypeptide backbone (Fig. 4, results shown for N-glycosidase). After 18 hours of incubation in the presence of N- or O-glycosidase, no change in the apparent M_r of the protein was detected by SDS-PAGE under reducing conditions. A control experiment with α_1-acid glycoprotein as a positive control showed that its molecular weight was reduced under these conditions, from 44 kd to 30 kd, in accordance with published observations.[1]

N-terminal amino acid sequencing of the isolated 15 kd protein purified from bile yielded a 19-mer including three blank cycles (Table I). Comparison of the sequence information with computer databases (PC/GENE, Swiss-Prot) showed no homology to any other protein.

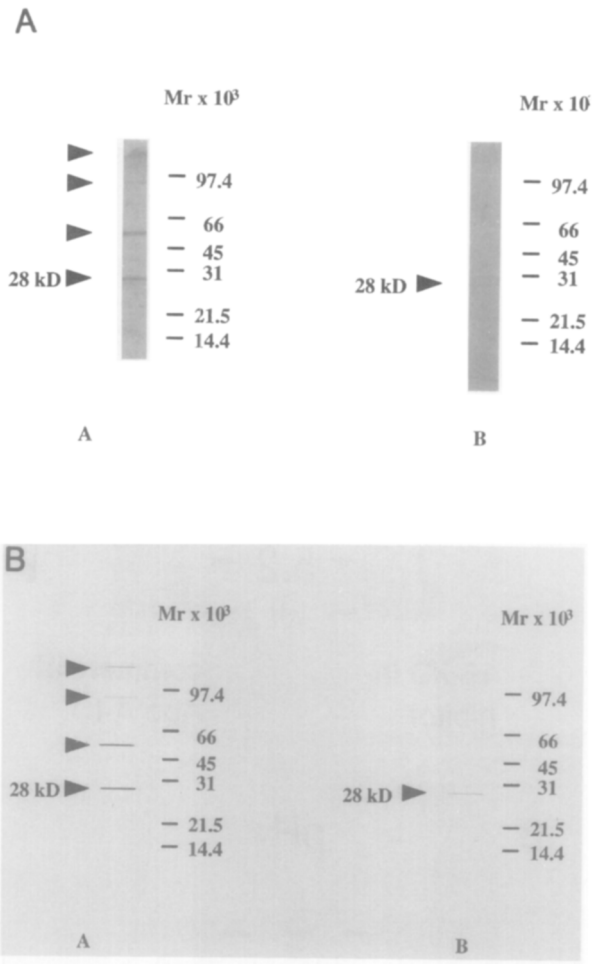

Fig. 2. A, *Lane A*, Immunoblot of the anti-apo A-I antibody-binding fraction of bile. Proteins separated as described in Fig. 1, A, were transferred to a nitrocellulose membrane and blotted with anti-apo A-I antibodies. Reactive bands are indicated by *arrowheads*. *Lane B*, Immunoblot of the bile-derived 15 kd inhibitor protein dimer. Protein was prepared as described in Fig. 1, B. After transfer to a nitrocellulose membrane, it was blotted with anti-apo A-I antibodies. Very little reactivity was detectable, as indicated by *arrowhead*. **B**, Schematic drawing of **A**.

Presence of the 15 kd inhibitor protein in human serum. The anti-apo A-I antibody-binding fraction from human serum was prepared by immunoaffinity chromatography. After ultrafiltration through a 30 kd cutoff membrane, SDS-PAGE under reducing conditions showed protein bands after silver staining at both 15 kd and 28 kd (Fig. 5, *A*). IEF of the filtrate revealed protein bands at pI = 6.1 as well as pI= 5.6 (Fig. 5, *B*).

Effect of the biliary and serum 15 kd protein on cholesterol crystallization. The effect of the new protein on cholesterol crystal growth was compared with that of apo A-I and a negative control (TBS) in the cholesterol crystal growth assay (Fig. 6). At a concentra-

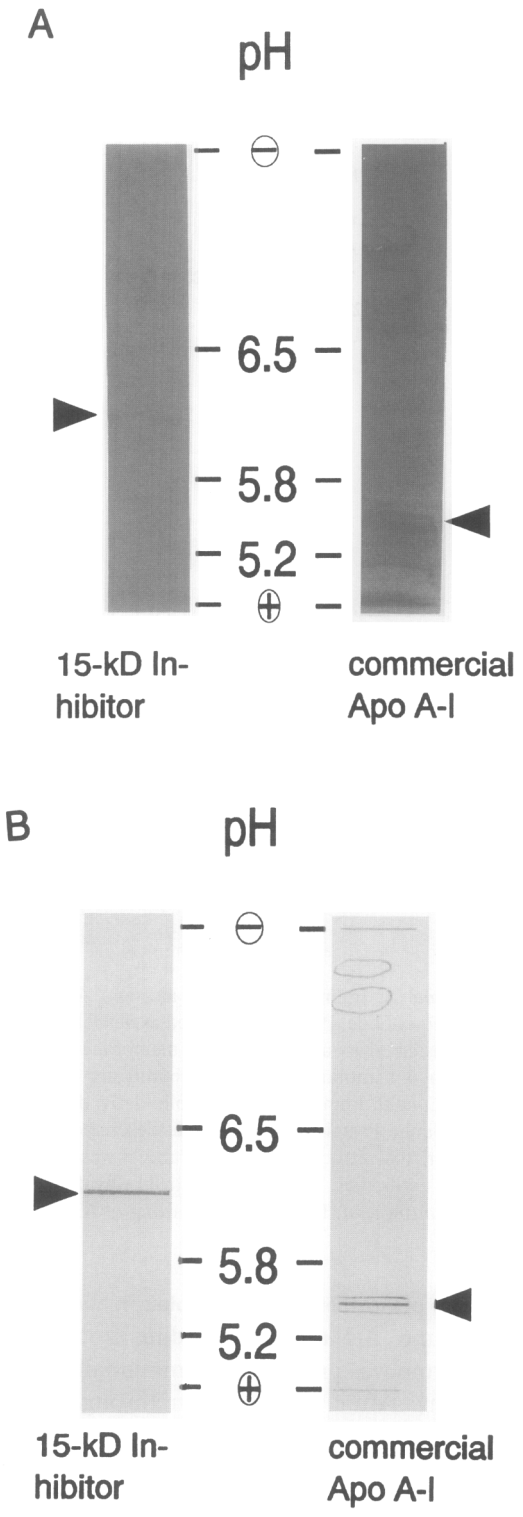

Fig. 3. **A**, IEF of the purified 15 kd inhibitor protein and apo A-I. A 0.1 mg sample of purified protein was applied to an IEF gel on a Bio-Rad model 111 mini IEF cell and stained with silver. Standard pH values are indicated. The 15 kd protein showed a pI of 6.1 without isoforms, different from the observed isoforms of apo A-I, which are in the published range of 5.5 to 5.7. Contact lines of the gel with the electrodes are marked as "+" and "−". **B**, Schematic drawing of **A**.

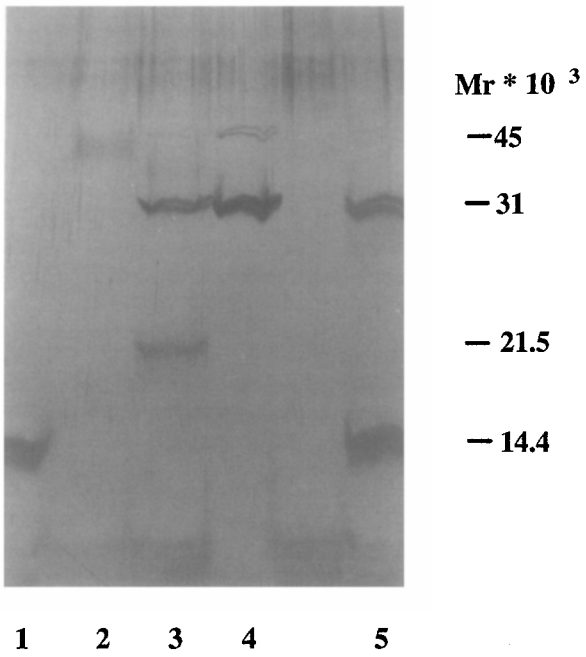

Fig. 4. N-enzymatic deglycosylation of the 15 kd inhibitor protein and α_1-acid glycoprotein. A 1 mg sample of the α_1-acid glycoprotein and a 1 mg sample of the 15 kd inhibitor protein were coincubated with N-glycosidase F for 18 hours at 37° C. While the molecular weight of α_1-acid glycoprotein is reduced because of deglycosylation, the 15 kd protein does not change its apparent molecular mass. *Lane 1*, The 15 kd inhibitor; *lane 2*, α_1-acid glycoprotein; *lane 3*, α_1-acid glycoprotein plus N-glycosidase F; *lane 4*, N-glycosidase F; *lane 5*, 15 kd inhibitor plus N-glycosidase F.

tion of 10 mg/ml, the 15 kd protein markedly delayed the onset of cholesterol nucleation ($I_t = 1.27$) and reduced the final crystal mass to 75% of the control. The effects were independent of the source of the protein (bile or serum). In contrast to this finding, apo A-I at the same concentration had no significant influence on either variable ($p < 0.05$ for crystal mass).

DISCUSSION

The new 15 kd protein inhibited cholesterol crystal growth in supersaturated model bile. From gallbladder bile and serum it can be copurified with apo A-I–containing lipid aggregates consisting of cholesterol and phospholipids (i.e., vesicles and high-density lipoprotein particles). Hence its purification relies apparently on an affinity to the same lipid-protein aggregates as is found with apo A-I and not on antigenic homology with apo A-I or specific binding of the protein itself to apo A-I. Like apo A-I, it has properties that differ from other, mostly hydrophilic, serum and bile proteins. In a recent study, apo A-I immunoaffinity chromatography was used

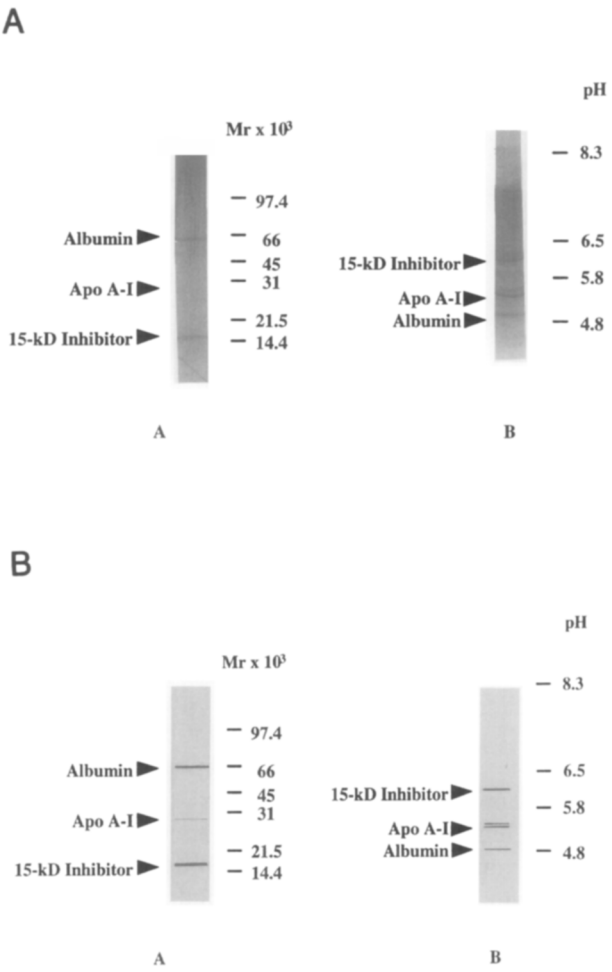

Fig. 5. A, Serum-derived 15 kd inhibitor protein. The 30 kd filtered anti-apo A-I bound fraction from serum was prepared. Silver-stained SDS-PAGE (*A*, reducing conditions) and IEF (*B*) show that in addition to a band with M_r = 15 kd and pI = 6.1, representing the 15 kd serum inhibitor, a 28 kd band with pI = 5.6, representing serum apo A-I, exists. **B**, Schematic drawing of **A**.

Table I. N-terminal amino acid sequence of the 15 kd biliary inhibitor glycoprotein

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Glu	Val	Pro	Glu	Arg	Val	Val	Lys	X	-	X	Glu	Arg	Phe	Glu	Arg	Val	X	Lys	Phe

X, Blank cycle.

to purify apo A-I–containing HDL from human serum.[29] In addition to apo A-I, SDS-PAGE showed that these fractions contained a series of protein bands of increasing M_r, among which albumin and α_1-antitrypsin were identified. An explanation for this unexpected finding was provided by evidence that a major fraction of apo A-I in serum does not exist in free monomeric form but rather in a complex association with lipids and other proteins present in the HDL fraction. Therefore, by using immunoaffinity chromatography to purify apo A-I, other associated proteins necessarily are obtained, unless the applied sample has been previously delipidated or the unbound fraction is eluted from the column with simultaneous delipidation.

As our results show, when this approach is used with bile, the outcome is similar despite the presence of bile salt detergents. The main band of apo A-I as determined by immunoblotting is at 56 kd, corresponding to a dimer. But some other bands that are not exact multiples of 28 kd also give a signal, indicating that apo A-I is associated with other proteins, lipids, or both. Many other bands showed no signal and therefore must be considered to be unrelated proteins.

For serum, our results confirm previously pub-

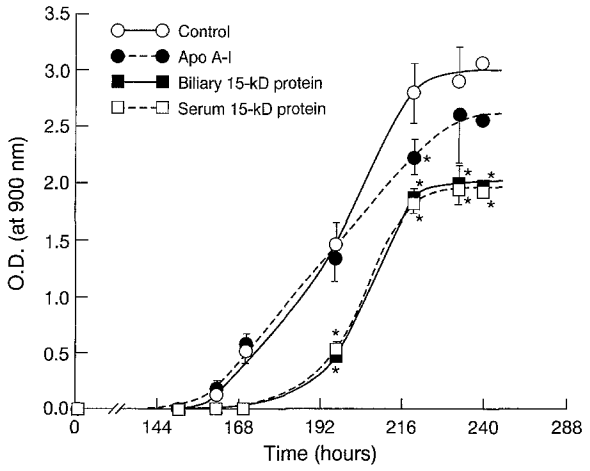

Fig. 6. Inhibiting effect of bile- and serum-derived 15 kd inhibitor protein and apo A-I on cholesterol crystal growth. Supersaturated bile model was prepared according to Carey's critical tables[24] (cholesterol saturation index = 1.4, bile acid/phospholipids = 4.4, total lipids = 12.5 gm/dl). A 10 mg/ml sample of purified protein was added. Values are expressed as mean ± SD (n = 5). Statistically significant differences versus control are marked by * ($p < 0.05$).

lished data.[29] In the 30 kd filtered apo A-I–bound fraction from serum, a considerable amount of monomeric apo A-I was recovered by immunoaffinity chromatography when examined with SDS-PAGE under reducing conditions. IEF showed the presence of bands with the pI of apo A-I, along with a band of pI = 6.1 that represents the serum 15 kd protein. The apo A-I antibody-binding fraction from serum has been shown to represent HDL.[29] Therefore, the copurification of the new inhibitor in the apo A-I–binding protein fraction shows that it is part of the HDL complex. However, not all proteins included in HDL (e.g., albumin) are apolipoproteins. Apolipoproteins are generally defined by their constant association with particular lipids in a fixed concentration range and by their structural localization (e.g., surface domains) in relation to the lipoprotein particulate species.[31] Whether the 15 kd protein in its serum form represents a new apolipoprotein cannot be determined from the present study. Further studies should indicate whether the new protein meets criteria for an apolipoprotein. The present protein could not have been observed in the earlier study simply because the investigators confined their studies to apolipoproteins of M_r greater than 17.5 kd (apo A-II).[29]

The absence of free monomeric apo A-I in bile is probably the reason why a 15 kd protein different from apo A-I was isolated and purified by simple ultrafiltration of the apo A-I binding fraction. Immunoblotting showed only minute amounts of apo A-I in the filtrate. Amino acid sequencing, however, yielded no apo A-I sequence[32] in the filtrate of proteins less than 30 kd. Because the N-terminus of apo A-I is not blocked,[31] its sequence would have been detected had it been present in more than negligible amounts.

Under nonreducing conditions the new protein is present at a molecular weight of 30 kd or 60 kd. We therefore conclude that under physiologic conditions it is present as a homodimer or a tetramer (or both). These structures may be formed by disulfide linkages; however, self-aggregation as seen in apo A-I cannot be excluded. The 60 kd complex may well consist of two aggregated disulfide-linked dimers.

Recently the balance between hydrophilic and hydrophobic domains in the amino acid sequence has been proposed to have a major influence on crystallization inhibition activity for a given protein.[14] Hydrophilic proteins such as chymotrypsin and biliary immunoglobulins tend to promote crystallization, whereas more hydrophobic ones (e.g., apolipoproteins) are more likely to inhibit crystallization. The present novel protein, similar to apo A-I, tends toward self-aggregation, a property typical of lipophilic proteins. Its copurification with apo A-I indicates its prevalence in the same protein-lipid structures. However, its high degree of purification from bile after 30 kd filtration probably is related to either a reduction in the number or extent of lipophilic domains, which would allow it to exhibit a more balanced equilibrium between its free monomeric and polymeric forms.

When using commercially available enzyme preparations for analysis, we were unable to find glycosylation of the new 15 kd protein. This approach should exclude the most prevalent forms of carbohydrate groups. However, some less-common but possible forms of glycosylation may not have been detected by application of the present methods.

The activity of the 15 kd protein was compared with the activity of apo A-I. We chose apo A-I as a standard because of the many similarities between the two proteins and because apo A-I represents the only established biliary crystallization growth inhibitor protein[33] that has a well-documented physiologic concentration (median, 10 to 20 μg/ml).[34,35] At a concentration of 10 μg/ml, the new protein reduced the final crystal mass by 30% and significantly delayed the onset time, whereas apo A-I had no effect. This is in keeping with previous reports by us and others that showed activity for apo A-I only at concentrations above 20 μg/ml.[15,33] Comparison

with other crystallization growth inhibitors is possible only by using published results. Such comparison is inherently difficult and unreliable because of probable differences in experimental conditions. However, the previously reported 120 kd dimer glycoprotein reduced cholesterol crystal growth by 10% to 15% at 30 μg/ml.[11] A lectin-purified group of inhibitor glycoproteins was recently described[12] that inhibited crystal growth variables. These proteins, in contrast to the present 15 kd protein, were isolated by using reducing and denaturing conditions, which may alter the native properties of a protein.

Because our purification approach is nonspecific and accordingly inefficient, our protein yield at present is only about 0.5 μg/ml gallbladder bile. Our estimate of a physiologic concentration near 10 μg/ml is based on the observation that a major 30 kd band remains in the apo A-I unbound fraction, indicating that the recovered amount under present circumstances considerably underestimates the true concentration. Because this estimate is not highly accurate, a more exact means of quantification is desirable. Developing an enzyme-linked immunosorbent assay[36,37] to measure the specific concentration of this protein will eventually help to clarify this point. However, a series of problems must be overcome in developing such an assay. Harvesting sufficient antigen for immunization (about 150 mg) is at present the most critical difficulty. Second, lipid-interference (e.g., antigen-shielding) is a common problem in enzyme-linked immunosorbent assay techniques.[38,39] This interference may lead to random inaccuracy and even to systematic underestimation.

In conclusion, the present 15 kd inhibitor protein is both novel and unique. It is probably not glycosylated, and it exhibits some properties typical of amphiphilic proteins. The mechanism of cholesterol crystallization inhibition of this protein is not yet clear. Although certain biliary effector glycoproteins are believed to directly interfere with crystal formation and growth,[12] for other proteins, such as α_1-acid-glycoprotein[40] or apo A-I,[14,15] modifications of biliary lipid carriers have been suggested. As has been shown for apo A-I, integration of the new protein into vesicle membranes based on its amphiphilicity may be feasible.[15] However, studies of the association of the new 15 kd protein with different forms of biliary lipids (e.g., vesicles and cholesterol crystals) will be needed to improve our understanding of both the target and mechanism of its primary functional activity.

We thank the staff of the Molecular Biology Core Facility, Case Western Reserve University Medical School, Cleveland, Ohio, for performing automated N-terminal amino acid sequencing. We also thank Janet Sperry for her assistance in preparing the manuscript.

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