Identification of Proteins Associated with Apolipoprotein A-I-Containing Lipoproteins Purified by Selected-Affinity Immunosorption†

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ABSTRACT: The isolation of apolipoprotein A-I-containing lipoproteins [Lp(A-I)] by selected-affinity immunosorption minimizes the loss of associated proteins that occurs during the isolation of high-density lipoproteins (HDL) by sequential ultracentrifugation. We have used two-dimensional gel electrophoretic analysis to separate the proteins associated with Lp(A-I). Using a combination of amino acid sequencing of transblotted proteins and Western blotting with specific antisera, we have identified a number of associated proteins. The positions of the apolipoproteins (apo) A-I, A-II, A-IV, C-III, D, and E were located on the gels. Lecithin–cholesterol acyltransferase and cholesteryl ester transfer protein were identified in association with Lp(A-I) to a greater extent than found associated with HDL after centrifugation. In addition to those proteins previously identified in association with HDL, we detected a number of plasma proteins associated with Lp(A-I), namely, fibrinogen, haptoglobin, proline-rich protein (C4b-binding protein), and apolipoprotein J (SP40,40 sulfated glycoprotein). The coisolation of these proteins with Lp(A-I) does not appear to be an artifact in that they have very low affinity for a sham column containing covalently bound preimmune goat IgG in place of the anti-apoA-I IgG. These findings suggest that in addition to apolipoproteins that exist largely in association with lipoproteins there is another class of proteins which exist both in lipoprotein-associated form and in the dispersed state. Detection and identification of these lipoprotein-associated proteins may aid in the mechanistic determination of a number of observed functions attributed to HDL.

High-density lipoproteins (HDL)[1] are thought to participate in a wide variety of biological functions. Prominent among these appears to be their participation in reverse cholesterol transport (Fielding & Fielding, 1982; Glomset, 1968). Additionally, they are also implicated in a number of other processes such as blood coagulation (Bareowcliffe et al., 1982; Carson, 1981), inhibition of viral infection (Kane et al., 1979; Levy et al., 1982), inhibition of infection by protozoal parasites (Ormerod & Venkatesan, 1982), delivery of cholesterol to steroidogenic cells (Chen et al., 1980; McNamara et al., 1981; Ohashi et al., 1981), degradation of bacterial endotoxins (Ulevitch et al., 1981), inhibition of LDL oxidation (Klimov, 1987; Kunitake et al., 1992; Ohta et al., 1989; Parthasarathy et al., 1990), and perhaps stimulation of vascular endothelium (Tauber et al., 1981).

One difficulty in elucidating the exact biochemical mechanisms by which HDL act is the particle heterogeneity that exists within this lipoprotein class. Until recently, the principal method of HDL isolation has been sequential ultracentrifugation. This method causes the loss of associated proteins (Curry et al., 1976; Fainaru et al., 1975; Fainaru et al., 1976; Kunitake & Kane, 1982; Mahley & Holcombe, 1977), either by dissociation of proteins from HDL complexes or by exclusion of complexes whose particle densities lie outside the traditional HDL density zone. Thus the natural association of proteins with HDL is obscured.

To avoid the use of sequential ultracentrifugation in isolation of HDL, we have developed a method, "selected-affinity immunosorption" (Kunitake et al., 1982; McVicar et al., 1984) allowing for the isolation of all apoA-I-containing lipoproteins [Lp(A-I)] with minimal perturbation of structure.

Immunoisolated Lp(A-I) retain greater amounts of associated proteins than HDL isolated by ultracentrifugation. Proteins known to interact with HDL, such as lecithin–cholesterol acyltransferase (LCAT) and cholesteryl ester transfer protein (CETP), have been found in greater abundance on immunoseparated lipoproteins than on ultracentrifugally isolated HDL (Cheung et al., 1986; Fielding & Fielding, 1980). Proteins, which have gone unnoticed in ultracentrifugally isolated HDL, have been detected in immunoisolated Lp(A-I): a subfraction of Lp(A-I)-containing transferrin (Kunitake et al., 1992), a subfraction of Lp(A-I)-containing apoJ (Burkey et al., 1992; de Silva et al., 1990b; Jenne et al., 1991; Stuart et al., 1992), and several unidentified proteins (James et al., 1988) have been reported.

We report here the association of a number of plasma proteins with Lp(A-I), characterized by two-dimensional gel electrophoresis, microsequencing, and Western blotting.

EXPERIMENTAL PROCEDURES

Isolation of Lipoproteins. Venous blood was drawn from fasting normolipidemic subjects (both female and male) and immediately cooled to 4 °C in the presence of the following preservatives and protease inhibitors (final concentrations): ethylenediaminetetraacetic acid (EDTA, 0.08%), sodium azide (0.1%), gentamycin sulfate (10 μg/mL), benzamidine (0.3 mg/mL), phenylmethanesulfonyl fluoride (10 μg/mL), and

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[1] Abbreviations: HDL, high-density lipoproteins; Lp(A-I), apolipoprotein A-I-containing lipoproteins; LDL, low-density lipoproteins; PRP, proline-rich protein.

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ϵ-aminocaproic acid (0.13%). The plasma was separated by centrifugation of the blood at 1000*g* and 4 °C for 45 min.

High-density lipoproteins were isolated by sequential ultracentrifugation from the density interval 1.063–1.21 gm/mL (Havel et al., 1955). The densities were adjusted by the addition of potassium bromide. The centrifugations were performed in a Beckman preparative ultracentrifuge with a 40.3 rotor at 36 000 rpm and 4 °C for 24 h. The centrifugations were repeated at each density. After isolation, the HDL were dialyzed against 5.0 mM Tris and 150 mM NaCl, pH 7.4 (TBS). The above preservatives, including α-2-macroglobulin (10 μg/mL), were added to the HDL.

The apoA-I-containing lipoproteins [Lp(A-I)] were isolated from plasma by selected-affinity immunosorption, as described previously (Kunitake et al., 1990, 1982; McVicar et al., 1984). Briefly, antibodies against apoA-I were isolated by binding them to an apoA-I–Sepharose column and eluting a subpopulation of antibodies that dissociated with a preselected elution buffer (0.2 M acetic acid, 0.15 M NaCl, pH 3.0). The selected antibodies were then cross-linked to CNBr-activated Sepharose (Pharmacia, Uppsala, Sweden) to form the anti-apoA-I–Sepharose column used to isolate the lipoproteins. Plasma was applied to the anti-apoA-I column, the unbound proteins were washed out with TBS (greater than 10 column volumes), and the elution buffer (the same buffer used to isolate the antibodies) was then used to recover all of the bound Lp(A-I). The eluate was immediately neutralized with 2 M Tris, and all preservatives were added. The Lp(A-I) were concentrated and exchanged into TBS containing the preservatives with an Amicon stirred ultrafiltration cell fitted with a YM 10 membrane. Finally, the Lp(A-I) were passed through protein A–Sepharose and anti-human albumin columns prior to analysis.

A sham immunosorption column was prepared in exactly the same fashion as the anti-apoA-I–Sepharose column except that nonreactive antibodies isolated from preimmune goat serum were bound to the Sepharose matrix instead of the purified anti-apoA-I antibodies. The eluate from the sham column was compared to the eluate from an anti-apoA-I–Sepharose column of exactly the same size, run in the same manner, and with both eluates concentrated to the same degree. Equal volumes of both eluates were analyzed for their contents of lipoprotein-associated proteins.

Protein Characterization. Two-dimensional gel electrophoresis was performed as described by Garrels (1983), except the concentration of urea in the isoelectric focusing gel was changed to 8.5 M. Sample preparation buffer consisted of 9.97 M urea, 0.31% SDS, 2% Nonidet P-40 (Sigma, St. Louis, MO), 100 mM dithiothreitol, and 2% ampholytes (Resolyte 4-8; Hoefer Scientific Instruments, San Francisco, CA). The apparatus used was the IsoDalt system (Hoefer Scientific Instruments). The second dimension gels were 11% acrylamide.

SDS–PAGE was performed according to the method of Laemmli (1970) employing 10% or 15% polyacrylamide gels run in a Bio-Rad (Richmond, CA) minigel system. Samples were boiled in sample buffer (2% SDS, 67 mM Tris, pH 6.8, 20% glycerol) for 1 min before loading. All chemicals used were of electrophoresis grade (Bio-Rad, Richmond, CA). Amersham Rainbow molecular weight markers (Arlington Heights, IL) were used.

Gels were stained either with silver nitrate, according to the procedure of Merrill as modified by Morrissey (1981), or with Coomassie brilliant blue R-250 (Sigma, St. Louis) by the precipitation method (Zehr et al., 1989).

Proteins were transferred onto nitrocellulose membranes (0.2 mm; Schleicher and Schuell, Keene, NH) according to the method of Towbin et al. (1979). For the 2-D gels, a semidry apparatus was employed (Hoefer Scientific, San Francisco); for the 1-D gels, a Bio-Rad tank system was used. The blotted membranes were soaked in wash buffer (40 mM sodium phosphate, pH 7.4, 0.1% Nonidet P-40, 150 mM NaCl) with 10 g/L nonfat dry milk (Lucerne, Oakland CA) and 10 g/L bovine serum albumin (RIA grade, Sigma, St. Louis) and then incubated with various antisera in the same buffer. Between antisera and before autoradiography the membranes were washed in four changes of wash buffer.

Antiserum directed against cholesteryl ester transfer protein (CETP) was provided by Yves Marcel and Alan Tall; against apoD and lecithin cholesterol acyltransferase (LCAT) by Yves Marcel; and against human proline-rich protein (PRP) by Clive Pullinger and Richard Havel. Goat antiserum directed against apoA-I was obtained from Tago (Burlingame, CA). Rabbit antiserum against human apoE was generated by the injection of purified apoE. Rabbit antisera against either the α- or β- chain of SP40,40 were generated by the injection of synthetic peptides corresponding to internal sequences of the α- and β- chains (ERKTLLSNLEEAKKKKEDAL and KLRRELDESLQAERLTRKY, respectively). Goat antiserum directed against apoA-II was from Boehringer-Mannheim (Indianapolis, IN). Antisera against fibrinogen and haptoglobin were from Zymed (South San Francisco, CA), as were rabbit anti-mouse IgG and rabbit anti-goat IgG. The rabbit anti-goat IgG was labeled with ^{125}I using Iodobeads (Pierce Chemical Co., Rockford, IL) and used as a second antibody for those antibodies raised in goats (fibrinogen, haptoglobin, A-I, and A-II). ^{125}I-Labeled goat anti-rabbit IgG (New England Nuclear, Boston, MA) was used as a second antibody for the remaining antibodies, either directly (apoE, SP40,40, and PRP) or after rabbit anti-mouse IgG (LCAT, CETP, and apoD).

Proteins were eluted from the 2-D gels using a Centrilutor apparatus (Amicon Division, C. R. Grace and Co., Danvers, MA). Gels run in parallel were stained briefly with Coomassie brilliant blue according to the procedure of Hunkapiller et al. (1983) and were lined up with an identical gel that had been silver stained, in order to determine which areas of the gel to excise. Slices of about 2 mm × 1 cm were placed in 400-μl polypropylene tubes and eluted into Centricon tubes (Amicon) in the presence of 10 mM ammonium bicarbonate and 0.02% SDS. The Centricon tubes were then centrifuged to concentrate the eluate to about 50 μL.

Sequencing was performed by automated Edman degradation with an Applied Biosystems Model 470A gas-phase microsequencer (Foster City, CA) (Hunkapiller & Hood, 1983). Because apoA-II has a blocked N-terminus, 1.6 μg of protein was first incubated with 0.2 μg of trypsin for 17 h at 37 °C, and then the fragments were separated by reverse-phase chromatography and finally sequenced.

The quantities of haptoglobin and fibrinogen in Lp(A-I) were measured by competitive ELISA assays similar to the procedure previously described (Kunitake et al., 1992), except purified haptoglobin and fibrinogen (Sigma, St. Louis) were used for standardization and rabbit anti-goat IgG conjugated to horseradish peroxidase (Pierce, Rockford, IL) was used to detect the anti-haptoglobin and anti-fibrinogen antibodies bound to the coated plates.

RESULTS

The proteins associated with immunoisolated Lp(A-I) were separated by high-resolution two-dimensional gel electro-

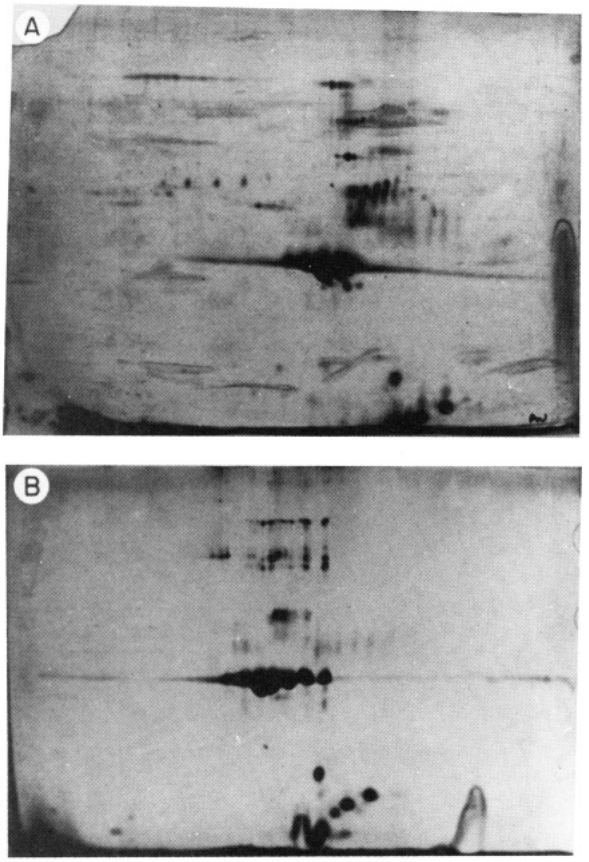

FIGURE 1: 2-D gels of the proteins of immunoisolated Lp(A-I) (70 μg of apoA-I; panel A) and ultracentrifugally isolated HDL (70 μg of apoA-I; panel B). Samples were separated in the first dimension by isoelectric focusing, pH 4–8, followed by SDS–PAGE in an 11% gel. After electrophoresis the gels were subjected to silver staining. While several protein species were common to both gels, a number of the proteins readily visible in panel A were absent or greatly diminished in panel B.

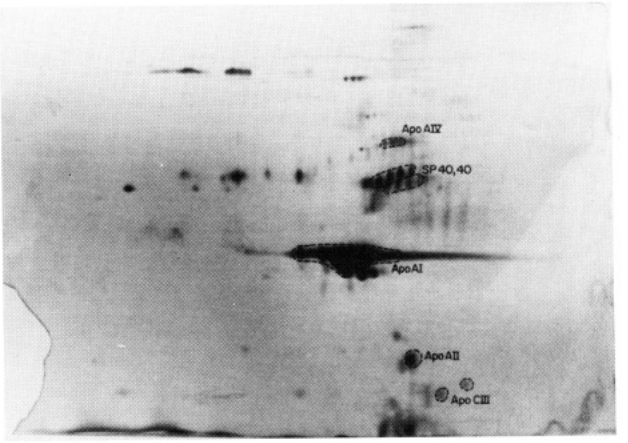

FIGURE 2: 2-D gel of proteins of Lp(A-I). 2-D gels of Lp(A-I) proteins were generated as described in Figure 1. From a 2-D gel run in tandem, a number of proteins were isolated by electroelution and identified by sequencing. The locations of apoA-I, apoA-II, apoA-IV, apoC-III, and SP40,40 (apoJ) are indicated.

Table 1: Amino Acid Sequences of Proteins Eluted from Two-Dimensional Gels of Lp(A-I)[a]

protein	determined sequence[b]
apoA-I	DEPPQ SPWDR VKDLA
apoA-II[c]	VKSPE LQAE
apoA-IV	XVXAX QVATV MXDY
apoC-III	SEAED ASLXX FMQG
SP40,40	
α-chain	SLMPF SPYEP LNFHA MFQP
β-chain	DQTVS DNELQ EMXNQ
fibrinogen	
β-chain	DNENV VNEYS S
γ	YVATR DNXXI LDERF

[a] All of the sequences reported completely matched the protein sequences listed in the SWISS-PROT data bank for the amino acids which were determined. [b] X = amino acid not determined. [c] Internal sequence determined after trypsin digestion of isolated protein; sequence corresponds to amino acids 29–37 of apoA-II.

phoresis (Figure 1, panel A), revealing a number of distinct components. While some of the components appear to represent isoforms of single proteins, it is nevertheless clear that several different proteins are associated with Lp(A-I). In contrast, only a limited number of proteins is associated with ultracentrifugally isolated HDL (Figure 1, panel B), even though the mass of apoA-I loaded on this gel equaled that of the Lp(A-I).

The locations of apoA-I, apoA-II, apoC-III, and apoA-IV on the 2-D gels were identified by automated Edman sequencing of the components excised and eluted from 10 identical gels (Figure 2, Table 1). ApoA-II was identified after trypsin digestion of the electroeluted protein and sequencing of the proteolytic fragments (digestion was necessary because the N-terminus was blocked in the intact protein). Western blotting of 1-D gels of Lp(A-I) and centrifugally isolated HDL was performed to confirm the presence of various apolipoproteins. ApoA-I, apoA-II, apoE, and apoD were found associated with both Lp(A-I) and HDL in roughly the same amounts (Figure 3), whereas LCAT and CETP were present on Lp(A-I) to a much greater extent than on centrifugally isolated HDL (Figure 3). The multiple bands observed in the anti-apoA-II and anti-apoE immunoblots were due to the presence of apoE–apoA-II heterodimers and apoE–apoE homodimers present in Lp(A-I). We cannot explain the appearance of the multiple bands observed in the apoD, LCAT, and CETP immunoblots.

The plasma proteins fibrinogen, haptoglobin, proline-rich protein (C4b-binding protein), and SP-40,40 (apoJ, clusterin) were found, by Western blotting of 1-D gels, to coisolate with Lp(A-I) but were not found with centrifugally isolated HDL (Figure 4). The percentages of fibrinogen and haptoglobin associated with Lp(A-I) were found to be 2.2 ± 0.2% and 0.9 ± 0.1%, respectively, of their total plasma contents. The locations on 2-D gels of the various associated proteins were determined by Western blotting as exemplified by proline-rich protein and SP40,40 (Figure 5). In the cases of SP40,40 and fibrinogen, amino acid sequencing of the electroeluted proteins verified their identities (Table 1).

The presence of these plasma proteins on Lp(A-I) was apparently not due to an artifact of the selected affinity immunosorption technique. A sham column constructed using preimmune goat antibodies instead of antibodies directed against apoA-I, and identical to the anti-apoA-I column in every other respect, was run using the identical procedure. Equal aliquots of human plasma were processed over each of these columns, and equal volumes of the eluates were compared by Western blotting of 1-D PAGE gels. Only trace amounts of these proteins appeared in the eluate from the sham column (Figure 4).

The association of these plasma proteins with Lp(A-I) was not unique to the Lp(A-I) isolated from a single subject. Lp-(A-I) isolated from the plasma of four different individuals had silver-staining components in the expected areas for SP40,-40, fibrinogen, haptoglobin, and proline-rich protein (C4b-binding protein) (Figure 6). However, the amount of these proteins relative to apoA-I appeared to vary widely among individuals.

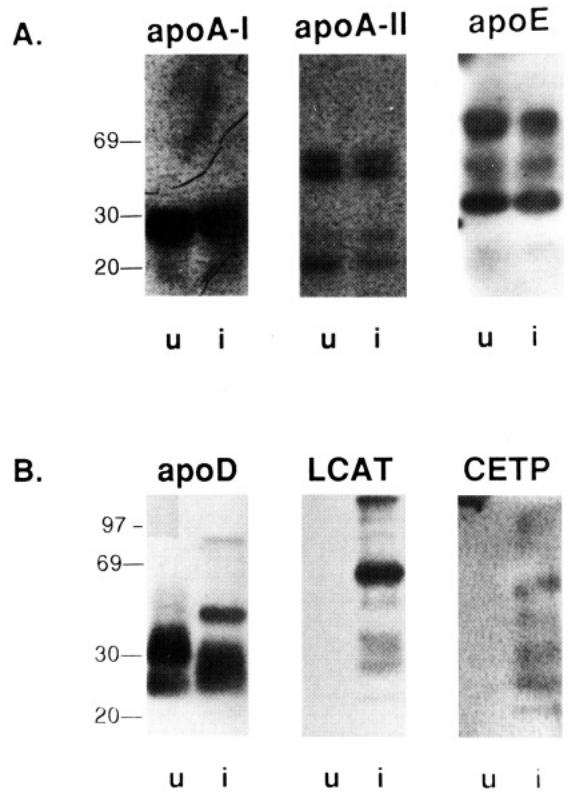

FIGURE 3: Western blots of Lp(A-I) and HDL. Both HDL (u, 30 μg) and Lp(A-I) (i, 25 μg) were resolved by 15% (panel A) and 10% (panel B) SDS–PAGE gels. Specific proteins were detected by immunoblot. Antisera used were against apoA-I, apoA-II, and apoE (panel A) and apoD, cholesteryl ester transfer protein (CETP), and lecithin cholesterol acyltransferase (LCAT) (panel B).

DISCUSSION

The immunoisolation of Lp(A-I) particles allows identification of specific lipoprotein-associated proteins that have not been detected previously primarily due to losses incurred during isolation by sequential ultracentrifugation. We found that proteins previously shown to associate with lipoproteins, LCAT, CETP, and apoA-IV, appeared to be enriched in Lp(A-I) as compared with ultracentrifugally isolated HDL, indicating that these proteins tend to be lost during ultracentrifugation. In addition to these proteins previously found to be associated with HDL, we found, by a combination of Western blotting and amino acid sequencing, that the plasma proteins PRP (C4B-binding protein), SP40,40 (apoJ), haptoglobin, and fibrinogen are also associated with Lp(A-I).

The coisolation of these proteins with Lp(A-I) does not appear to be artifactual, as indicated by the persistence of binding during extensive washing of the selected-affinity immunosorption columns and the virtual absence of these proteins in eluates from sham immunoaffinity columns. The ability of these proteins to remain associated with Lp(A-I) during extensive washing and at high dilutions suggests that the interaction between these proteins and Lp(A-I) is not weak. Furthermore, the observation that only a small portion of the plasma content of these proteins is bound to Lp(A-I) suggests that they interact with specific Lp(A-I) species.

Our observations have provided potential identification for several of the HDL-associated protein spots observed by James et al. (1988) from 2-D gel electrophoresis of Lp(A-I). Our findings also support previous findings of the enrichment of LCAT, CETP (Cheung et al., 1986), and apoA-IV (Duverger et al., 1993; Lagrost et al., 1989) in Lp(A-I) and emphasize the advantage of immunoisolation of HDL over sequential ultracentrifugal isolation.

Our detection of SP40,40 (apoJ, clusterin), likewise, is confirmatory of other reports. This newly discovered apolipoprotein has been characterized by de Silva and associates (de Silva et al., 1990a,c). Its association with Lp(A-I) has been documented by a number of groups. Harmony and colleagues found the association between apoJ and apoA-I using antibodies directed against apoJ (de Silva et al., 1990b) and have characterized these lipoproteins (Burkey et al., 1992; Stuart et al., 1992). James et al. also identified SP40,40 as one of the previously unidentified proteins they described when they viewed the lipoprotein-associated proteins of Lp(A-I) by two-dimensional electrophoresis (James et al., 1991). Jenne et al., using antibodies directed against clusterin, have isolated a clusterin–apoA-I lipoprotein complex (Jenne et al., 1991). SP40,40 was originally isolated as the major protein secreted by rat Sertoli cells (Griswold et al., 1986; Sylvester et al., 1984), and its sequence was determined (Collard & Griswold, 1987). The human protein was cloned by two separate groups, having been found both in blood (Kirszbaum et al., 1989), and in seminal fluid (Jenne & Tschopp, 1989). This same protein has been found in secretions from bovine chromaffin granules (Palmer & Christie, 1990), and elevated levels of expression have been described in both scrapie-infected hamster brain and hippocampus from Alzheimer's patients (Duguid et al., 1989).

There are two possible explanations for the enrichment of these proteins in Lp(A-I) as compared with centrifugally isolated HDL. First, centrifugation could cause the dissociation of the lipoprotein-associated proteins from HDL. A number of reports have documented the loss of proteins from HDL by ultracentrifugation (Curry et al., 1976; Fainaru et al., 1975, 1976; Kunitake & Kane, 1982; Mahley & Holcombe, 1977). We have demonstrated that the repeated ultracen-

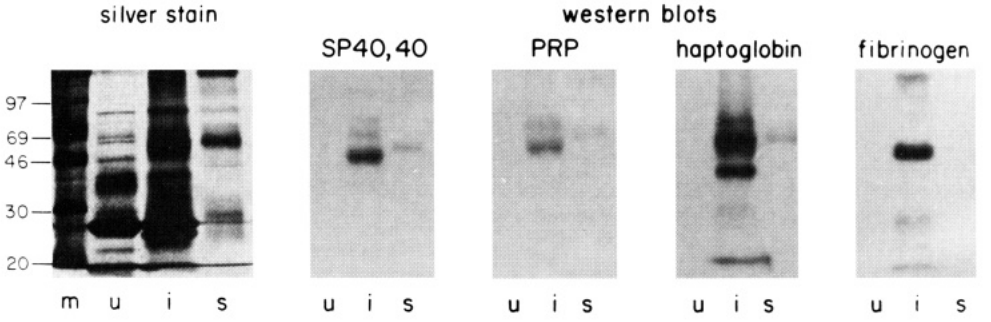

FIGURE 4: Western blots of 10% SDS–PAGE gels. Western blots of ultracentrifugally isolated HDL (u, 30 μg), Lp(A-I) (i, 25 μg), and "sham" eluate [s, same volume of column eluate as Lp(A-I), see Experimental Procedures] were performed to determine the presence of selected proteins in the three samples. Antisera used were directed against SP40,40, PRP, fibrinogen, and haptoglobin. A gel containing the samples and molecular weight markers (m) was silver stained for comparison (left).

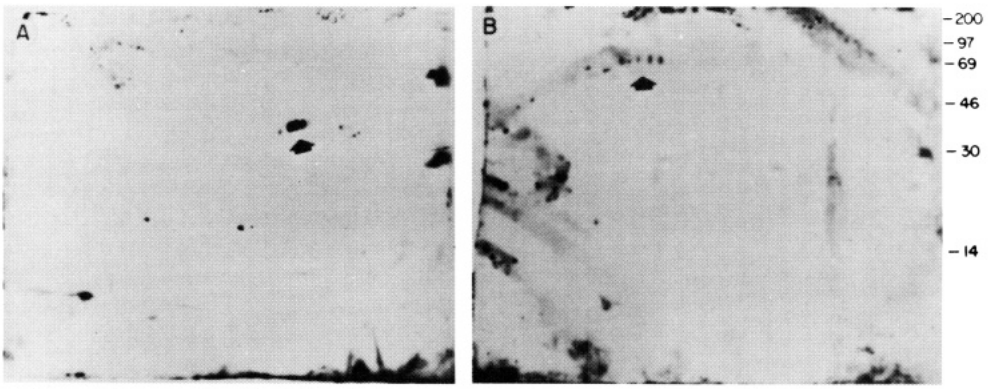

FIGURE 5: Western blots of 2-D gels. The identities of the components observed after 100 μg of Lp(A-I) proteins was resolved by 2-D gel electrophoresis were verified by immunoblotting (indicated by arrows). Antibodies used were against SP40,40 (panel A) and proline-rich protein (PRP) (panel B).

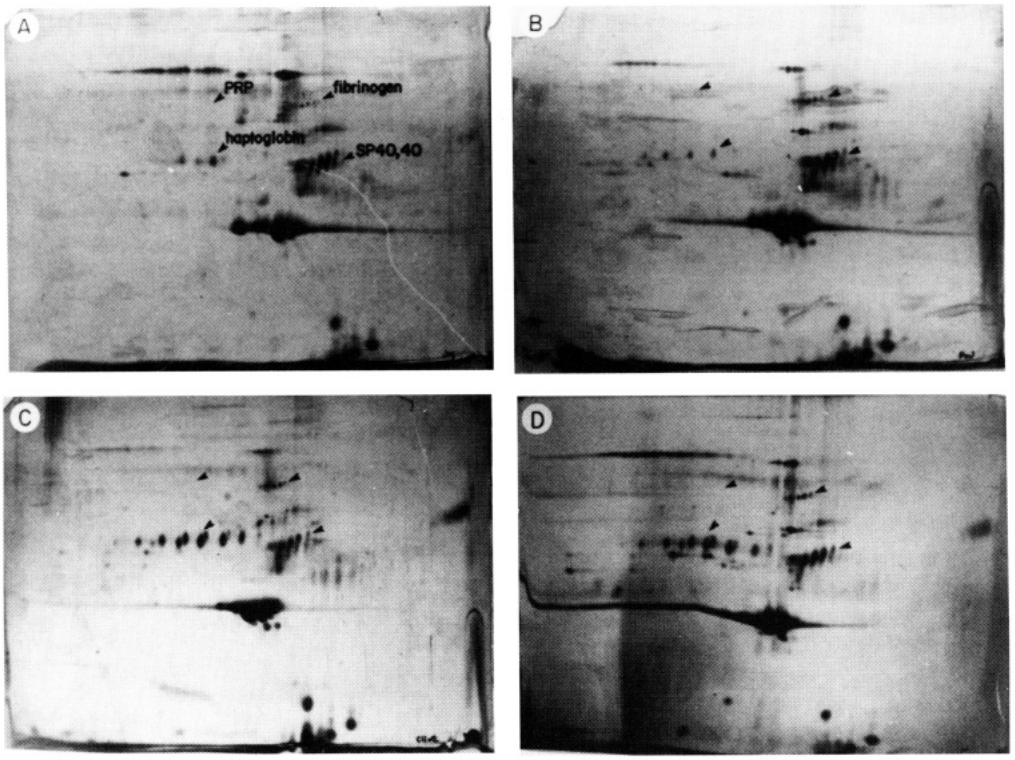

FIGURE 6: Silver-stained 2-D gels of Lp(A-I). The Lp(A-I) proteins (100 μg) isolated from four different individuals were resolved by 2-D gel electrophoresis. Arrows indicate positions of SP40,40, fibrinogen, haptoglobin, and PRP. The intensity of staining of these spots relative to the intensity of apoA-I varied substantially between the four individuals.

trifugation of HDL causes the continued loss of apoA-I from the intact lipoprotein (Kunitake & Kane, 1982). Second, the Lp(A-I) particles containing these proteins may have buoyant densities which are outside the density interval used to centrifugally isolate HDL. ApoA-I-containing lipoproteins both less dense and more dense than typical HDL have been described (Alaupovic et al., 1966; Schmitz & Assmann, 1982). A transferrin-containing Lp(A-I) that we have isolated by selected-affinity immunosorption appears to have a particle density greater than 1.21 g/mL and falls into this second category (Kunitake et al., 1992).

The association of C4b-binding protein (PRP), haptoglobin, and fibrinogen with Lp(A-I) is a novel finding. Only a small portion of these plasma proteins are bound to Lp(A-I), suggesting that only selected HDL species may be capable of binding to these proteins. The physiological importance of the association of the plasma proteins SP40,40 (apoJ), PRP, fibrinogen, and hapotglobin with Lp(A-I) is unknown. On the basis of a few of the reported functions attributed to HDL we can, however, speculate about their roles.

SP40,40 is a component of the SC5b-9 complex (Choi et al., 1989; Murphy et al., 1988; Podack & Muller-Eberhard, 1979), the nonlytic complement complex. The association of such a protein with Lp(A-I) raises the possibility that HDL could interact with the immune system.

Proline-rich protein, originally found associated with triglyceride-rich lipoproteins (Sata et al., 1976), has been shown to be identical to C4b-binding protein (Matsuguchi et al., 1989; C. Pullinger, personal communication). This association also suggests a relationship between HDL and the complement system.

HDL have been found to inhibit coagulation. Studies indicate that HDL interact with factor Xa to block the clotting cascade (Bareowcliffe et al., 1982; Carson, 1981). However, direct interaction of Lp(A-I) with fibrinogen could indicate another point of involvement in this process.

Haptoglobin binds hemoglobin which has been released from red blood cells. The possible physiologic importance of haptoglobin association with HDL is not obvious. However, aerobic bacteria require iron for growth. Haptoglobin binds

iron, effectively inhibiting bacterial growth. Perhaps the observed antiparasitic activity of HDL (Ormerod & Venkatesan, 1982) could involve haptoglobin.

Our findings lead to the concept that lipoprotein–protein interactions can be divided into two categories. Certain proteins (apolipoproteins) have very high affinity for the lipid constituents of lipoproteins and are found predominately in the lipoprotein-associated state in circulation. Other proteins are partitioned between complexes with specific lipoproteins and the dispersed (unbound) state. These associations do not appear to be the result of weak protein-protein interactions. Biological roles for the lipoprotein-associated forms of these plasma proteins should be sought.

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