Identification of a Critical Lysine Residue in Apolipoprotein B-100 That Mediates Noncovalent Interaction with Apolipoprotein(a)*

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We have previously shown that lipoprotein(a) (Lp(a)) assembly involves an initial noncovalent interaction between sequences within apolipoprotein(a) (apo(a)) kringles IV types 5–8 and the amino terminus of apolipoprotein B-100 (sequences between amino acids 680 and 781 in apoB-100), followed by formation of a disulfide bond. In the present study, citraconylation of lysine residues in apoB-100 abolished the ability of the modified low density lipoprotein to associate with apo(a), thereby demonstrating a direct role for lysine residues in apoB in the first step of Lp(a) assembly. To identify specific lysine residues in the amino terminus of apoB that are required for the noncovalent interaction, we initially used an affinity chromatography method in which recombining forms of apo(a) (r-apo(a)) were immobilized on Sepharose beads. Assessment of the ability of carboxy-terminal truncations of apoB-18 to bind to r-apo(a)-Sepharose revealed that a 25-amino acid sequence in apoB (amino acids 680–704) bound specifically to apo(a) in a lysine-dependent manner; citraconylation of the lysine residues in the apoB derivative encoding this sequence abolished the binding interaction. Using fluorescence spectrometry, we found that a synthetic peptide corresponding to this sequence bound directly to apo(a); the peptide also reduced covalent Lp(a) formation. Lysine residues present in this sequence (Lys680 and Lys690) were mutated to alanine in the context of apoB-18. We found that the apoB-18 species containing the Lys690 mutation was incapable of binding to r-apo(a)-Sepharose columns, whereas the apoB-18 species containing the Lys690 mutation exhibited slightly reduced binding to these columns. Taken together, our data indicate that Lys690 is critical for the noncovalent interaction of apo(a) and apoB-100 that precedes covalent Lp(a) formation.

Epidemiological studies have identified elevated plasma concentrations of human lipoprotein(a) (Lp(a))1 as a risk factor for the development of a variety of atherosclerotic disorders, including coronary heart disease (reviewed in Ref. 1). Lp(a) is similar to low density lipoprotein (LDL) both in lipid composition and in the presence of apolipoprotein B-100 (apoB-100). Lp(a) is distinguishable from LDL, however, by the presence of a unique glycoprotein termed apolipoprotein(a) (apo(a)), which is attached to apoB-100 by a single disulfide bond. The presence of apo(a) likely confers the unique structural and functional properties attributable to Lp(a). Apo(a) contains tandem repeats of a sequence that is highly similar to plasminogen kringle IV, followed by sequences that are homologous to the kringle V and protease domains of plasminogen (2). Apo(a) contains 10 distinct subclasses of kringle IV; the kringle IV type 2 domain (KIV2) is present in variable copy number, which forms the basis for the observed isoform size heterogeneity of Lp(a) (3, 4). An unpaired cysteine in apo(a) KIV9 (Cys67) is involved in disulfide linkage with apoB-100 to form Lp(a) particles (5, 6).

Lp(a) assembly is thought to proceed via a two-step process (7) in which an initial noncovalent interaction between apo(a) and apoB-100 results in the correct orientation of the two proteins that is required for subsequent disulfide bond formation; validation of this model was provided by studies that clearly demonstrated that the efficiency of the noncovalent step dictates the extent of covalent Lp(a) formation (8). Early studies have shown that the process of Lp(a) formation can be inhibited by lysine, lysine analogs such as ε-aminocapric acid (ε-ACA), and proline (8–10); studies by our group have specifically demonstrated that the noncovalent step of Lp(a) assembly is also sensitive to the addition of arginine and phenylalanine (8, 11).

Our previous studies have focused on defining the sequence requirements in both apo(a) and apoB-100 that are required for their noncovalent association. Using truncated derivatives of recombinant apo(a) (r-apo(a)), we have shown that sequences within apo(a) kringle IV types 6–8 (each of which are thought to contain weak lysine-binding sites (LBS)) are required for noncovalent interaction with LDL and that this interaction can be inhibited by lysine, lysine analogs, proline, arginine, and phenylalanine (8). With respect to identification of sequences in apoB-100 that are required for noncovalent association with apo(a), we previously analyzed a series of carboxyl-terminally truncated apoB species for their ability to bind apo(a) (12). The results demonstrated that sequences between apoB-18 (N-terminalsequence) and citraconylation of lysine residues in the apoB derivative encoding this sequence abolished the binding interaction. Using fluorescence spectrometry, we found that a synthetic peptide corresponding to this sequence bound directly to apo(a); the peptide also reduced covalent Lp(a) formation. Lysine residues present in this sequence (Lys680 and Lys690) were mutated to alanine in the context of apoB-18. We found that the apoB-18 species containing the Lys690 mutation was incapable of binding to r-apo(a)-Sepharose columns, whereas the apoB-18 species containing the Lys690 mutation exhibited slightly reduced binding to these columns. Taken together, our data indicate that Lys690 is critical for the noncovalent interaction of apo(a) and apoB-100 that precedes covalent Lp(a) formation.

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electrophoresis (PAGE), followed by staining with Coomassie Blue; LDL was purified by the appearance of a single band upon SDS-polyacrylamide gel electrophoresis (PAGE). All proteins were judged to be pure by the appearance of a single band upon SDS-polyacrylamide gel electrophoresis (PAGE). To remove free citraconic anhydride, and the protein concentration was determined by a modified Bradford assay using bovine serum albumin (BSA) as a standard.

In this study, we initially sought to define conclusively a role for lysine residue(s) in apoB-100 in mediating noncovalent association with apo(a). Our second goal was to precisely identify sequences within the region spanning apoB-15 and apoB-18 that mediate noncovalent binding to apo(a) using novel carboxyl-terminal truncations of apoB-18 for noncovalent assembly assays. Our third goal was to identify lysine residues(s) in apoB-100 directly interact with lysine residue(s) in apo(a) by chemical modification of lysine residues with citraconic anhydride. However, the notion that lysine-binding sites in apo(a) directly interact with lysine residues in apoB-100 remains to be substantiated.

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MATERIALS AND METHODS
Production and Purification of LDL and Recombinant Apo(a) Derivatives—LDL was purified as previously described (13). Briefly, whole blood obtained from a normolipidemic volunteer (in accordance with ethics procedures set forth by Queen’s University) was collected into ethylenediaminetetraacetic acid (EDTA) (5 mM final concentration). Plasma was isolated by low speed centrifugation of whole blood (710 g for 20 min) and supplemented with 1 mM phenylmethylsulfonyl fluoride. LDL (in the 1.02–1.063 density range) was isolated from plasma by sequential flotation. The final LDL (pH 5) was then incubated at 37 °C for 5 h, followed by overnight incubation at 4 °C. The pH of the solution was adjusted to 7.4

Modification of LDL with Citraconic Anhydride and Reversal of Modification—Purified LDL was modified with citraconic anhydride as previously described (17); this procedure has been reported to result in the modification of 50% of the lysine residues within apoB-100 (17). Briefly, an equal volume of a saturated sodium acetate solution was added to 1 mg of purified LDL, and the mixture was incubated on a rocker at 4 °C for 15 min. At this time, a 50-fold excess (with respect to lysine concentration in apoB-100) of citraconic anhydride was added in two equal portions with a 15-min incubation at 4 °C between additions. The modified LDL was then dialyzed exhaustively against HBS to remove free citraconic anhydride, and the protein concentration was determined by a modified Bradford assay using BSA as the standard.
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by a stepwise addition of 1 M NaOH. The LDL was dialyzed against HBS, and the protein concentration was determined by a modified Bradford assay using BSA as the standard. LDL modification and de-modification were qualitatively verified by subjecting native, modified, and de-modified LDLs to SDS-PAGE. Sample lanes were loaded with 20 µl of loading dye (50% sucrose and bromophenol blue in HBS) and loaded onto a 1% agarose gel made up in 0.5x TBE buffer containing 5 mM Tris-HCl (pH 8.5), 45 mM boric acid, and 1 mM EDTA. The samples were electrophoresed at 4 °C for 2 h at 100 V, and lipoproteins were visualized by staining with Coomassie Blue. The native, modified, and de-modified LDLs were also subjected to SDS-PAGE, followed by staining with Coomassie Blue; these analyses revealed no fragmentation of apoB-100 by these procedures.

Binding of Native and Citraconic Anhydride-modified LDLs to r-apo(a)—An enzyme-linked immunosorbent assay (ELISA)-based binding assay was utilized to monitor the binding of native LDL, LDL modified with citraconic anhydride, and de-modified LDL to immobilized r-apo(a). Purified 17K r-apo(a) was coated onto microtiter wells (polyvinyl chloride 96-well plates, Costar Corp.) at a concentration of 2 µg/ml in 0.1 M NaHCO₃ (pH 9.6) overnight at 4 °C. After extensive washing with a solution of PBS containing 0.1% (v/v) Tween 20 (PBST), the microtiter wells were blocked overnight at 4 °C with 150 µl of a 2.5% (w/v) solution of BSA in HBS. Blocked wells were washed with PBST and incubated with varying concentrations (starting at 500 nM, serially diluted in diluent buffer (1% (w/v) BSA and 0.1% (v/v) Tween 20 in HBS)) of native, citraconic anhydride-modified, or de-modified LDL for 18 h at 4 °C. At this time, the wells were washed with PBST and incubated with a 333 pg/µl solution of anti-apoB antibody monoclonal 1D1 (epitope within apoB-26) (18) in diluent buffer for 1 h at room temperature. After another washing step with PBST, the microtiter wells were incubated with a solution of a sheep anti-mouse horseradish peroxidase-conjugated antibody (Sigma; 200 pg/µl in diluent buffer) for 1 h at room temperature. A final wash with PBST was performed, after which the wells were developed for 5 min with 100 µl of developing solution containing the substrate o-phenylenediamine dihydrochloride (0.42 mg/ml). The reactions were stopped by the addition of 50 µl of 2 M H₂SO₄ solution, and the absorbance was read at 490 nm (less the background absorbance at 650 nm) was measured using a TiterTek plate reader.

Covalent Lp(a) Assembly Assays—In vitro covalent Lp(a) assembly assays were performed as previously described (10). Briefly, 17K r-apo(a) was transiently expressed in HEK 293 cells, and its concentration in CM was quantified by ELISA using purified 17K r-apo(a) as a standard. Purified native LDL (50 nm) was incubated with CM (diluted with HBS) containing 2 nM 17K r-apo(a) at 37 °C in a total volume of 300 µl. At selected times (0, 0.5, 1, 2, 4, and 6 h), a 30-µl aliquot was removed from the incubation, added to an equal volume of 2x Laemmli sample buffer (19) in the absence of a reducing agent, and heated at 95 °C for 5 min. Samples were then subjected to SDS-PAGE on a 5% polyacrylamide gel, followed by Western blot analysis using anti-apo(a) monoclonal antibody a-6. The extent of recombinant Lp(a) (r-Lp(a)) assembly was then quantified by densitometric analysis of the blots by dividing the density of the Lp(a) band by the sum of the densities of the Lp(a) and free apo(a) bands. To assess the effect of modification of LDL with citraconic anhydride on covalent Lp(a) formation, LDL modified with citraconic anhydride (50 nm) was used in place of native LDL.

Construction and Expression of ApoB Truncations and Site-directed Mutants—The r-apoB derivatives utilized in this study are shown schematically in Fig. 1: details of the construction of the corresponding expression plasmids are described below. All constructs were assembled in the pRK5 expression plasmid, which contains the cytomegalovirus promoter and SV40 transcription termination sequences (14). To create an apoB-18 expression construct in pRK5, a 2600-base pair EcoRI/BamHI fragment was excised from apoB-18-pCMV5 (21) and ligated into the pRK5 vector, which had been digested with these enzymes. Using apoB-18 in pRK5 as a template for polymerase chain reaction-directed mutagenesis, carboxyl-terminal truncations of apoB-18 were created by the introduction of a premature stop codon at amino acid 685, 705, 730, and 755 to generate apoB-15, apoB-15.8, apoB-16.5, and apoB-17.5, respectively. Polymerase chain reaction-mediated mutagenesis was also utilized to generate two mutants of apoB-18 in which Lys1680 or Lys1690 was mutated to alanine, resulting in the apoB-18(K1680A) and apoB-18(K1690A) derivatives, respectively. McArdle rat hepatoma 7777 (RH7777) cells expressing each of the r-apoB derivatives were generated as previously described (14). Briefly, these cells were transfected by calcium phosphate coprecipitation using 10 µg of each r-apoB expression plasmid and 1 µg of a plasmid encoding the neomycin resistance gene per 100-mm plate. Stable transformants were selected by culturing cells in the presence of 0.8 mg/ml G418 (Life Technologies, Inc.) until cellular foci developed. At this time, foci were picked and screened for r-apoB expression by ELISA and Western blot analysis using the 1D1 anti-apoB monoclonal antibody.

Apo(a) Affinity Chromatography—Purified r-apo(a) corresponding to the 17K or KIV_8 derivative were immobilized on CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech) (12); affinity chromatography using r-apo(a) derivatives coupled to Sepharose columns corresponding to either the 17K or KIV_8 derivative. After a 20-min incubation period at room temperature, the flow-through and wash (5 x 1 ml of PBS containing 0.5 M NaCl) fractions were collected. Specifically bound r-apoB was eluted in 3 x 1 ml fractions with PBS containing 0.5 M NaCl and 0.2 M e-ACA. All column fractions were then immunoprecipitated (12) with 1 µg of a sheep polyclonal antibody raised against human LDL (Roche Molecular Biochemicals). Immune complexes were subjected to SDS-PAGE on 7.5% polyacrylamide gels under reducing conditions, followed by Western blot analysis using the 1D1 anti-apoB monoclonal antibody. Binding was quantified by densitometric analysis as previously described (12). In some experiments, CM containing apoB-15.8 was treated with citraconic anhydride using a methodology identical to that described for purified LDL. Binding to 17K and KIV_8, r-apo(a)-Sepharose was performed as described above.
Analysis of ApoB-(680–704) Binding to 17K r-apo(a) by Intrinsic Fluorescence—A synthetic peptide (apoB-(680–704)) corresponding to amino acids 680–704 (N-KQGFPFDVSYKALYVWNNGQYDPDVG-C) in the primary sequence of apoB-100 was generated using an Applied Biosystems 431A peptide synthesizer by Fmoc (N-(9-fluorenyl)carbonyl) chemistry, purified by high performance liquid chromatography, and analyzed by mass spectroscopy. The preparation was assessed to be ~95% pure and was solubilized in HBS for use in this study. Intrinsic fluorescence measurements of r-apo(a) were performed using a PerkinElmer Life Sciences LS550B luminescence spectrometer. The 17K r-apo(a) derivative (100 nm) was titrated with apob-(680–704). Titrations were performed in HBS and 0.1% Tween 20 in a quartz cuvette that had been conditioned with this buffer prior to use. Tween 20 was included to minimize nonspecific interactions between apo(a) and/or peptide and the quartz cuvette. Apo(a) tryptophan excitation was performed using a wavelength of 280 nm and a slit width of 2.5 nm, whereas tryptophan emission was detected at a wavelength of 340 nm and a slit width of 5 nm with a 290-nm cutoff filter placed in the emission beam. Ligand solutions (containing 100 nm 17K r-apo(a) to eliminate dilution effects) were added in a stepwise manner until saturation of the fluorescence change was attained. Fluorescence directly attributable to apob-(680–704) was controlled for by performing a titration of the peptide in the absence of apo(a) and subtracting the appropriate fluorescence values from those obtained in the presence of r-apo(a). To estimate $K_D$ and $M_{max}$ for the r-apo(a)/apoB-(680–704) interaction, titration curves were subjected to nonlinear regression analysis using Equation 1,

$$
\Delta I = \frac{M_{max} [P]}{K_D + [P]} + P_B
$$

where $\Delta I$ is the absolute change in fluorescence, $\Delta I_{max}$ is the absolute change in fluorescence at saturating ligand concentrations, $K_D$ is the dissociation constant, and $[P]$ is the total concentration of apob-(680–704). Based on initial regression analyses in which the number of apob-(680–704)-binding sites on apo(a) was included in the model as a fit parameter and was calculated to be ~1, a 1:1 r-apo(a)/apob-(680–704) binding stoichiometry was assumed.

Modification of LDL with 5-(iodoacetamido)fluorescein and Binding to 17K r-apo(a)—Purified LDL (300 μg) was incubated with a 50-molar excess of 5-(iodoacetamido)fluorescein overnight at 4°C. To remove free 5-(iodoacetamido)fluorescein, fluorescein-labeled LDL was passed over a 1-ml DEAE-cellulose column (Sigma) and eluted with HBS. Protein-containing fractions were pooled and dialyzed extensively against 0.01% Tween 20 (HBS); the concentration of fluorescein-labeled LDL was determined using a modified Bradford assay, and the protein was stored at 4°C for no longer than 3 days prior to use.

Fluorescein fluorescence measurements of fluorescein-labeled LDL were performed using the LS550B luminescence spectrometer. Fluorescein-labeled LDL (50 μl) was titrated with 17K r-apo(a). Titrations were performed in HBS and 0.01% Tween 20 in a quartz cuvette that had been conditioned with this buffer prior to use. Tween 20 was included to minimize nonspecific interactions between apo(a) and/or LDL and the quartz cuvette. Fluorescein was excited at a wavelength of 495 nm and a slit width of 2.5 nm, whereas fluorescein emission was detected at a wavelength of 530 nm and a slit width of 5 nm with a 510-nm cutoff filter placed in the emission beam. Ligand solutions (containing 50 μl fluorescein-labeled LDL to eliminate dilution effects) were added in a stepwise manner until saturation of the fluorescence change was attained. To estimate $K_D$ for the fluorescein-labeled LDL/r-apo(a) interactions, titration curves were subjected to nonlinear regression analysis using Equation 1 (with a total concentration of r-apo(a) substituting for the total concentration of the peptide).

Effect of ApoB-(680–704) on Covalent Lp(a) Assembly—To determine the effect of apob-(680–704) on the second step of Lp(a) assembly, in vitro covalent Lp(a) assembly assays were performed as described above. In the first experiment, Lp(a) assembly was allowed to proceed for a fixed time (t = 4 h) in the presence of varying apob-(680–704) concentrations (0, 1, 10, 100, and 1000 μM). Following Western blotting, densitometry was performed to calculate the percent r-Lp(a) assembly. Lp(a) detached at time 0 does not correspond to contaminating Lp(a) in the purified LDL fraction. As such, the percent r-Lp(a) was corrected for contaminating Lp(a) by subtracting the value at time 0. Relative r-Lp(a) formation was calculated by dividing the percent r-Lp(a) formed in the presence of apob-(680–704) (t = 4 h) by the percent r-Lp(a) formed in the absence of the peptide (t = 4 h). In the second experiment, a time course assay (t = 0, 0.5, 1, 2, 4, and 6 h) was performed in the absence or presence of either 1 mM peptide or 2 mM ε-ACA. The Western blot was subjected to densitometric analysis to determine the percent r-Lp(a) formed, and the data were fit to Equation 2,

$$
\% Lp(a) = b (1 - e^{-c t})
$$

where $b$ is the maximal percent r-Lp(a) formed, $c$ is a constant, and $t$ is the time of the reaction. This equation describes exponential rise to a maximum and was derived from first principles with the assumption that Lp(a) assembly occurs as a two-step process.

RESULTS

Citraconylation of LDL Inhibits Its Ability to Bind to Apo(a)—To demonstrate that lysine residue(s) within apoB are specifically involved in the first step of Lp(a) assembly, we treated purified LDL with citraconic anhydride (which specifically modifies primary amines) and assayed the ability of the modified LDL to noncovalently bind 17K r-apo(a). Modification of LDL was qualitatively detected by the appearance of a mobility shift on a nondenaturing 1% agarose gel (Fig. 2 lane 1). The modified LDL (lane 2) migrated faster than native LDL (lane 3) as a consequence of changes in the surface charge of the molecule. An ELISA-based binding experiment was performed to measure the noncovalent association between immobilized 17K r-apo(a) and modified LDL. The results indicate that although native LDL exhibited binding to 17K r-apo(a) in this system, the modified LDL did not (Fig. 2A). An in vitro Lp(a) assembly assay was used to examine whether the citraconylation of LDL affected its ability to form covalent Lp(a) particles. Covalent Lp(a) particle formation was observed with native LDL, whereas the ability of the citraconic anhydride-modified LDL to form covalent Lp(a) was completely abolished (Fig. 2B). The antibody reactivity of the modified or de-modified LDL species did not differ from that of native LDL as assessed by Western blot analysis (data not shown). Citraconylation of LDL
Table I
Analysis of noncovalent binding of apoB variants to KIV_{5-8}-r-apo(a) Sepharose affinity columns

| ApoB varianta | Binding to KIV_{5-8} b  |
|----------------|-------------------------|
| Wild-type apoB-18 | 53.4 %  |
| ApoB-17.3 | 35.1 %  |
| ApoB-16.5 | 52.3 %  |
| ApoB-15.8 | 48.5 %  |
| Citra-apoB-15.8 | 0 %  |
| ApoB-15 | 0 %  |
| ApoB-18/K690A | 19.2 %  |
| ApoB-18/K680A | 0 %  |

a ApoB variants are shown schematically in Fig. 1.
b Binding was quantified by densitometric analysis of Western blots of column fractions and are representative of at least two independent experiments.

was reversed by incubation at low pH and verified by native agarose electrophoresis (Fig. 2A, inset). Although incomplete de-modification was observed, this partial reversal of the LDL modification was adequate for the complete recovery of noncovalent binding to apo(a) (Fig. 2A), which may reflect the more efficient de-modification of lysine residues specifically important for apo(a) binding. Collectively, these results indicate that lysine residues in apoB-100 are essential for the binding LDL to apo(a) and hence for the assembly of covalent Lp(a) particles.

Binding of Truncated ApoB-18 Derivatives to KIV_{5-8}-Sepharose—We have previously demonstrated that sequences between apoB-18 (N-terminal 18% of apoB-100) and apoB-15 are important for the first step of Lp(a) assembly (12). To identify a region within apoB-18 that mediates the first step of Lp(a) assembly, we generated carboxyl-terminal truncations of apoB-18 (Fig. 1A) and assessed their ability to bind noncovalently to apo(a). The truncated apoB derivatives (apoB-17.3, apoB-16.5, and apoB-15.8) were stably expressed in McArdle RH7777 cells, and the integrity of the resulting proteins was verified by Western blot analysis (Fig. 2B). Noncovalent binding of the apoB derivatives to apo(a) KIV_{5-8} was analyzed by affinity chromatography wherein CM containing each apoB derivative was incubated with KIV_{5-8} immobilized on Sepharose 4B beads. Nonspecifically bound proteins were eluted by extensive washing with 0.5 M NaCl, whereas specifically bound proteins were eluted with 0.2 M ε-ACA. Column fractions were subjected to Western blot analysis, and amounts of apoB in the column fractions were quantified by densitometric analysis (Table 1). In keeping with our previous observations (12), apoB-15 did not bind the affinity column, whereas ~50% of the apoB-18 pool was competent to bind apo(a) (data not shown). Although the amount of the bound fraction for each derivative varied slightly (Table 1), apoB-15.8 (Fig. 3A), apoB-16.5 (data not shown), and apoB-17.3 (data not shown) were all capable of interacting with immobilized KIV_{5-8} r-apo(a). Interestingly, treatment of CM containing apoB-15.8 with citraconic anhydride abolished the interaction between this derivative and apo(a) (Fig. 3B), suggesting that lysine residues between apoB-15 and apoB-15.8 (corresponding to amino acids 680–704 in the primary apoB sequence) play a critical role in the interaction between apoB-15.8 and KIV_{5-8} r-apo(a).

LDL and ApoB-680–704 (Fig. 4A) Binding to 17K r-apo(a)—A peptide corresponding to amino acids 680–704 in the primary sequence of apoB-100 was titrated with 17K r-apo(a), and intrinsic fluorescence measurements were performed to quantitate the binding. The apoB-derived peptide bound the 17K r-apo(a) derivative in a specific and saturable manner (Fig. 4A) with a $K_D$ of 83.4 nM. In corroborative experiments, ELISA-based binding experiments indicated that the peptide bound both 17K and KIV_{5-8} r-apo(a) in an ε-ACA- and proline-dependent manner (data not shown). We also compared the affinity of apo(a) for full-length apoB-100 with that for the apoB-680–704 peptide. Purified LDL was labeled with 5'-iodoacetamido)fluorescein and titrated with 17K r-apo(a); binding was detected as a decrease in fluorescence. Specific and saturable binding was observed between fluorescence-labeled LDL and 17K r-apo(a) (Fig. 4B). The $K_D$ for the interaction between fluorescence-labeled LDL and 17K r-apo(a) was determined to be 56.8 nM, which is comparable to that observed for the binding of 17K r-apo(a) to the peptide (83.4 nM; see above).
Inhibition of Covalent Lp(a) Formation by ApoB-(680–704)—Having demonstrated that apoB-(680–704) bound 17K r-apo(a) with high affinity, we assessed the ability of this peptide to inhibit the second step of Lp(a) formation by performing in vitro covalent Lp(a) assembly assays as described under “Experimental Procedures.” Initially, a dose-response experiment was performed to determine the effective concentration range at which the apoB-derived peptide affected covalent Lp(a) formation. Although modest reductions in Lp(a) formation were observed with lower peptide concentrations (1, 10, and 100 μM), maximal inhibition of Lp(a) assembly was attained using a peptide concentration of 1000 μM (Fig. 5A). As such, we fixed the apoB-(680–704) concentration at 1000 μM and performed a time course assay to more rigorously examine the effect of the peptide on covalent Lp(a) formation. The results indicated that compared with the control (i.e., assembly observed in the presence of 2 mM ε-ACA), the apoB-(680–704) peptide inhibited both the initial rate and maximal extent of r-Lp(a) formation (Fig. 5B) as determined by nonlinear regression of the assembly data to Equation 2. The control experiment was conducted in the presence of 2 mM ε-ACA to discount the possibility that the two lysine residues in the peptide could inhibit Lp(a) assembly in the absence of specific flanking sequences. Higher apoB-(680–704) concentrations could not be tested as a consequence of the relative insolubility of the peptide.

Identification of a Lysine Residue Mediating Binding of ApoB-18 to Apo(a)—The sequence spanning amino acids 680–704 in apoB-100 contains two lysine residues (at positions 680 and 690). Using site-directed mutagenesis, we mutated each of these lysines to alanines and expressed these mutations in the context of apoB-18 to generate apoB-18(K680A) and apoB-18(K690A) (Fig. 1A). We monitored the binding of these mutant derivatives to KIV<sub>5</sub>-r-apo(a) by affinity chromatography and quantified the binding by Western blot analysis, followed by densitometry. As previously reported (12), ~50% of the wild-type apoB-18 pool was competent to noncovalently associate with immobilized KIV<sub>5</sub>-r-apo(a) (Fig. 6A and Table I). Although apoB-18(K690A) bound the apo(a) affinity column less efficiently compared with apoB-18 (Fig. 6B and Table I), mutation of the lysine residue at position 680 completely abolished noncovalent association with KIV<sub>5</sub>-r-apo(a) (Fig. 6C and Table I). Comparable results were obtained using 17K r-apo(a)-Sepharose for the chromatography (data not shown).

**DISCUSSION**

Lp(a) assembly is thought to proceed through a two-step process in which initial noncovalent interactions between apo(a) and apoB-100 precede specific disulfide bond formation. The first step likely results in the correct orientation of the
respective cysteine residues on both apo(a) and apoB-100, thereby facilitating spontaneous disulfide bond formation. Evidence for this two-step model stems from data showing that inhibition of the noncovalent interaction between apo(a) and apoB-100 results in disruption of covalent Lp(a) particle formation (8). In this regard, Lp(a) assembly is sensitive to the addition of lysine and lysine analogs such as ε-ACA (8–11), which has been interpreted to suggest that lysine residue(s) on apoB-100 are important in the initial noncovalent interaction. Lp(a) assembly can, however, also be inhibited with other amino acids, including proline, arginine, and phenylalanine (8, 11), which brings the specific role of apoB lysine residues in this process into question. In this study, we demonstrate that the modification of lysine residues on apoB-100 with citraconic anhydride abolishes both noncovalent and covalent Lp(a) assembly, a result that is consistent with the two-step model for Lp(a) assembly. Moreover, we have pinpointed a specific lysine residue in apoB (Lys680) that is crucial for the noncovalent interaction between apo(a) KIV_{7,8} and apoB-18. Together, these findings constitute the first demonstration that lysine residue(s) on apoB-100 are directly involved in the first step of Lp(a) assembly.

Numerous studies have focused on identifying sequence determinants in both apo(a) and apoB-100 that are required for Lp(a) assembly. Studies using truncated derivatives of apo(a) have suggested a role for KIV types 6 and 7 in Lp(a) formation (22, 23); studies by Trieu and McConathy (7) have shown a role for apo(a) KIV type 6 and possibly type 7 in the initial noncovalent association of apo(a) and apoB-100. Our own studies using truncated r-apo(a) variants have shown a role for KIV types 6–8 in noncovalent binding to apoB-100 (8). Most recently, we have introduced point mutations into 17K r-apo(a) to elucidate the Lp(a) assembly. Moreover, we have demonstrated that the two lysine residues (amino acids 680 and 690) present within this sequence are important for the noncovalent association between apoB-18 and apo(a), with a critical role for Lys680. Importantly, all of the r-apo(a) derivatives tested bound both 17K and KIV_{7,8} r-apo(a) in a similar fashion, suggesting that N-terminal lysines in apoB are specifically recognized by sequences within apo(a) KIV_{7,8}.

It is important to stress that the potential availability for interaction with apo(a) of the amino-terminal apoB-18 domain is supported by the findings of other studies. For example, apoB-18 is predicted to assume a globular conformation (28) and is capable of mediating the interaction of LDL with biological substrates, including microsomal triglyceride transfer protein (17), lipoprotein lipase (29), and heparin (30). The interaction of apoB-18 with microsomal triglyceride transfer protein is particularly illuminating because this association occurs prior to the completion of translation and lipidation of LDL and is therefore dependent upon the folding of apoB-18 into an independent domain (31).

To demonstrate the overall importance of the lysine residues identified by truncation and mutation analysis, we generated a synthetic peptide corresponding to amino acids 680–704 in apoB-100. Solution-phase binding experiments indicated that the affinity of the apo(a)/peptide interaction was comparable to the affinity of the apo(a)/LDL interaction. The affinity of the peptide for the 17K r-apo(a) derivative (83.4 nM) was over a 1000-fold higher than that of ε-ACA for lysine-binding kringle (apo(a)), indicating that sequences flanking the lysine residues in apoB provide an important context required for this high affinity noncovalent interaction. Importantly, the peptide was capable of inhibiting covalent Lp(a) assembly. The concentrations of peptide required to inhibit Lp(a) assembly were considerably higher than the $K_d$ for the binding of the peptide to r-apo(a). It should be noted, however, that these two experiments are not directly comparable. We speculate that numerous nonproductive interactions (i.e. not resulting in the formation of a disulfide bond) between the large apo(a) and LDL molecules exist such that the concentration of free apo(a) available to interact with the peptide is quite low under these conditions. In addition, covalent Lp(a) assembly is not a reversible process, so complexes accumulate that are refractory to the effects of the peptide. Finally, we cannot exclude the possibility that significant quantities of the (relatively nonpolar) peptide are removed from the system by adsorption to LDL.

The generation of a peptide aimed at inhibiting covalent Lp(a) formation is not an unprecedented approach. A previous study has attempted to identify the free cysteine in apoB responsible for covalent bond formation by screening an apoB-derived peptide for its ability to inhibit covalent Lp(a) formation (24). In this report, however, the peptide sequence included a potentially free cysteine residue surrounded by accompanying sequences. What distinguishes our study is that the peptide used did not contain a free cysteine residue, and it was not representative of sequences surrounding any one of the C-terminal free cysteine residues in apoB-100. As such, the data generated underscore the notion that noncovalent interactions between apo(a) and apoB-100 may involve sequences that are not proximate (in the primary sequence) to the site of disulfide bond linkage.

Our study suggests a model for Lp(a) assembly in which the first step of Lp(a) formation is mediated by an interaction between lysines 680 and 690 in apoB with the weak LBS of two apoB kringle IV domains (within apo(a) KIV types 6–8). We hypothesize that this initial noncovalent interaction tethers apoB-100 and apo(a) and allows for subsequent noncovalent interaction between C-terminal sequences in apoB-100 (possibly proximate to the free cysteine involved in disulfide bond formation as suggested by Cheesman et al. (26)) and apo(a);

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2 T. G. Wright and M. L. Koschinsky, unpublished data.

M. N. Rahman and M. L. Koschinsky, unpublished data.
this secondary interaction may involve KIV$_9$ (25). This revised model for Lp(a) assembly is supported by scanning atomic force microscopy data, which demonstrate that in Lp(a) particles, apo(a) is bound to LDL at two distant sites, one of which appears to correspond to the amino terminus of apoB (27). The importance of the lysine-dependent interaction between apo(a) KIV$_{5-8}$ and apoB-18 in Lp(a) assembly is underscored by the ability of lysine analogs to inhibit Lp(a) assembly as well as the compromised assembly observed with truncations of apo(a) lacking KIV$_7$ and KIV$_9$ (10). Further biochemical studies are required to verify this model and to determine the relative affinities of each of the interactions that contribute to the assembly process.

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REFERENCES
1. Marcovina, S. M., Hegele, R. A., and Koschinsky, M. L. (1999) Curr. Cardiol. Rep. 1, 105–111
2. McLean, J. W., Tomlinson, J. E., Kuan, W. J., Eaton, D. L., Chen, E. Y., Fless, G. M., Scann, A. M., and Lawn, R. M. (1987) Nature 330, 132–137
3. Lackner, C., Cohen, J. C., and Hobbs, H. H. (1993) Hum. Mol. Genet. 2, 903–940
4. van der Hoek, Y. Y., Wittekoek, M. E., Beisiegel, U., Kastelein, J. J., Koschinsky, M. L. (1993) Hum. Mol. Genet. 2, 361–366
5. Brunner, C., Kraft, H.-G., Utermann, G., and Muller, H.-J. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 11643–11647
6. Koschinsky, M. L., Cote, G., Gabel, B., and van der Hoek, Y. Y. (1993) J. Biol. Chem. 268, 19819–19825
7. Trieu, V. N., and McConathy, W. J. (1995) J. Biol. Chem. 270, 15471–15474
8. Gabel, B. R., and Koschinsky, M. L. (1998) Biochemistry 37, 7892–7898
9. Frank, S., Durovic, S., Kostner, K., and Kostner, G. M. (1995) Arterioscler. Thromb. Vasc. Biol. 15, 1774–1780
10. Gabel, B. R., May, L. F., Marcovina, S. M., and Koschinsky, M. L. (1996) Arterioscler. Thromb. Vasc. Biol. 16, 1559–1567
11. Koschinsky, M. L., Marcovina, S. M., May, L. F., and Gabel, B. R. (1997) Clin. Genet. 52, 338–346
12. Gabel, B. R., McLeod, R. S., Yao, Z., and Koschinsky, M. L. (1998) Arterioscler. Thromb. Vasc. Biol. 18, 1738–1744
13. Havel, R. J., Eder, H. A., and Bragdon, J. D. (1955) J. Clin. Invest. 34, 1345–1353
14. Koschinsky, M. L., Tomlinson, J. E., Zionscheck, T. F., Schwartz, K., Eaton, D. L., and Lawn, R. M. (1991) Biochemistry 30, 5044–5051
15. Keesler, G. A., Gabel, B. R., Devlin, C. M., Koschinsky, M. L., and Tabas, I. (1996) J. Biol. Chem. 271, 32096–32104
16. Graham, F. L., Smiley, J., Russell, W. C., and Marcel, Y. L. (1983) Arterioscler. Thromb. Vasc. Biol. 3, 132–137
17. Bakillah, A., Jamil, J., and Hussain, M. M. (1998) Biochemistry 37, 3727–3734
18. Milne, R. W., Theolis, R., Jr., Verderly, R. B., and Marcel, Y. L. (1983) Arteriosclerosis 3, 23–30
19. Laemmli, U. K. (1970) Nature 227, 680–685
20. Marcovina, S. M., Albers, J. J., Gabel, B., Koschinsky, M. L., and Gaur, V. P. (1995) Clin. Chem. 41, 246–255
21. Yao, Z., Blackhart, B. D., Linton, M. F., Taylor, S. M., Young, S. G., and McCarthy, B. J. (1991) J. Biol. Chem. 266, 3300–3308
22. Frank, S., Durovic, S., and Kostner, G. M. (1994) Biochem. J. 304, 27–30
23. Frank, S., and Kostner, G. M. (1997) Protein Eng. 11, 291–298
24. Sharp, R. J., Pollock, R. C., Bowron, M. E., and McCormick, S. P. A. (1999) Circulation 100, I-109
25. Rahman, M., Jia, Z., Gabel, B. R., Marcovina, S. M., and Koschinsky, M. L. (1998) Protein Eng. 11, 1249–1256
26. Cheeseman, E. J., Sharp, R. J., Zlot, C. H., Liu, C. Y.-Y., Taylor, S., Marcovina, S. M., Young, S. G., and McCormick, S. P. A. (2000) J. Biol. Chem. 275, 28195–28200
27. Xu, S. (1998) Biochemistry 37, 9284–9294
28. Segrest, J. P., Jones, M. K., Mishra, V. K., Anantharamaiah, G. M., and Garber, D. W. (1994) Arterioscler. Thromb. 14, 1674–1685
29. Sivaram, P., Choi, S. Y., Curtiss, L. K., and Goldberg, I. J. (1994) J. Biol. Chem. 269, 9409–9412
30. Golberg, I. J., Wagner, W. D., Pang, L., Faka, L., Curtiss, L. K., DeLozier, J. A., Shelness, G. S., Young, C. S., and Pillarisetti, S. (1998) J. Biol. Chem. 273, 35355–35366
31. Ingram, M. F., and Shelness, G. S. (1997) J. Biol. Chem. 272, 10279–10286