Sequences within Apolipoprotein(a) Kringle IV Types 6−8 Bind Directly to Low-Density Lipoprotein and Mediate Noncovalent Association of Apolipoprotein(a) with Apolipoprotein B-100[†]

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ABSTRACT: Lipoprotein(a) [Lp(a)] particle formation is a two-step process in which initial noncovalent interactions between apolipoprotein(a) [apo(a)] and the apolipoprotein B-100 (apoB-100) component of low-density lipoprotein (LDL) precede disulfide bond formation. To identify kringle (K) domains in apo(a) that bind noncovalently to apoB-100, the binding of a battery of purified recombinant apo(a) [r-apo(a)] species to immobilized human LDL has been assessed. The 17K form of r-apo(a) (containing all 10 types of kringle IV sequences) as well as other truncated r-apo(a) derivatives exhibited specific binding to a single class of sites on immobilized LDL, with K_d values ranging from ∼340 nM (12K) to ∼7900 nM (KIV_{5-8}). The contribution of kringle IV types 6−8 to the noncovalent interaction of r-apo(a) with LDL was demonstrated by the decrease in binding affinity observed upon sequential removal of these kringle domains (K_d ∼ 700 nM for KIV_{6-P}, K_d ∼ 2000 nM for KIV_{7-P}, K_d ∼ 5100 nM for KIV_{8-P}, and no detectable specific binding of KIV_{9-P}). Interestingly, KIV_9 also appears to participate in the noncovalent binding of apo(a) to LDL since the binding of KIV_{5-8} (K_d ∼ 7900 nM) was considerably weaker than that of KIV_{5-9} (K_d ∼ 2000 nM). Finally, it is demonstrated that inhibition of Lp(a) assembly by proline, lysine, and lysine analogues, as well as by arginine and phenylalanine, is due to their ability to inhibit noncovalent association of apo(a) and apoB-100 and that these compounds directly exert their effects primarily through interactions with sequences contained within apo(a) kringle IV types 6−8. On the basis of the obtained data, a model is proposed for the interaction of apo(a) and LDL in which apo(a) contacts the single high-affinity binding site on apoB-100 through multiple, discrete interactions mediated primarily by kringle IV types 6−8.

Lipoprotein(a)[1] [Lp(a)] is a major focus of study since elevated levels of this lipoprotein (greater than a risk threshold of ∼25−30 mg/dL) have been identified as an independent risk factor for a variety of atherosclerotic disorders (*1*, *2*). Lp(a) levels vary over 1000-fold in the human population, ranging from <0.1 mg/dL to >100 mg/dL. Lp(a) contains a low-density lipoprotein (LDL)-like moiety but is distinguishable from LDL by the presence of apolipoprotein(a) [apo(a)], which is covalently linked to apoB-100 by a single disulfide bridge (*3*, *4*). Human apo(a) contains multiply repeated copies of a sequence that closely resembles that of plasminogen kringle IV, followed by sequences which bear a high degree of similarity to the kringle V and protease domains of plasminogen (*5*). Differently sized apo(a) isoforms all contain 10 different classes of kringle IV sequences; the apo(a) kringle IV type 2 domain (the major repeat kringle) is present in different numbers of identical copies, which constitutes the basis of Lp(a) isoform size heterogeneity (*6*, *7*). Interestingly, no binding function(s) has (have) been ascribed to the major repeat kringle sequence to date.

Of the kringle IV sequences in apo(a), the sequence of apo(a) kringle IV type 10 most closely resembles that of plasminogen kringle IV. Like plasminogen kringle IV, apo(a) kringle IV type 10 has a strong lysine-binding pocket (*8*), which has been postulated to mediate the interaction of Lp(a) with lysine residues present in biological substrates such as fibrin (*9*−*11*). Weak lysine-binding sites have also been identified in apo(a) kringle IV types 5−8 (*12*, *13*), some of which may mediate the interaction of apo(a) and apoB-100 to form Lp(a) particles (see below).

In the Lp(a) molecule, apo(a) is covalently linked to apoB-100 by a single disulfide bond that involves Cys^{4057} (*3*, *4*), which is present in apo(a) kringle IV type 9. Using recombinant expression systems for apo(a), strong evidence has been presented by a number of different investigators to suggest that the assembly of Lp(a) particles occurs extracellularly (*3*, *4*, *12*−*15*). Further studies using baboon primary hepatocytes have provided evidence that Lp(a) assembly may occur on the hepatocyte cell surface (*16*). Interestingly, however, a recent study using HepG2 cells transfected with a 6-kringle-containing form of recombinant

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[1] Abbreviations: Lp(a), lipoprotein(a); apoB-100, apolipoproteinB-100; LDL, low-density lipoprotein; apo(a), apolipoprotein(a); r-apo(a), recombinant apo(a); r-Lp(a), recombinant lipoprotein(a); PBS, phosphate-buffered saline; PBST, PBS containing 0.1% Tween 20; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HBS, HEPES-buffered saline; HBST, HBS containing 1% BSA and 0.1% Tween 20; ϵ-ACA: ϵ-aminocaproic acid.

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apo(a) [r-apo(a)] demonstrated that covalent recombinant Lp(a) [r-Lp(a)] particles containing the 6K r-apo(a) species formed intracellularly (*17*). However, in other studies intracellular covalent Lp(a) particles could not be demonstrated using human liver (*18*), baboon liver (*19*), or HepG2 cells transfected with a 17-kringle r-apo(a) derivative (*3*).

Several groups have assessed the role of specific apo(a) kringles in the process of Lp(a) formation: studies by Frank and co-workers suggested a role for apo(a) kringle IV types 6 and 7 in this process (*14*, *20*), while Trieu and McConathy demonstrated that apo(a) kringle IV type 6 and, possibly, type 7 are involved in the noncovalent association of apo(a) and apoB-100 (*21*). Generally, studies to date suggest that Lp(a) formation is a two-step process involving initial noncovalent interactions between apo(a) and apoB-100 that precede specific disulfide bond formation. It is not clear, however, to what extent these noncovalent interactions ultimately influence the efficiency of covalent complex formation. Although it has been demonstrated that lysine, lysine analogues, and proline can inhibit the formation of Lp(a) particles (*13*, *22*), the mechanism of action of these inhibitors is currently unknown.

In the present study, we have examined in detail the noncovalent association of apo(a) and apoB-100 using a battery of r-apo(a) derivatives. Our results indicate a key role for sequences within apo(a) kringle IV types 6−9 in mediating the noncovalent interaction of apo(a) and apoB-100 and demonstrate for the first time that sequences within apo(a) kringle IV types 6−8 bind noncovalently to LDL and directly mediate inhibition of the first step of Lp(a) formation by a variety of compounds.

EXPERIMENTAL PROCEDURES

Construction and Expression of r-Apo(a) Derivatives. With the exception of the 17KΔCys construct (see below), the construction of expression plasmids encoding all of the r-apo(a) derivatives used in this study (Figure 1) has been detailed elsewhere (*13*, *23*−*25*). The 17KΔCys expression plasmid, in which the free cysteine at amino acid 67 in the wild-type kringle IV type 9 sequence is replaced with a tyrosine, was generated using a PCR-based mutagenesis strategy using synthetic oligonucleotides. Oligo pairs Cys1 (5′GAAGCACCAACGGAGCAAAG3′) and Cys2 (5′CCTCAC**GT**ACGGATCGGTTG3′) and Cys3 (5′CGATCCGT**AC**GTGAGGTGGG3′) and Cys4 (5′GAAGAGGATGCACAGAGAGG3′) were utilized to amplify overlapping 1244 and 714 bp fragments, respectively, that span the sequence encoding the free cysteine in apo(a) kringle IV type 9. The boldface nucleotides indicate substitutions that convert the Cys codon at position 67 to a Tyr codon. The fragment amplified using Cys1 and Cys2 was digested with *Xma*I and *Bsi*WI; the fragment amplified using oligos Cys3 and Cys4 was digested with *Bsi*WI and *Eco*RV. The restriction fragments were then cloned as a three-part ligation into pRK5ha17 [encoding the 17K r-apo(a) species; ref *23*] that had been digested with *Xma*I and *Eco*RV. The 342 bp *Xma*I fragment [spanning sequences encoding apo(a) kringle IV types 5 and 6] removed from pRK5ha17 by *Xma*I digestion was subsequently replaced to generate the final expression plasmid encoding the 17KΔCys derivative.

Stably transformed human embryonic kidney (293; ref *26*) cells expressing each of the r-apo(a) derivatives were generated by calcium phosphate coprecipitation (*27*) using 10 μg of each r-apo(a) expression plasmid and 1 μg of a plasmid encoding the neomycin resistance gene (*28*) per 100 mm plate. Stable transformants were selected by culturing cells in the presence of G418 (800 μg/mL of media) as previously described (*13*, *23*−*25*).

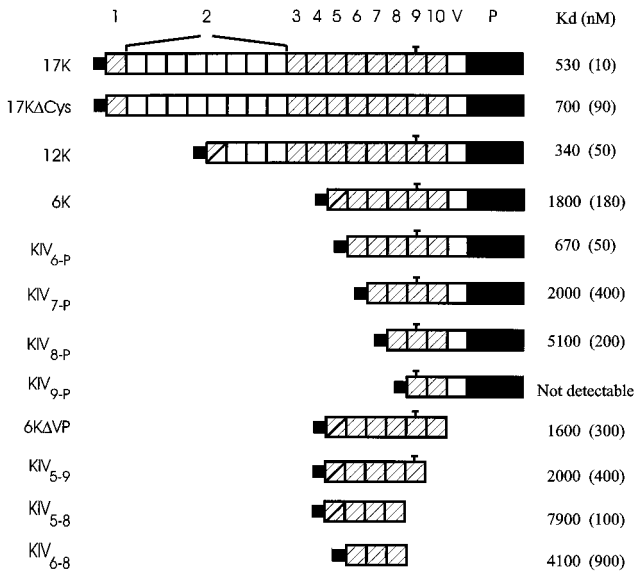

FIGURE 1: Schematic representation of r-apo(a) derivatives. The r-apo(a) derivatives used to examine the noncovalent association of apo(a) with LDL are shown. The uppermost graphic illustrates the organization of the 17-kringle r-apo(a) species (17K), which was derived from the published cDNA as previously described (*19*). The 17K r-apo(a) contains a single copy of sequences corresponding to each of the 9 unique kringle IV domains of apo(a) (KIV types 1 and 3−10; shaded boxes) and also contains 8 copies of the major repeat kringle (KIV type 2; open boxes) and a single copy of kringle V (V) and inactive protease domain (P). The position of the free cysteine in apo(a) kringle IV type 9, which forms the disulfide bond with a cysteine residue in the apoB-100 moiety of LDL, is shown by a bar. The presence of a hybrid kringle at the amino terminus of a number of the r-apo(a) derivatives is indicated by a diagonal line. These hybrid kringles consist of an in-frame fusion of the amino-terminal one-third of apo(a) kringle IV type 1 with either the carboxyl-terminal two-thirds of apo(a) kringle IV type 2 [for the 12K r-apo(a) derivative] or apo(a) kringle IV type 5 [for the r-apo(a) derivatives 6K, 6KΔVP, KIV_{5-9}, and KIV_{5-8}]. Mean K_d values for the noncovalent binding of the respective derivatives to immobilized human LDL are indicated; numbers in parentheses correspond to the standard deviation of three independent experiments.

Protein Purification. All of the r-apo(a) derivatives shown in Figure 1 were purified from the conditioned medium (CM) of stably expressing cell lines by lysine−Sepharose (Pharmacia) affinity chromatography as previously described (*25*). Briefly, CM (OptiMEM; Gibco/BRL) harvested from stably transfected cells was loaded over 50 mL lysine−Sepharose CL-4B columns equilibrated with phosphate-buffered saline (PBS). Columns were washed extensively with PBS containing 0.5 M NaCl, and r-apo(a) protein was eluted from the columns with 0.2 M ϵ-aminocaproic acid (ϵ-ACA) in the same buffer. Protein-containing fractions were pooled, dialyzed against HEPES-buffered saline (HBS; 20 mM HEPES, pH 7.4, containing 0.15 M NaCl), and concentrated against PEG-20000 (Fluka). Protein concentrations for each r-apo(a) derivative were determined by measurement of absorbance at 280 nm using corresponding extinction coefficients (*25*) determined by the tyrosine difference spectral

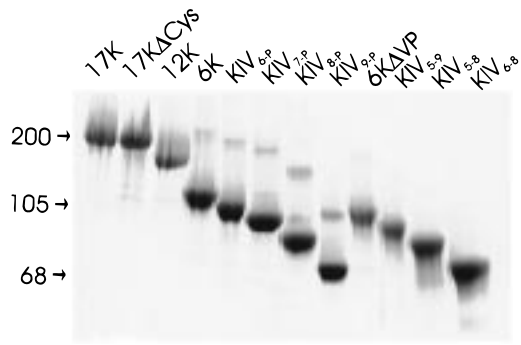

FIGURE 2: SDS−PAGE analysis of purified r-apo(a) derivatives. Recombinant apo(a) was purified by lysine−Sepharose affinity chromatography from conditioned medium harvested from 293 cells stably expressing each derivative. The indicated recombinant proteins (10 μg) were analyzed by SDS−PAGE under nonreducing conditions using a 3−15% polyacrylamide gradient gel and stained with Coomassie Brilliant Blue. The positions of molecular weight markers (Bio-Rad) are indicated to the left of the gel.

method as previously described (*29*). Aliquots of the purified proteins were stored at −70 °C prior to use.

Purified protein corresponding to each r-apo(a) derivative was analyzed by SDS−PAGE, followed by staining with Coomassie Brilliant Blue (Figure 2).

Lipoprotein Isolation. LDL ($1.02 < d < 1.05$ g/mL) was isolated according to the method of sequential flotation (*30*) from fresh frozen plasma obtained from a single normolipidemic volunteer with Lp(a) levels <5 mg/dL. Briefly, plasma was adjusted to $d = 1.02$ g/mL with sodium bromide and centrifuged for 2 h at 100 000 rpm in a TL-100 rotor (Beckman) at 10 °C. The supernatant was discarded, and the infranatant was adjusted to $d = 1.05$ g/mL with sodium bromide and centrifuged for a further 2 h at 100 000 rpm. The LDL-containing fraction was harvested and recentrifuged for a final 2 h at $d = 1.05$ g/mL. The LDL was subsequently harvested, and the protein content was determined by using a modified Bradford assay (Bio-Rad) using bovine serum albumin (BSA; ICN) as a reference standard. All buffers used during the isolation procedure were supplemented with 1 mM EDTA, 0.02% (w/v) sodium azide, and 1 mM phenylmethanesulfonyl fluoride (PMSF). Isolated LDL was stored at 4 °C and used within 5 days of purification.

Iodination of r-Apo(a) Derivatives. Recombinant apo(a) derivatives were iodinated using Iodo-Beads (Pierce Chemicals). One Iodo-Bead was incubated for 5 min with 250 μCi of $Na^{125}I$ (ICN) in 0.5 mL of iodination buffer (200 mM Tris-HCl, pH 7.4, containing 0.1 M NaCl). The supernatant was then transferred to a microcentrifuge tube containing 200−250 μg of protein, and the reaction was allowed to proceed for 2 min at room temperature. The iodination reaction was stopped by the addition of sodium metabisulfite (5−10 mM final concentration). Free ^{125}I was separated from ^{125}I-labeled proteins by passage over a 10DG desalting column (Bio-Rad) that had been pre-equilibrated with 20 mM Tris-HCl, pH 7.4, containing 0.15 M NaCl. The protein concentration was determined by using a modified Bradford assay (Bio-Rad) using the corresponding purified r-apo(a) derivative as a standard. The specific activity of the radiolabeled r-apo(a) derivatives ranged from 400 to 1000 cpm/ng; >95% of the radioactivity was TCA-precipitable. Labeled r-apo(a) derivatives were stored at 4 °C and used within 5 days of iodination. The integrity of iodinated proteins was routinely assessed by SDS−PAGE and autoradiography.

Immobilized Binding Assays. LDL isolated as described above was coated onto microtiter wells (Immulon-4; Dynatech) at a concentration of 1 μg/mL in 0.1 M $NaHCO_3$, pH 9.6, overnight at 4 °C. Microtiter wells were then washed with PBS containing 0.1% Tween 20 (v/v) (PBST) and incubated overnight at 4 °C with 200 μL of a 2.5% solution of BSA (w/v) in HBS to block nonspecific binding sites. Blocked wells were washed with PBST and incubated for 16−20 h at 4 °C with various concentrations [up to 10 μM, depending on the r-apo(a) species] of the iodinated r-apo(a) derivatives serially diluted in HBS supplemented with 1% BSA (w/v) and 0.1% Tween 20 (v/v) (HBST). Following incubation, microtiter wells were washed three times with 200 μL of PBST and the well-associated radioactivity was determined using a γ-radiation counter. For each r-apo(a) derivative, with the exception of KIV_{9-P}, nonspecific binding was considered to be that observed in the presence of 0.2 M ϵ-ACA and was subtracted from total binding to yield specific binding values. For KIV_{9-P}, nonspecific binding was determined using a 50-fold molar excess of unlabeled protein. In all cases, dissociation constants (K_d) were determined by Scatchard transformation of the specific binding data.

In some experiments the effect of various amino acids and competitor r-apo(a) proteins on the binding of r-apo(a) to immobilized LDL was examined. For these assays, LDL immobilized onto microtiter wells was incubated with either 17K (250 nM) or KIV_{6-8} (1−2 μM) r-apo(a) derivatives for 16 h at 4 °C in the presence of amino acids (50−200 mM) or competitor r-apo(a) proteins (4 μM each) as indicated in the corresponding figure legends. Following incubation, the wells were washed three times with 200 μL of PBST. Well-associated radioactivity was determined using a γ-radiation counter. The inhibition of binding of r-apo(a) derivatives to LDL by the various amino acids or r-apo(a) proteins was expressed as a percentage of the total r-apo(a) bound in the absence of amino acids or proteins.

To determine if apo(a) binding to LDL was reversible in our assay system, radioiodinated 17K r-apo(a) (500 nM) was incubated with LDL immobilized on microtiter wells for 16 h at 4 °C. Wells were subsequently washed three times with 200 μL of PBST and incubated for a further 16 h with either 100 μL of HBST, HBST containing either 0.2 M ϵ-ACA or 0.2 M proline, or HBST containing 5 μM unlabeled r-apo(a) (17K). Following incubation the wells were washed three times with 200 μL of PBST and the amount of bound r-apo(a) was determined by counting the wells in a γ-radiation counter. Data were expressed as a percentage of the amount of r-apo(a) bound relative to that in wells which received only buffer following the initial 16 h incubation and subsequent washes.

RESULTS

Binding of Recombinant Apo(a) to Immobilized LDL. The binding of the purified radioiodinated r-apo(a) derivatives shown in Figure 1 to human LDL immobilized in microtiter wells was assessed. In an initial experiment we observed that the r-apo(a) derivative 17KΔCys (lacking the free cysteine in KIV_9 that mediates disulfide bond linkage to the

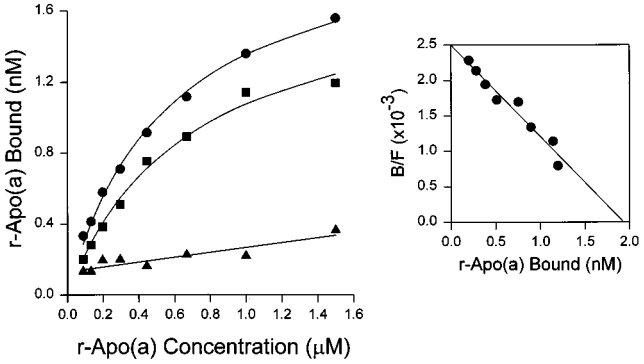

FIGURE 3: Specificity of binding of 17KΔCys r-apo(a) to immobilized LDL. LDL immobilized onto microtiter wells was incubated with increasing concentrations of the ^{125}I-labeled r-apo(a) derivative 17KΔCys overnight at 4 °C in the absence or presence of 0.2 M ϵ-ACA. At the conclusion of the incubation the microtiter wells were washed and the well-associated radioactivity was determined by γ-radiation counting. The amount of r-apo(a) bound was plotted as a function of the r-apo(a) concentration to obtain the total binding values (circles), nonspecific binding values (triangles; determined in the presence of 0.2 M ϵ-ACA), and specific binding values [squares; determined by subtraction of the nonspecific from the total binding values at each input apo(a) concentration]. The data shown represent the mean of an experiment done in duplicate. The Scatchard transformation (right panel) of the specific binding isotherm revealed a single class of binding sites with a K_d of 700 nM.

apoB moiety of LDL) was able to bind to immobilized LDL in a saturable manner and that this binding is inhibited >70% by addition of the lysine analogue ϵ-ACA to a final concentration of 0.2 M (Figure 3, left panel). Scatchard transformation of the specific binding isotherm indicated a single class of binding sites with a dissociation constant (K_d) of ~700 nM (Figure 3, right panel).

This result indicated that the 17KΔCys derivative was able to bind to immobilized LDL with relatively high affinity and that specific binding could be abolished by the addition of ϵ-ACA. We therefore sought to determine if r-apo(a) containing the free cysteine in KIV$_9$ also bound to immobilized LDL in a noncovalent manner. Figure 4 shows the specific binding isotherm obtained for the binding of radioiodinated 17K r-apo(a) derivative to immobilized LDL with the corresponding Scatchard transformation. The 17K r-apo(a) derivative was also able to bind to LDL in a saturable and specific manner. Scatchard transformation indicated the presence of a single class of binding sites with a K_d of ~530 nM, which is similar to the results obtained for the 17KΔCys derivative shown in Figure 3. The results obtained for the binding of the iodinated 17K derivative to immobilized LDL suggested that the presence of the free cysteine in KIV$_9$ did not preclude reversible binding of r-apo(a) and LDL.

To more rigorously demonstrate that the iodinated 17K r-apo(a) derivative was binding to LDL in a reversible manner, we conducted the experiment presented in Figure 5. Briefly, following overnight incubation of radioiodinated 17K r-apo(a) (500 nM) with LDL immobilized in microtiter wells, the wells were washed and incubated with ϵ-ACA, proline, or a 10-fold molar excess of unlabeled r-apo(a) for a further 16 h, at which time unbound r-apo(a) was removed by aspiration and the amount of bound r-apo(a) was determined by counting of the wells in a γ-radiation counter. The remaining amount of r-apo(a) bound following treatment

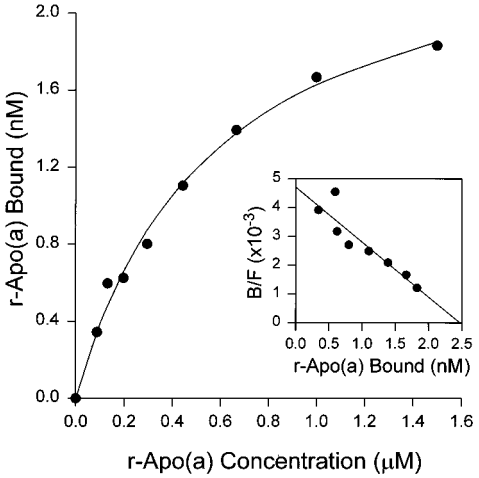

FIGURE 4: Specific binding of the 17K r-apo(a) derivative to immobilized LDL. Increasing concentrations of ^{125}I-labeled 17K r-apo(a) were incubated overnight at 4 °C in the absence or presence of 0.2 M ϵ-ACA in microtiter wells in which LDL had been immobilized. The assay was terminated by aspirating the wells followed by three washes with PBST. The amount of radioactivity present in each well was determined by counting the wells in a γ-radiation counter. The concentration of r-apo(a) specifically bound was determined as described in the legend to Figure 3; specific binding values were plotted as a function of the input r-apo(a) concentration. Scatchard transformation of the binding isotherm (inset) for 17K revealed a single class of binding sites with a K_d value of ~530 nM.

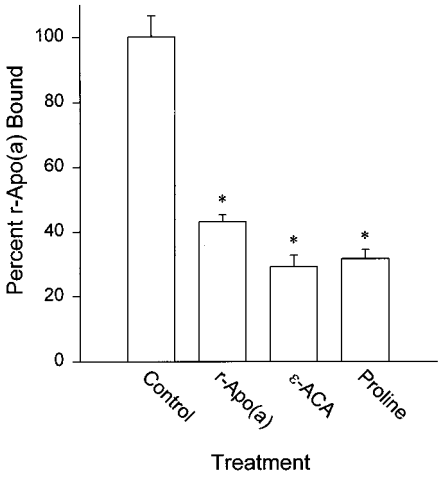

FIGURE 5: Binding of the 17K r-apo(a) derivative to immobilized LDL is reversible. ^{125}I-r-apo(a) (17K; 500 nM final concentration) was incubated with human LDL immobilized in microtiter wells. Following a 16 h incubation at 4 °C the microtiter wells were aspirated and washed three times with 200 μL of PBST. The wells were then incubated for a further 16 h at 4 °C with either ϵ-ACA or proline (both 0.2 M final concentration dissolved in HBST) or a 5 μM solution of the unlabeled r-apo(a) derivative 17K in HBST. At the conclusion of the incubation all wells were aspirated and washed three times with PBST, and the amount of bound radioactivity was determined using a γ-radiation counter. The amount of r-apo(a) bound under the indicated conditions was expressed as a percentage of the total amount of bound r-apo(a) observed in control wells to which only buffer was added following the initial 16 h incubation and wash steps. The results shown represent the mean of an experiment done in triplicate; error bars correspond to the standard deviation. Values with asterisks are significantly different from the control ($p < 0.01$; determined using the paired Student' t test).

with 0.2 M ϵ-ACA and proline or a 10-fold molar excess of unlabeled 17K r-apo(a) was reduced to <45% for r-apo(a)

and 30% for ϵ-ACA and proline relative to control wells. The values obtained for ϵ-ACA and proline correspond approximately to the nonspecific binding component at this concentration of input apo(a) shown in Figure 3. This experiment demonstrated that under the conditions employed in the present study, r-apo(a) is able to bind to LDL immobilized onto microtiter wells in a reversible manner, thereby indicating that the binding experiments were performed under equilibrium conditions.

Having demonstrated the reversible and saturable nature of the binding of the 17KΔCys and 17K r-apo(a) derivatives to immobilized LDL, we determined K_d values for each of the r-apo(a) derivatives represented schematically in Figure 1 (the corresponding dissociation constants are also presented in this figure). Scatchard transformation of the specific binding isotherms indicated that each of the derivatives bound to a single class of sites within the apoB-100 moiety of LDL (data not shown). The K_d values ranged from ~340 nM (12K) to ~7900 nM (KIV_{5-8}) (see Figure 1). No specific binding of the r-apo(a) derivative KIV_{9-P} was observed using concentrations of this derivative up to 10 μM. The lack of involvement of apo(a) kringle IV type 10 and the kringle V- and protease-like domains is further emphasized by the similar K_d values obtained for the r-apo(a) derivatives 6K (K_d ~ 1800 nM), 6KΔVP (K_d ~ 1600 nM), and KIV_{5-9} (K_d ~ 2000 nM). Interestingly, however, KIV_9 may also contribute to the noncovalent association of apo(a) to LDL since the binding of KIV_{5-8} (K_d ~ 7900 nM) was considerably weaker than that of KIV_{5-9} (K_d ~ 2000 nM) (see Figure 1). Our results clearly demonstrate the important contribution of apo(a) kringle IV types 6−8 to the noncovalent association of apo(a) with LDL. The successive removal of each of these kringles resulted in a decrease in the affinity of the r-apo(a) derivatives for LDL (compare K_d values of ~670, ~2000, and ~5100 nM for KIV_{6-P}, KIV_{7-P}, and KIV_{8-P} respectively; see Figure 1). Taken together, our data therefore suggest that the initial noncovalent association of r-apo(a) with apoB-100 is mediated predominantly by a core of kringle domains consisting of KIV_{6-8} and to a lesser degree KIV_9.

Inhibitors of the Noncovalent Association of Apo(a) and ApoB-100. To date, a number of inhibitors of in vitro formation of Lp(a) have been identified, which include lysine, lysine analogues, and proline (*13*, *22*), although the mechanism of action of these inhibitors has not been thoroughly examined. As such, we chose to analyze the mechanism underlying the inhibitory effect of a number of different amino acids on noncovalent association of radiolabeled r-apo(a) (17K and KIV_{6-8} derivatives; see Figure 1) and immobilized LDL using the methodology described above. The results of this experiment (Figure 6) demonstrate that phenylalanine and arginine (in addition to ϵ-ACA, lysine, and proline) significantly inhibited the noncovalent association of 17K (Figure 6, top) with immobilized LDL, while glycine had no significant effect. Similar results were obtained using the r-apo(a) derivative KIV_{6-8} (Figure 6, bottom).

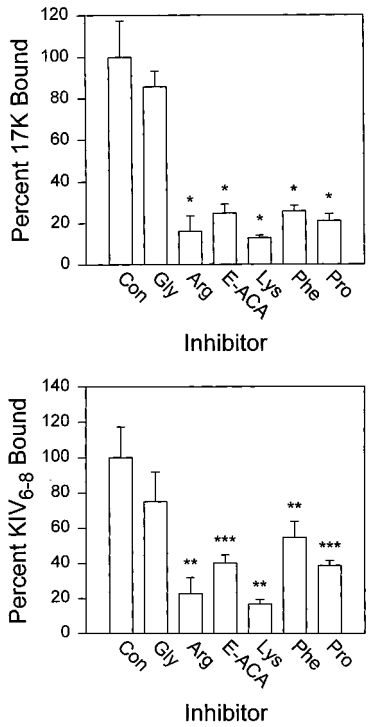

FIGURE 6: Inhibition of binding of the r-apo(a) derivatives 17K and KIV_{6-8} to immobilized LDL by amino acids. LDL was immobilized onto microtiter wells and subsequently incubated with either the r-apo(a) derivative 17K (250 nM final concentration; top panel) or KIV_{6-8} (1 μM final concentration; bottom panel) in the absence (Con) or presence of a number of amino acids (all 50 mM final concentration) for 16 h at 4 °C. Upon completion of the incubation, the wells were aspirated and washed three times with 200 μL of PBST, and the well-associated radioactivity was determined by γ-radiation counting. The amount of r-apo(a) bound was expressed as a percentage of the amount of r-apo(a) observed in the absence of amino acids. The results represent the mean of an experiment done in triplicate; error bars correspond to the standard deviation. Values with asterisks are significantly different from the controls (single asterisk, $p < 0.005$; double asterisk, $p < 0.02$; triple asterisk, $p < 0.05$; determined using the paired Student' *t* test).

DISCUSSION

In this study we have quantified the noncovalent binding of a variety of recombinant apo(a) derivatives to immobilized human LDL to identify kringle domains in apo(a) that are directly involved in mediating this process. As described under Experimental Procedures, the binding assays were performed using immobilized LDL and radioiodinated r-apo(a) derivatives. Using the 17K r-apo(a), we demonstrated that the binding to immobilized LDL was reversible (i.e., noncovalent), even though this derivative readily forms covalent r-Lp(a) particles in solution (*13*). This is not surprising since it is has been reported that a side reaction of iodination results in oxidation of thiol groups of cysteine residues (*31*). With respect to the integrity of the iodinated r-apo(a), we have found that the labeled derivatives retain full lysine-binding properties and migrate as discrete bands on SDS−PAGE (data not shown).

Scatchard transformation of the binding data obtained for each r-apo(a) derivative demonstrates that apo(a) interacts with a single class of binding sites on immobilized LDL; comparable results were obtained in binding assays using immobilized r-apo(a) derivatives and radiolabeled human LDL (data not shown). Removal of amino-terminal sequences of r-apo(a) corresponding to apo(a) kringle IV types 1−5 did not severely affect the binding of apo(a) to apoB-100, suggesting that these kringle domains are not essential for the noncovalent association of apo(a) with apoB-100. This

is further supported by our previous study in which we demonstrated that kringle IV types 1−5 were not required for the efficient formation of r-Lp(a) particles in vitro (*13*). Further amino-terminal truncation of r-apo(a) resulted in a stepwise decrease in the affinity of the various r-apo(a) derivatives for LDL. Importantly, the contribution of apo(a) kringle IV types 6−8 to the noncovalent association of apo(a) with LDL was clearly demonstrated; the successive removal of each of these kringles resulted in a decrease in the affinity of the r-apo(a) derivatives for LDL (see Figure 1). Additionally, no specific binding of the KIV_{9-P} derivative was detectable at concentrations up to 10 μM, suggesting that the carboxyl-terminal sequences of apo(a) do not contribute significantly to the noncovalent association of apo(a) with LDL. The reduced affinities observed for KIV_{8-P} and KIV_{9-P} relative to that of KIV_{7-P} are in accordance with the dramatic reduction in the in vitro formation of r-Lp(a) particles in solution using these derivatives which we have previously reported [89, 57, and 19% r-Lp(a) formed using the KIV_{7-P}, KIV_{8-P}, and KIV_{9-P} species, respectively; ref *13*]. These data are also in agreement with a previous paper by Trieu and McConathy in which a significant role for KIV_6 and possibly KIV_7 was suggested in Lp(a) formation (*21*). In addition to kringle IV types 6 and 7, our study indicates a previously unreported role for the KIV_8 sequence in mediating the first noncovalent step of Lp(a) assembly. It is likely that the weak lysine-binding pockets within each of these kringles bind independently to specific sequences within the apoB-100 moiety of LDL such that kringle IV types 6−8 each contribute to noncovalent binding of apo(a) to apoB-100. In this model, the strength of the noncovalent interaction of apo(a) and apoB-100 reflects the sum of the binding energies for the individual kringle interactions. Furthermore, as our previous study suggests (*13*), the specific noncovalent interaction of each of apo(a) kringles 6−8 with their cognate sites on LDL is required for maximally efficient covalent Lp(a) formation. The identification of sequences in apoB-100 that are able to interact directly with these kringle domains will aid in the evaluation of these hypotheses.

Analysis of the noncovalent interaction of the 6K r-apo(a) derivative with immobilized LDL revealed that this derivative bound with lower affinity ($K_d \sim 1800$ nM) than the KIV_{6-P} species ($K_d \sim 670$ nM). It should be noted that the amino-terminal kringle of the 6K species is a hybrid kringle of sequences encoding apo(a) kringle IV types 1 and 5 (see Figure 1). Interestingly, the presence of this hybrid kringle in KIV_{5-8} also reduces its binding affinity to LDL ($K_d \sim$ 7900 nM compared with $K_d \sim 4100$ nM for the KIV_{6-8} derivative; see Figure 1). Taken together, these data suggest that this hybrid kringle serves to decrease noncovalent interactions between apo(a) and apoB-100, although the mechanism underlying this observation is unclear at present.

The absence of detectable specific binding of the KIV_{9-P} derivative to immobilized LDL in the present study is in agreement with results obtained by Trieu and McConathy (*21*), indicating that the carboxyl terminus of apo(a) is unnecessary for its noncovalent association with LDL. This is further underscored by our results in which similar K_d values were obtained for the 6K, 6KΔVP, and KIV_{5-9} derivatives (see Figure 1). Clearly, sequences corresponding to apo(a) kringle IV type 10 and the kringle V and protease-like domains can be eliminated without adversely affecting the noncovalent interaction of apo(a) and apoB-100. Interestingly, however, removal of the apo(a) kringle IV type 9 sequence resulted in a significantly decreased binding affinity to immobilized LDL (compare K_d for KIV_{5-8} of ~7900 nM versus ~2000 nM for the KIV_{5-9} derivative; see Figure 1). This suggests that the KIV type 9 sequence may provide contacts which contribute to the noncovalent association of apo(a) and LDL. This novel observation supports a model in which the initial interaction of apo(a) with the apoB-100 moiety of LDL is mediated through one or more of the kringles present in KIV types 6−8 and that this initial contact is essential for the recognition of KIV type 9 binding sites in apoB and subsequent specific disulfide bond formation.

We also sought to determine if inhibitors of Lp(a) assembly exert their effects by eliminating the noncovalent interaction between apo(a) and apoB-100 that precedes specific disulfide bond formation. We examined the ability of known inhibitors of Lp(a) assembly (lysine, ϵ-ACA, and proline) to inhibit the noncovalent binding of apo(a) and apoB-100; we also tested the effect of additional amino acids (glycine, arginine, and phenylalanine) on this process. All of the amino acids with the exception of glycine significantly reduced the amount of r-apo(a) (either 17K or KIV_{6-8} derivatives) bound to immobilized LDL. These data provide the first demonstration that inhibition of Lp(a) formation by these amino acids is mediated by their direct interaction with binding sites present in KIV_{6-8}, thereby eliminating the initial noncovalent interaction of apo(a) and apoB-100. Our data suggest that in addition to known inhibitors of Lp(a) assembly, which include lysine, lysine analogues, and proline (*13*, *22*), phenylalanine and arginine also appear to inhibit the noncovalent interaction of apo(a) and apoB-100 by binding directly within apo(a) kringle IV types 6−8. We have observed the interaction of both phenylalanine and arginine with apo(a) in solution as determined by the measurement of changes in intrinsic fluorescence (B.R.G. and M.L.K., unpublished observations). Substitutions of amino acids within the lysine-binding pockets in apo(a) kringle IV types 5−8 (compared with the lysine-binding site in KIV_{10}) may facilitate the binding of arginine within these binding pockets.

To demonstrate that the direct binding of apo(a) kringle IV types 6−8 to LDL in the context of the KIV_{6-8} derivative was representative of the behavior of these sequences present within a larger r-apo(a) derivative such as 17K, we assessed the ability of the 17K and KIV_{6-8} r-apo(a) derivatives to mutually compete for binding to LDL. We found that a 2-fold excess of unlabeled 17K r-apo(a) was able to effectively inhibit the binding of radiolabeled KIV_{6-8} to immobilized LDL ($p < 0.005$; data not shown), whereas a similar concentration of KIV_{9-P} had no effect. This finding indicates that 17K and KIV_{6-8} do in fact bind to analogous sites on LDL, albeit with different affinities since the 17K derivative also contains additional sequences (i.e., KIV_9) which serve to increase its overall affinity for LDL.

In summary, our study provides important new information on the initial noncovalent step of Lp(a) formation. Our analysis of the binding of a variety of r-apo(a) derivatives to immobilized LDL suggests a key role for apo(a) kringle IV types 6−8 in this process and provides the first demonstration that these sequences interact directly with apoB-100. The latter observation is supported by our data

showing that the inhibition of Lp(a) assembly by a variety of inhibitors specifically prevents the noncovalent association of apo(a) kringle IV types 6−8 with LDL. Finally, the present study also suggests a novel role for sequences in apo(a) kringle IV type 9 in mediating noncovalent interactions with LDL. Clearly, further studies are required to more precisely identify interactive sequences in apo(a) and apoB-100 that mediate noncovalent interactions between these two proteins.

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