Sequences Within the Amino Terminus of ApoB100 Mediate Its Noncovalent Association With Apo(a)

Brent R. Gabel, Roger S. McLeod, Zemin Yao, Marlys L. Koschinsky

Abstract—Although sequences within the C terminus of apolipoprotein B (apoB) have been implicated in the formation of covalent lipoprotein(a) [Lp(a)] particles, sequences in apoB that mediate initial noncovalent interaction with apo(a) remain to be characterized. To address this question, we have used an affinity chromatography method in which 2 recombinant forms of apo(a) [r-apo(a); either a 17-kringle form (17K) or a derivative containing apo(a) kringle IV types 5-8] have been immobilized onto Sepharose beads. Conditioned media from rat hepatoma (McA-RH7777) cell lines stably expressing various carboxyl-terminally truncated forms of human apoB (ranging from full-length apoB to apoB15) were applied to the r-apo(a) affinity columns; the columns were subsequently washed and eluted with e-aminocaproic acid (e-ACA). Specific binding was quantified by Western blot analysis of column fractions. Of the apoB truncations examined, apoB94, apoB42, apoB37, and apoB29 exhibited complete specific binding to 17K r-apo(a). Only \( \approx 50\% \) binding was observed for apoB18, whereas essentially no detectable binding was observed with apoB15. In all cases, similar results were obtained when the r-apo(a) kringles IV types 5-8-Sepharose column was used. Additionally, substitution of proline for e-ACA as the eluent resulted in similar column profiles with either r-apo(a) affinity column. We also demonstrated that apoB48 present in chylomicrons bound completely to the 17K column in an e-ACA-dependent manner. Taken together, these results represent the first demonstration that N-terminal sequences in apoB between amino acid residues 680 (apoB15) and 781 (apoB18) are essential for noncovalent association with apo(a) and that these sequences interact with domain(s) present within apo(a) kringles IV types 5-8. (Arterioscler Thromb Vasc Biol. 1998;18:1738-1744.)

Key Words: lipoprotein(a) • apolipoprotein(a) • apolipoprotein B-100 • LDL • Lp(a) particle assembly

lipoprotein(a) [Lp(a)] is a unique lipoprotein that closely resembles LDL both in lipid composition and the presence of the protein moiety apolipoprotein B100 (apoB). The distinguishing feature of Lp(a) is the presence of the highly glycosylated, polymorphic protein apolipoprotein(a) [apo(a)], which is covalently attached to apoB by a single disulfide bond.\(^1\) Lp(a) has been the focus of considerable research attention owing to a number of epidemiological studies that have identified elevated levels of Lp(a) as a risk factor for the development of atherosclerosis.\(^2,3\) However, the mechanism(s) by which Lp(a) contributes to the atherosclerotic process remains unclear.\(^2,3\)

The process of Lp(a) assembly has been the subject of intensive investigation, with specific emphasis on the sequence requirements in both apo(a) and apoB that are required for Lp(a) formation. Several lines of evidence indicate that Lp(a) formation is predominantly an extracellular event: intracellular Lp(a) was undetectable in human liver homogenates,\(^4\) in the lysates of human hepatoma (HepG2) cells transfected with a 17-kringle (17K) form of recombinant apo(a) [r-apo(a)],\(^5\) or in cellular lysates of primary cultures of baboon hepatocytes expressing high levels of apo(a).\(^6\) Furthermore, there are recent data to suggest that Lp(a) assembly may occur on the hepatocyte cell surface.\(^7\) Although the aforementioned reports strongly suggest that Lp(a) formation occurs extracellularly, intracellular Lp(a) has been observed in HepG2 cells stably transfected with a 6-kringle form of r-apo(a).\(^8\)

The generation of a number of recombinant expression systems for apo(a) has allowed for significant advances in our understanding of the process of Lp(a) formation. Studies examining Lp(a) assembly in vitro have led to the identification of inhibitors of the assembly process, including lysine, lysine analogues, and proline,\(^9,10\) as well as a thorough examination of the contribution of specific kringles domains of apo(a) to the process of Lp(a) formation. It is now well accepted that Lp(a) formation is a 2-step process in which the initial noncovalent association of apo(a) with the apoB moiety of LDL is mediated by the weak lysine-binding sites present in kringles IV types 5–8 (KIV5–8) of apo(a)\(^10–12\) and that this interaction likely precedes specific disulfide bond formation. Within this core sequence containing apo(a) KIV5–8, variable contributions of apo(a) kringles IV types 6, 7, and 8 in Lp(a) formation have been demonstrated.\(^10,12–14\)
A relative paucity of information exists concerning the sequences in apoB that are necessary for Lp(a) formation. Examination of Lp(a) assembly in vitro by using carboxyl-terminally truncated forms of apoB expressed in the rat hepatoma cell line McA-RH7777 demonstrated that removal of the carboxyl-terminal 6% of apoB eliminated covalent Lp(a) formation. Moreover, plasma from mice expressing human apoB90 from a transgene did not support Lp(a) assembly in vitro, and no Lp(a) was detectable in mice expressing human apoB90 from a transgene did not support Lp(a) assembly in vitro, and no Lp(a) was detectable in mice expressing human apoB90 from a transgene did not support Lp(a) assembly in vitro, and no Lp(a) was detectable in mice expressing human apoB90 from a transgene did not support Lp(a) assembly in vitro, and no Lp(a) was detectable in mice expressing human apoB90 from a transgene did not support Lp(a) assembly in vitro, and no Lp(a) was detectable in mice expressing human apoB90 from a transgene did not support Lp(a) assembly in vitro, and no Lp(a) was detectable in mice expressing human apoB90 from a transgene did not support Lp(a) assembly in vitro, and no Lp(a) was detectable in mice expressing human apoB90 from a transgene did not support Lp(a) assembly in vitro, and no Lp(a) was detectable in mice expressing human apoB90 from a transgene did not support Lp(a) assembly in vitro, and no Lp(a) was detectable in mice expressing human apoB90 from a transgene did not support Lp(a) assembly in vitro, and no Lp(a) was detectable in mice expressing human apoB90 from a transgene did not support Lp(a) assembly in vitro, and no Lp(a) was detectable in mice expressing human apoB90 from a transgene did not support Lp(a) assembly in vitro, and no Lp(a) was detectable in mice expressing human apoB90 from a transgene did not support Lp(a) assembly in vitro, and no Lp(a) was detectable in mice expressing human apoB90 from a transgene did not support Lp(a) assembly in vitro, and no Lp(a) was detectable in mice expressing human apoB90 from a transgene did not support Lp(a) assembly in vitro, and no Lp(a) was detectable in mice expressing human apoB90 from a transgene did not support Lp(a) assembly in vitro, and no Lp(a) was detectable in mice expressing human apoB90 from a transgene did not support Lp(a) assembly in vitro, and no Lp(a) was detectable in mice expressing human apoB90 from a transgene did not support Lp(a) assembly in vitro, and no Lp(a) was detected in mice transgenic for both apoB90 and apo(a). Taken together, these results suggested that sequences essential for Lp(a) formation, including the carboxyl-terminal cysteine of apoB (Cys4326), may reside within the carboxyl-terminal 6% of apoB. Site-directed mutagenesis of Cys4326 (in the context of full-length apoB) and subsequent expression of this mutant apoB species in mice have demonstrated that this cysteine residue is essential for both in vitro and in vivo Lp(a) formation. More recently, further studies using transgenic mice expressing the C-terminally truncated apoB derivatives apoB95 and apoB97 have shown that although apoB97 supports efficient Lp(a) formation, apoB95 assembles Lp(a) inefficiently both in vitro and in vivo. These findings suggest that amino acids 4331 to 4397 of apoB may directly contribute to the covalent association of apoB and apo(a) by allowing the proper positioning of the respective cysteine residues in these proteins that are involved in this process.

The observation that apoB95 supports Lp(a) assembly, albeit with greatly reduced efficiency, suggests that additional sequences contained within apoB95 are capable of mediating the noncovalent association of apoB with apo(a). To identify these sequences, we have examined the ability of a number of carboxyl-terminally truncated apoB variants contained in conditioned medium (CM) harvested from stably transfected McA-RH7777 cells to bind to r-apo(a) coupled to Sepharose 4B. Our results indicate that sequences between amino acids 680 (apoB15) and 781 (apoB18) are involved in noncovalent association with apo(a) and that these sequences bind specifically to 1 or more kringle sequences present within apo(a) KIVs-s.

Methods

Cloning and Expression of Recombinant Proteins

The details of the construction of the r-apo(a) derivatives 17K and KIVs-s and the generation of human embryonic kidney (293) cell lines stably expressing these proteins have been previously described. The generation of expression plasmids encoding apoB100 and carboxyl-terminal truncations of apoB100, including apoB94, apoB42, apoB37, and apoB29 as well as apoB18 and apoB15, has been previously described as indicated. All apoB constructs were used to stably transfect the rat hepatoma cell line McA-RH7777 (American Type Culture Collection, Rockville, Md; No. CRL 1601) as previously reported. The organization of derivatives of apo(a) and apoB used in this study is shown schematically in Figure 1.

Human embryonic kidney (293) cells stably expressing either the 17K r-apo(a) or the r-apo(a) derivative KIVs-s were routinely cultured in minimal essential medium (MEM, GIBCO/BRL) supplemented with 5% FBS (ICN). For the purpose of protein purification, confluent cell monolayers were maintained in serum-free medium (OptiMEM, GIBCO/BRL). CM was harvested and replaced every 48 hours and was stored at −20°C before use in protein purification (see below).

Isolation of LDL

Whole blood obtained from a normolipidemic volunteer was collected into EDTA (final concentration, 1 mmol/L). Plasma was isolated by low-speed centrifugation of whole blood (710g for 15 minutes) and supplemented with 1 mmol/L PMSF. Plasma was then adjusted to a density of 1.02 g/mL with NaBr and centrifuged for 20 hours at 30,000 rpm (10°C) in a Beckman 60 Ti rotor. The infranatant was collected and the density adjusted to 1.063 g/mL.

Figure 1. Schematic representation of recombinant proteins. 17K form of r-apo(a) contains each of the 10 unique KIV domains (indicated with arabic numerals), including 8 copies of sequences corresponding to KIVs-s, as well as sequences corresponding to KV and inactive protease domains. r-Apo(a) derivative KIVs-s contains sequences encoding apo(a) KIVs-s. Diagonal line in KIVs indicates hybrid kringle sequence containing amino-terminal one third of apo(a) KIV type 1 and C-terminal two thirds of apo(a) KIV type 5. Bar indicates position of free cysteine in apo(a) KIV type 9, which forms the disulfide bond with apoB moiety of LDL. Also shown are recombinant-apoB and C-terminally truncated forms of apoB that have been stably expressed in McA-RH7777 cells. C-terminal truncations of apoB are expressed as percentage of full-length apoB100. Position of amino and carboxyl termini are indicated; numbers in parentheses indicate carboxyl-terminal amino acid position for each derivative.

Purification of r-Apo(a) Derivatives

r-Apo(a) derivatives were purified by affinity chromatography by using lysine–Sepharose CL-4B (Pharmacia) and a previously described procedure. CM harvested from stably expressing cell lines was loaded onto 50-mL lysine–Sepharose CL-4B columns equilibrated with PBS, pH 7.4. Columns were washed with PBS containing 0.5 mol/L NaCl, pH 7.4, and eluted with 0.2 mol/L e-aminocaproic acid (e-ACA) in the same buffer. Protein-containing fractions were pooled and dialyzed extensively against HEPES-buffered saline (20 mmol/L HEPES, pH 7.4, containing 0.15 mol/L NaCl). The dialed protein was concentrated against PEG-20 000 (Fluka). Protein concentrations were determined by measurement of absorbance at 280 nm using corresponding molar extinction coefficients determined by the method of tyrosine difference spectroscopy. Aliquots of the purified proteins were stored at −70°C before use.
with NaBr and recentrifuged as described above. The floating LDL was harvested and recentrifuged at a density of 1.063 g/mL for a further 20 hours at 30,000 rpm. The floating LDL was removed and passed over a Bio-Rad 10DG desalting column equilibrated with PBS. The protein concentration was determined by measuring the absorbance at 280 nm of the pooled LDL-containing fractions. The isolated LDL was found to be devoid of contaminating proteins by SDS–polyacrylamide gel electrophoresis (PAGE) analysis.

Isolation of Chylomicrons
Whole blood was collected into EDTA (final concentration, 1 mmol/L) from a normal lipidemic volunteer 90 minutes after ingestion of a fatty meal. Plasma was isolated by low-speed centrifugation of whole blood (710g for 15 minutes) and supplemented with 1 mmol/L PMSF. Plasma was then centrifuged for 30 minutes at 20,000 rpm in a 70.1 Ti rotor (Beckman) at 4°C. The floating chylomicrons were removed and centrifuged again under the same conditions as described above. The supernatant from this latter spin was isolated and concentrated by centrifugation for 2 hours at 100,000 rpm in a Beckman TL-100 rotor at 4°C. The floating chylomicrons were removed and the protein content determined by using a modified Bradford assay (Bio-Rad) with BSA (Sigma) as the standard.

Im mobilization of r-Apo(a) Derivatives Onto Sepharose 4B
CNBr-activated Sepharose 4B (1 g, Pharmacia) was resuspended in 1 mmol/L HCl and washed extensively with 200 mL of 1 mmol/L HCl in a sintered glass funnel. The washed resin was resuspended in 5 mL of coupling buffer (0.1 mol/L NaHCO₃, pH 8.3, containing 0.5 mol/L NaCl). Purified r-apo(a) (2 mg) corresponding to either the 17K or KIV₅₋₈ derivative was added to 1 mL of washed gel and incubated overnight at 4°C. The slurry was washed 3 times with 5 mL of coupling buffer to remove uncoupled r-apo(a), and any remaining active groups were blocked by incubation of the coupled gel over night at 4°C with 10 mL of 0.1 mol/L Tris-HCl, pH 8. The following day the gel was washed extensively with PBS containing 0.02% NaN₃, and stored at 4°C. Under the conditions described above, >95% of each of the r-apo(a) derivatives was immobilized onto the Sepharose 4B resin.

Recombinant Apo(a)–Sepharose 4B Affinity Chromatography
CM (0.25 mL) harvested from the apoB100 or carboxyl-terminally truncated apoB cell lines was applied to 1.0 mL r-apo(a)–Sepharose columns corresponding to either the 17K form of r-apo(a) or the r-apo(a) derivative KIV₅₋₈. The CM was allowed to adsorb to the r-apo(a)–Sepharose columns for 30 minutes at room temperature. At this time, the flow-through was collected and the columns were washed with 5 to 6 mL of PBS containing 0.5 mol/L NaCl, and 1-mL fractions were collected. Specifically bound protein was then eluted with either 0.2 mol/L e-ACA or 0.2 mol/L proline dissolved in PBS containing 0.5 mol/L NaCl, and 1-mL fractions were collected. For immunoprecipitations, all column fractions were incubated overnight at 4°C with 10 μg of a sheep polyclonal antibody raised against human LDL (Calbiochem). Protein A–Sepharose (Pharmacia) was then added to the samples and the mixtures were incubated for a further 30 minutes at 4°C. The Sepharose was then pelleted by brief centrifugation at 16,000g and washed twice with 500 μL of RIPA buffer (50 mmol/L Tris-HCl, pH 7.4; 150 mmol/L NaCl; 1% Triton X-100; 20 mmol/L EDTA; 0.5% sodium deoxycholate; and 0.1% SDS) containing 0.5 mol/L NaCl and once with 500 μL of PBS. The pellets were then resuspended in 2× Laemmlie sample buffer containing 10 mmol/L DTT. Samples were boiled for 5 minutes, and solubilized proteins were subjected to SDS-PAGE and Western blot analysis as described below.

Isolated chylomicrons were diluted to a concentration of 25 μg/mL with PBS, and 250 μL was adsorbed to the r-apo(a)–Sepharose 4B columns for 30 minutes at room temperature. The columns were washed with 6 volumes of PBS containing 0.5 mol/L NaCl and eluted with 0.2 mol/L e-ACA in this buffer. Column fractions were immunoprecipitated with 10 μg of a sheep polyclonal antibody raised against human LDL as described above. The resulting immune complexes were resolved by SDS-PAGE on a 6% polyacrylamide gel under reducing conditions and subjected to Western blot analysis as described below.

In control experiments, purified LDL or BSA (100 μg each) was chromatographed over Sepharose 4B columns to which either no protein (mock) or the r-apo(a) derivatives 17K or KIV₅₋₈ had been immobilized as described above. In all cases, the columns were washed with PBS containing 0.5 mol/L NaCl (6× 1-mL washes), and specifically bound protein was eluted from the columns with this buffer supplemented with 0.2 mol/L e-ACA (6× 1 mL). The protein concentration in each fraction was determined spectrophotometrically by measurement of the absorbance at 280 nm. In chromatography experiments using the apoB derivatives, each derivative was assessed for its ability to bind to mock Sepharose columns; no binding was observed in all cases.

Western Blot Analysis
Immunoprecipitates of r-apo(a) affinity column fractions were electrophoresed on SDS-PAGE gels (5%, 6%, or 7.5% polyacrylamide gels, depending on the size of the apoB species being examined) under reducing conditions. After electrophoresis the gels were transferred onto Immobilon P (ICN) membranes at 100 V for 1 hour in transfer buffer containing 25 mL/L Tris-HCl, 192 mL/L glycine, and 20% methanol. Membranes were blocked overnight at room temperature with a 6% solution of skim milk powder dissolved in NET buffer (50 mmol/L Tris-HCl, pH 7.4, containing 0.15 mol/L NaCl, 50 mmol/L EDTA, and 0.05% Triton X-100). Blocked membranes were probed with the apoB monoclonal antibody 1D1 (kind gift of Dr Ross Milne, Ottawa Heart Institute) (200 ng/mL in NET) for 1 hour and then washed extensively with several changes of NET over the course of an hour. Membranes were then incubated for 1 hour with a horseradish peroxidase–conjugated anti-murine IgG raised in sheep (Amersham, 1:5000 dilution in NET). The membrane was then washed with NET, and immunoreactive proteins were detected with an enhanced chemiluminescence (ECL) kit (Amersham) according to the manufacturer’s recommendations. Densitometric analysis of the resulting immunoblot was performed by using a Hewlett-Packard Scanjet 3c flatbed scanner and analyzed by using Corel Photopaint (version 7.0, Corel Corp) and Sigmagel (version 1.0, Jandel Scientific) software. The percentage of apoB specifically eluted by e-ACA or proline was determined by dividing the sum of the density of e-ACA or proline fractions by the total amount of apoB (either full-length or C-terminally truncated species) observed. For each apoB truncation, 2 independent column chromatography experiments were performed with each of the 17K- and KIV₅₋₈-Sepharose columns.

Results and Discussion
To identify regions in apoB that mediate the first step of Lp(a) assembly, we have compared the ability of a number of C-terminally truncated apoB derivatives (Figure 1) to bind covalently to affinity columns containing either immobilized 17K or KIV₅₋₈ r-apo(a) species (Figure 1). We utilized the KIV₅₋₈ derivative in these experiments on the basis of recent studies in which we have shown that core sequences within apo(a) KIV₅₋₈ directly mediate the e-ACA–dependent noncovalent interaction of apo(a) with apoB. 25 To validate our experimental system, we initially demonstrated that LDL isolated from human plasma bind completely to either 17K- or KIV₅₋₈-Sepharose columns, whereas no detectable binding was observed with BSA (Figure 2). In each case, by the direct measurement of protein in column fractions, we determined that the recovery of LDL and BSA from the columns was >95% of the total protein applied (Figure 2). This suggests
that we can achieve quantitative recovery of the material applied to the columns under these conditions, and this is supported by the observation that treatment of the columns with the denaturant 0.1 mol/L glycine (pH 2.3) did not result in the removal of any additional protein (see below).

In the current study, we observed that apoB100 present in the CM harvested from stably transfected McA-RH7777 cells was able to bind completely to a 17K r-apo(a)–Sepharose affinity column in the presence of 0.5 mol/L NaCl. Furthermore, we found that the bound apoB100 could be specifically eluted from the 17K affinity column by the addition of 0.2 mol/L $\epsilon$-ACA in the same buffer (6 × 1 mL). Amount of protein present in each fraction was determined by measurement of absorbance at 280 nm, and percentage of total amount of protein in each fraction was calculated. Profiles for LDL and BSA are shown in A and B, respectively.

The ability of the apoB48-like species to bind to the KIV$_{5-8}$ affinity column demonstrates that sequences in the N-terminal 48% of apoB are sufficient for noncovalent association with core sequences present in apo(a) KIV$_{5-8}$, which is characterized by sensitivity of this interaction to the addition of $\epsilon$-ACA.$^{10,11}$ To exclude the possibility that the observed noncovalent association of the apoB48-like species was the result of either sequence differences relative to plasma-derived apoB48$^{22}$ or differences in the composition of the lipid core, we assessed the binding of chylomicrons isolated from human plasma to either the KIV$_{5-8}$ or 17K affinity column.

<table>
<thead>
<tr>
<th>ApoB Derivative*</th>
<th>17K, % Bound†</th>
<th>KIV$_{5-8}$, % Bound†</th>
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<tr>
<td>ApoB100</td>
<td>100</td>
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<td>ApoB94</td>
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<td>ApoB48‡</td>
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<td>86</td>
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<td>ApoB18</td>
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<td>ApoB15</td>
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*Carboxyl-terminally truncated apoB derivatives are shown schematically in Figure 1.
†Binding was quantified by densitometric assessment of Western blots corresponding to r-apo(a) affinity-column fractions containing either unbound or specifically bound (ie, $\epsilon$-ACA–elutable) apoB (see Methods for details). Results presented are in each case representative of 2 independent experiments for which identical results were obtained.
‡Corresponds to apoB48-like protein that results from posttranscriptional processing of apoB in McA-RH7777 cells. Identical results were obtained with apoB48 in plasma-derived chylomicrons (see Figure 4).
r-apo(a) affinity column. As shown in Figure 4, chylomicron-derived apoB48 bound completely to the 17K Sepharose column, and the specifically bound material was eluted from the column by the addition of e-ACA. Additionally, apoB100 corresponding to contaminating VLDL present in the chylomicron preparation also bound completely to this column (see Figure 4); similar results were obtained with VLDL purified from human plasma by density gradient ultracentrifugation (data not shown). Chylomicron-derived apoB48 also bound completely to the KIV5–8 column in an e-ACA–dependent manner (the Table and data not shown). Taken together, these results suggest that sequences present within the amino-terminal half of apoB are sufficient to mediate complete noncovalent association with apo(a) and that this binding interaction is mediated by sequences within apo(a) KIV5–8. Additionally, our data suggest that the noncovalent association of apoB and apo(a) is not affected by the composition of the apoB48-containing particle with respect to triglyceride and cholesteryl ester content. Whereas chylomicrons are triglyceride-rich, cholesteryl ester–poor particles of density <1.006 g/mL, the apoB48-containing particles secreted by McA-RH7777 cells are smaller in diameter, triglyceride poor, and present at densities between 1.10 and 1.19 g/mL.22 Clearly, the presentation of sequences in apoB that interact noncovalently with apo(a) is not affected by the size or lipid composition of the apoB-containing particles.

To exclude the possibility that the observed binding of apoB48 to apo(a) was due solely to conformational constraints specific to apoB48, we assessed the binding of the apoB94 species that lacks the C-terminal 6% of apoB. As shown in Figure 5, apoB94 present in the CM of stably transfected McA-RH7777 cells bound specifically to 17K Sepharose. ApoB94 present in the CM also bound completely to the KIV5–8 affinity column and could be specifically eluted with e-ACA (the Table and data not shown). These results demonstrate that despite its inability to form covalent Lp(a) particles,13 apoB94 is able to associate noncovalently with both r-apo(a) derivatives, thereby providing further evidence for the role of amino-terminal sequences in apoB in mediating its interaction with apo(a).

To more precisely define the sequences within the amino-terminal half of apoB that mediate noncovalent association with apo(a), we assessed the noncovalent binding of other carboxyl-terminally truncated apoB species (represented schematically in Figure 1) present in the CM of stably transfected cells to either 17K or KIV5–8 affinity columns. Both apoB42 and apoB37 bound specifically and completely to the 17K Sepharose and KIV5–8 Sepharose (the Table and data not shown). Although the apoB29 species bound completely to the 17K affinity column, a fraction (≈15%) did not bind the KIV5–8 Sepharose column (Figure 6 and the Table). Interestingly, with the apoB18 species, we observed an
apparent 50% decrease in specific binding to either the 17K or KIV5–8 column, whereas no specific binding to the r-apo(a) columns was observed with CM containing the apoB15 species (Figure 6 and the Table). It appears that the reduced binding of apoB18 to the r-apo(a) columns relative to apoB29 reflects a functionally distinct pool of apoB18 that does not bind to the column, as opposed to a reduced binding affinity of apoB18 for the immobilized apo(a). This was determined by reapplication of the unbound apoB18 fraction to the 17K r-apo(a) column; we observed that the apoB18 that did not bind to the column in the first passage also showed no binding when it was reapplied to the same r-apo(a) column (data not shown). No additional apoB18 could be removed from the r-apo(a) column on the addition of denaturant (0.1 mol/L glycine, pH 2.3) (data not shown). The nature of the apoB18 species that shows no binding to the apo(a) column is unclear at present. It is possible that the nonbinding pool of apoB18 may differ in conformation or in glycosylation relative to the binding fraction. No difference in electrophoretic mobility on a variety of gel systems was observed (data not shown).

Our observation that apoB15 did not bind at all to either 17K or KIV5–8 r-apo(a) columns indicates a drastically reduced affinity of this variant for apo(a). Taken together, our data suggest that sequences contained within a region corresponding approximately to amino acids 680 to 781 of apoB18 mediate its noncovalent association with apo(a) and that these sequences interact directly with sequences present within KIV5–8 of apo(a). It should be noted that this result is not an artifact of the column system used in the current study, since we also observed that apoB18, but not apoB15, could be coimmunoprecipitated with 17K r-apo(a) by a monoclonal anti-apo(a) antibody (data not shown). This suggests that the interactions observed on using r-apo(a)–Sepharose affinity chromatography also occur when apo(a) and apoB are free in solution.

To further characterize the interaction of apo(a) with the C-terminally truncated apoB variants, we examined whether proline, a known inhibitor of the in vitro formation of Lp(a), could elute specifically bound truncated apoB species from either of the 2 r-apo(a) affinity columns. As shown in Figure 7, both apoB42 and apoB18 could be specifically eluted from KIV5–8-Sepharose by proline; identical results were obtained with the 17K-Sepharose column. These data, taken together with those obtained with the use of e-ACA for column elution, indicate that both proline and e-ACA disrupt the noncovalent association of the apoB variants with apo(a) by binding to 1 or more of the kringle pockets within KIV5–8. Furthermore, since we have found that the specific binding of 17K and KIV5–8 to LDL immobilized onto microtiter wells can be abolished by the addition of either e-ACA or proline,28 the present study strongly suggests that amino acids 680 to 781 of apoB may in fact contribute to the high-affinity noncovalent binding of apo(a) and apoB that precedes specific disulfide bond formation.

Previous studies by our group15 and others16 have demonstrated that the C-terminal 6% to 10% of apoB is required for the formation of covalent Lp(a) particles. In the context of our current study, however, we find that apoB94 is capable of noncovalent association with r-apo(a). This suggests that lack of covalent Lp(a) formation when using this species arises owing to a defect in the second step of Lp(a) assembly involving disulfide bond formation. This is likely due to the absence of Cys4326 in apoB, which has been shown to be the cysteine residue in apoB that is responsible for disulfide bond formation with apo(a).17,18 Additionally, apoB94 lacks additional C-terminal sequences located between apoB95 and apoB97 that have been reported to be essential for efficient Lp(a) formation both in vitro and in vivo in transgenic mouse models.19 These latter sequences may be potential sites for the interaction of apo(a) KIV5–8 and apoB, which is necessary for disulfide bond formation, or may affect the presentation of Cys4326 for disulfide bond formation.

In conclusion, the current study clearly demonstrates a novel role for sequences in the amino terminus of apoB in mediating its noncovalent binding with apo(a). Since the noncovalent interaction of apo(a) and apoB is almost certainly a prerequisite for the formation of covalent Lp(a) particles, the sequences that we have identified in this study can be expected to play an important role in Lp(a) assembly in vivo. Clearly, further studies aimed at identifying specific amino acid(s) in apoB that are essential in mediating this noncovalent interaction will be of interest, particularly in the context of developing therapeutic strategies aimed at lowering plasma Lp(a) levels by inhibiting Lp(a) assembly.

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References


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