Baboon Lipoprotein(a) Binds Very Weakly to Lysine−Agarose and Fibrin Despite the Presence of a Strong Lysine-Binding Site in Apolipoprotein(a) Kringle IV Type 10[†]

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ABSTRACT: Human apolipoprotein(a) kringle IV type 10 [apo(a) KIV_{10}] contains a strong lysine-binding site (LBS) that mediates the interaction of Lp(a) with biological substrates such as fibrin. Mutations in the KIV_{10} LBS have been reported in both the rhesus monkey and chimpanzee, and have been proposed to explain the lack of ability of the corresponding Lp(a) species to bind to lysine and fibrin. To further the comparative analyses with other primate species, we sequenced a segment of baboon liver apo(a) cDNA spanning KIV_9 through the protease domain. Like rhesus monkey apo(a), baboon apo(a) lacks a kringle V (KV)-like domain. Interestingly, we found that the baboon apo(a) KIV_{10} sequence contains all of the canonical LBS residues. We sequenced the apo(a) KIV_{10} sequence from an additional 10 unrelated baboons; 17 of 20 alleles encoded Trp at position 70, whereas only two alleles encoded Arg at this position and thus a defective LBS. Despite the apparent presence of a functional KIV_{10} LBS in most of the baboons, none of the Lp(a) in the plasma of the corresponding baboons bound specifically to lysine−Sepharose (agarose) even upon partial purification. Moreover, baboon Lp(a) bound very poorly to plasmin-modified fibrinogen. Expression of baboon and human KIV_{10} in bacteria allowed us to verify that these domains bind comparably to lysine and lysine analogues. We conclude that presentation of KIV_{10} in the context of apo(a) lacking KV may interfere with the ability of KIV_{10} to bind to substrates such as fibrin; this paradigm may also be present in other non-human primates.

Since the discovery of lipoprotein(a) [Lp(a)][1] by Berg (*1*), numerous studies have demonstrated that elevated plasma concentrations of this lipoprotein are a risk factor for the development of coronary heart disease in the human population (reviewed in ref *2*). However, the true physiological role of Lp(a) as well as the mechanism by which it exerts its pathogenic effects remains unclear (*2*, *3*). Lp(a) is similar to low-density lipoprotein (LDL) both in its lipid composition and in the presence of apolipoprotein B-100 (apoB). The distinguishing feature of Lp(a) is the presence of the glycoprotein apolipoprotein(a) [apo(a)] that is covalently linked to apoB by a single disulfide bridge, and that likely confers the unique properties attributed to Lp(a). On the basis of cloning of the entire human apo(a) cDNA, it was found that apo(a) bears a striking homology to the fibrinolytic proenzyme plasminogen (*4*) which has prompted speculation concerning the evolution of these two proteins, and the role of Lp(a) in fibrin clot lysis.

Plasminogen has been characterized from several species, including human and bovine (*5*), rhesus monkey (*6*), and the European hedgehog (*Erinaceus europaeus*) (*7*). Plasminogen from all of these species contains a preactivation (tail) peptide followed by five kringle (K) motifs (designated K1−5) and a trypsin-like protease domain. Human apo(a) contains tandemly repeated copies of sequences that closely resemble plasminogen kringle 4 (KIV), followed by sequences that are homologous to the K5 and protease domains of plasminogen. The plasminogen K4-like domains of apo(a) can be classified on the basis of amino acid sequence into 10 types which, with the exception of KIV type 2 (KIV_2), are each present in a single copy in all individuals (*8*); the KIV_2 domain is present in varying numbers of identically repeated copies which forms the basis of the apo(a) isoform size heterogeneity observed in the human population (*9*). Interestingly, the protease-like domain of apo(a) is catalytically inactive, because of the substitution of a Ser for an Arg at the site of cleavage by tissue-type plasminogen activator (tPA), and the presence of a nine-amino acid (aa) deletion in the apo(a) protease domain relative to plasminogen which alters the conformation of the active site (*10*). Numerous studies have demonstrated that Lp(a), through its apo(a) component, binds to a variety of biological substrates via

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[1] Abbreviations: Lp(a), lipoprotein(a); apo(a), apolipoprotein(a); LDL, low-density lipoprotein; apoB, apolipoprotein B-100; K, kringle; LBS, lysine-binding site; tPA, tissue-type plasminogen activator; ϵ-ACA, ϵ-aminocaproic acid; DTE, dithioerythreitol; PBS, phosphate-buffered saline; HBS, HEPES-buffered saline.

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Table 1: Primers Utilized in These Studies

application	primer name	sequence
PCR of baboon KIV_9 protease	KIV_9 forward primer	5′-GATTCTGGGAAACAACCCTGG-3′
	protease reverse primer	5′-ATAGACACCAGGCTTATTGGG-3′
PCR of genomic DNA encoding baboon KIV_{10}	KIV_{10} exon 1, forward	5′-CTGTGGTCCAGCAGTGCTAC-3′
	KIV_{10} exon 1, reverse	5′-TCATTTGGGTGGTTTTCCG-3′
	KIV_{10} exon 2, forward	5′-AGATGCTGACACAGGCCCTT-3′
	KIV_{10} exon 2, reverse	5′-CTAGGCTTGGAACCGGGATA-3′
PCR of baboon KIV_{10} for *E. coli* expression	KIV_{10}/pET16b, forward	5′-GGACTGACATATGGCACCAACTGAGCAAACCCC-3′[a]
	KIV_{10}/pET16b, reverse	5′-GGACTGACATATGTTATTGTTCAGAAGGAGCC-3′[a]
mutagenesis of baboon KIV_{10}	$Q^{32}R$, sense	5′-CTTATGC**CT**GTGTGG**C**GTCATGGATGACC-3′[b]
	$Q^{32}R$, antisense	5′-CATGAC**G**CCACAC**AG**GCATAAGAGGACC-3′[b]

[a] The underlined sequence represents the *Nde*I site incorporated for insertion of the PCR product into pET16b. [b] The underlined sequence represents the *Bgl*I site incorporated for fusion of the overlapping PCR products; the bold nucleotides are substitutions relative to the wild-type baboon KIV_{10} sequence.

lysine-dependent mechanisms. Apo(a) KIV type 10 (KIV_{10}) contains a canonical lysine-binding site (LBS) similar to that in plasminogen KIV; the LBS in KIV_{10} has been proposed to mediate the lysine-dependent binding of Lp(a) to biological substrates such as fibrin (*3*).

To date, Lp(a) has been identified in only humans (*1*), rhesus monkeys (*6*), chimpanzees (*11*), baboons (*12*), and the European hedgehog (*13*). Hedgehog apo(a) is characterized by multiply repeated copies of sequence that are homologous to plasminogen KIII (*14*). Like human apo(a), rhesus monkey apo(a) contains multiply repeated copies of a sequence that is similar to plasminogen KIV (*6*); these are directly followed by a sequence which is homologous to the plasminogen protease domain such that rhesus apo(a) lacks sequences corresponding to plasminogen KV (*6*). A single-base substitution in the KIV_{10}-like sequence of the rhesus monkey ($Trp^{70} \rightarrow Arg$, where the first Cys in the kringle is designated as residue 1) (*6*) has been proposed to explain the relative inability of rhesus Lp(a) to bind to lysine−Sepharose (*15*). Because of substitutions in two of the three members of the catalytic triad as well as in the tPA cleavage site, rhesus apo(a) is also proteolytically inactive (*6*). A partial sequence of chimpanzee apo(a) has been reported (*16*); apo(a) from this species contains multiple plasminogen KIV-like domains followed by sequences which are highly homologous to the plasminogen KV and protease domains. A substitution in the KIV_{10} sequence of the chimpanzee ($Asp^{57} \rightarrow Asn$, where the first Cys in the kringle is designated as residue 1) has also been postulated to abolish the lysine binding properties of the corresponding Lp(a). Interestingly, the chimpanzee apo(a) protease-like domain is also catalytically inactive, because of the presence of the same nine-amino acid deletion in human apo(a), as well as the Arg → Ser substitution corresponding to the tPA cleavage site which is also found in human apo(a) (see above).

In this study, we have characterized a partial liver cDNA sequence that corresponds to the carboxyl-terminal end of baboon apo(a); we have also analyzed the sequence encoding the KIV_{10} domain from genomic DNA isolated from 10 unrelated baboons. We have examined the lysine binding properties of baboon Lp(a) and recombinant baboon KIV_{10}. Our findings show that (i) a functional LBS in baboon KIV_{10} is encoded by the majority of alleles in our study, (ii) there is a T → C polymorphism which results in a $Trp^{70} \rightarrow Arg$ substitution within the LBS of some baboon apo(a) KIV_{10} species, and (iii) baboon Lp(a) containing a functional LBS does not bind specifically to lysine−Sepharose (agarose), and binds only weakly to fibrin and plasmin-modified fibrin surfaces. These results have a considerable impact on our understanding of the lysine binding properties of Lp(a) and suggest a key role for apo(a) KV in this process.

EXPERIMENTAL PROCEDURES

Materials. L-Lysine and the lysine analogues ϵ-aminocaproic acid (ϵ-ACA), N^{α}-acetyl-L-lysine, and N^{ϵ}-acetyl-L-lysine were from Sigma-Aldrich Canada Ltd. All DNA-modifying and restriction enzymes were from Promega, New England Biolabs Ltd., and MBI Fermentas. The Sequenase version 2.0 DNA sequencing kit was from Amersham Life Sciences Inc. Lysine−Sepharose (agarose) CL-4B resin was from Amersham Biosciences, and P60 resin was from Bio-Rad Laboratories. Chemiluminescence kits (ECL kits) for the development of Western blots were purchased from Amersham Biosciences. D-Val-Phe-Lys-chloromethyl ketone dihydrochloride was purchased from CalBiochem. Purified human fibrinogen and human plasmin were generously provided by M. Nesheim (Department of Biochemistry, Queen's University). L. Harker (Division of Hematology and Yerkes Primate Research Center, Emory University School of Medicine, Atlanta, GA) kindly provided blood samples from unrelated baboons.

Baboon Liver cDNA Preparation and Analysis. Total RNA was extracted according to the method of Cathala *et al.* (*17*) from a single baboon liver that had been removed and snap-frozen in liquid nitrogen. Briefly, 1 g of liver tissue was homogenized in the presence of guanidine isothiocyanate, and total RNA was isolated using lithium chloride extraction. Poly A^+ mRNA was isolated by affinity chromatography of total RNA over an oligo-dT cellulose column. mRNA (1 μg) was used to generate randomly primed first-strand cDNA using AMV (avian myeloblastosis virus) reverse transcriptase (Promega) in the presence of 0.1 mM random hexanucleotide primers, 1 mM dNTPs, and 1 unit of RNase inhibitor. Oligonucleotide primers (Table 1) were designed according to regions of sequence identity in kringle IV_9 and the protease-like domains between the human apo(a) cDNA sequence (*4*) and the rhesus monkey apo(a) cDNA sequence (*6*). PCR conditions, using 100 ng of cDNA template, were as follows: 30 cycles of denaturation (96 °C for 1 min), primer annealing (48 °C for 1 min), and elongation (72 °C for 1 min) followed by a final elongation (72 °C for 15 min).

PCR products were made blunt-ended using T4 DNA polymerase; fragments were inserted into the *Eco*RV site of pBluescript SK+ for DNA sequence analysis.

Genomic DNA Extraction and Analysis. Whole blood from 10 individual unrelated baboons was obtained; plasma and white blood cells were isolated from 10 mL of blood by centrifugation at 2000*g* in a preparative benchtop centrifuge for 15 min. The plasma fraction was removed and stored at −70 °C in 1 mL aliquots prior to use. The white blood cells were transferred into a fresh tube and suspended in 11 mL of fresh solution I [10 mM Tris-HCl (pH 7.6), 10 mM KCl, and 10 mM $MgCl_2$]. Nonidet P40 was added, and the tubes were mixed by gentle inversion. Nuclei were lysed by the addition of solution II [10 mM Tris-HCl (pH 7.6), 10 mM KCl, 10 mM $MgCl_2$, 0.4 M NaCl, 0.5% SDS, and 2 mM EDTA] followed by gentle mixing for 10 min. Following phenol/chloroform extraction, genomic DNA was ethanol precipitated and resuspended in TE. The purified DNA was kept at 4 °C prior to use.

The oligonucleotide primers used for PCR amplification of the two exons encoding baboon apo(a) KIV_{10} are listed in Table 1. The PCR conditions were the same as described above for amplification of baboon liver cDNA, using 100 ng of genomic DNA as the template; each PCR was independently repeated four times for each animal with each set of primers. All PCR fragments were cloned into pBluescript for DNA sequence analysis.

Lysine−Sepharose Chromatography of Baboon Lp(a). Lp(a) was partially purified from plasma by density ultracentrifugation. Briefly, baboon plasma (200 μL) was added to a centrifuge tube containing 300 μL of 1.2 mg/mL sodium bromide followed by centrifugation at 87 000 rpm for 2 h at 15 °C in a TL-100 ultracentrifuge using a TLA-100.2 rotor. The top 50 μL (i.e., $d < 1.2$ g/L) was removed and microdialyzed against PBS (phosphate-buffered saline) for 20 min using a 0.025 μm filter (Millipore). Dialyzed fractions were brought up to a volume of 400 μL with PBS and subsequently applied to a lysine−Sepharose CL-4B column (bed volume of 0.4 mL) that had been pre-equilibrated with PBS. The column was washed with 5 column volumes of PBS, followed by 5 column volumes of PBS containing 1 M NaCl. Specifically bound proteins were eluted by the addition of 0.2 M ϵ-ACA. Samples of the column flow-through, PBS, and NaCl washes as well as ϵ-ACA elution fractions were collected. Laemmli sample buffer (20 μL) was added to each fraction, and proteins were separated under reducing conditions by SDS−agarose gel electrophoresis on a 1.5% agarose gel as previously described (*18*). Proteins were transferred to an Immobilon-P membrane, and the blots were probed with an anti-apo(a) polyclonal antibody raised in sheep and affinity-purified over an apo(a)−Sepharose column (AB35). The antibody was the kind gift of H. Hoogendorn (Affinity Biologicals Inc., Hamilton, ON). Immunoreactive bands were visualized by chemiluminescence.

Analysis of Binding to a Plasmin-Modified Fibrinogen Surface. Binding assays were performed essentially as previously described (*19*). Microtiter wells were coated with 100 μL of fibrinogen [10 μg/mL in HEPES-buffered saline (HBS)] for 90 min at 37 °C. Wells were washed four times with 150 μL of PBS containing 0.1% (v/v) Tween 20 (PBST) after this and subsequent incubations. The fibrinogen-coated wells were treated with 100 μL of plasmin [30 ng/mL in HBS containing 0.1% (v/v) Tween 20 (HBST)] for 30 min at 37 °C. Wells were washed twice with 150 μL of HBST containing 0.5 M NaCl and 0.2 M ϵ-ACA prior to PBST washes. Residual plasmin was inactivated by incubation with 100 μL of D-Val-Phe-Lys-chloromethyl ketone dihydrochloride per well (1 μM in HBST) for 40 min at room temperature. Following PBST washes, nonspecific binding sites were blocked overnight at 4 °C by treatment with 150 μL of PBS containing 2% (w/v) BSA. Into wells containing immobilized plasmin-modified fibrinogen were placed increasing concentrations of total lipoprotein fractions from either a normolipidemic human volunteer or baboon 10, each adjusted to contain Lp(a) at a concentration of 10 mg/dL; binding was allowed to proceed for 2 h at room temperature. After washing, the bound protein was detected by incubation with the apo(a) monoclonal antibody 2G7 (*20*) for 1 h at room temperature, followed by incubation for 1 h with a secondary rabbit anti-mouse antibody conjugated to horseradish peroxidase. Binding of the secondary antibody was detected by addition of 100 μL of development buffer per well containing 0.42 mg/mL *o*-phenylenediamine dihydrochloride (OPD). Color development was arrested by the addition of 50 μL of 2 M H_2SO_4 per well, and the absorbance at 490 nm (minus the background absorbance at 650 nm) was determined using a Titertek plate reader. Assays were performed in duplicate and the resulting values averaged. Specific binding was assessed by subtracting the signal obtained for wells not coated with fibrinogen. In an additional set of experiments, binding assays were performed in the presence of 0.2 M ϵ-ACA.

Expression of Baboon Apo(a) KIV_{10} in Escherichia coli. Sequences corresponding to baboon apo(a) KIV_{10} containing a Trp at position 70 were amplified by PCR from the baboon apo(a) cDNA obtained above; primers used for the PCR amplification are shown in Table 1. The fragment encoding baboon KIV_{10} was cloned into the *Nde*I site of pET16b (Novagen) and transformed into *E. coli* strain BL21(DE3) (Novagen). A mutant version of the kringle containing a Gln → Arg substitution at position 32 was constructed as follows: overlapping PCR products were produced using the flanking primers and mutagenic primers shown in Table 1. The PCR products were fused using the *Bgl*I site in the overlapping region, and the resultant fragment encoding the mutant kringle was cloned into the *Nde*I site of pET16b.

The baboon KIV_{10} constructs were expressed in *E. coli* and purified as previously described for human apo(a) KIV_{10} (*19*). Briefly, the recombinant protein was released from inclusion bodies by denaturation with guanidine, followed by refolding in the presence of oxidized and reduced glutathione (1.25 mM each) at 4 °C for 2 days prior to passage over a Ni^{2+}−Sepharose column (His-Bind resin, Novagen). Fractions from the column containing baboon KIV_{10} were pooled and dialyzed against 20 mM Tris-HCl (pH 7.9) for 2 days prior to chromatography over a gel filtration column (300 mL Bio-Gel P60 column, Bio-Rad Laboratories). Kringle-containing fractions were pooled and dialyzed against 20 mM Tris-HCl (pH 7.9) prior to refolding as described above. The purity of the baboon KIV_{10} preparation was assessed by SDS−PAGE using a 15% polyacrylamide gel followed by silver staining. The purified protein was aliquoted, flash-frozen in liquid nitrogen, and stored at

−70 °C prior to use.

Lysine−Sepharose binding properties of purified baboon KIV_{10} (2 μg) were determined using lysine affinity chromatography on a 0.5 mL column as described above for baboon Lp(a); column fractions corresponding to the flow-through, washes, and ϵ-ACA elution were analyzed by SDS−PAGE and Western blotting using the Ab35 antibody.

Equilibrium Binding Analyses by Measurement of Intrinsic Fluorescence. Wild-type or mutant baboon KIV_{10} [4 μM in 20 mM Tris-HCl (pH 7.9) and 0.1% (v/v) Tween 20] was placed in a quartz cuvette in a Perkin-Elmer LS50B luminescence spectrometer. The intrinsic fluorescence was measured at an excitation wavelength of 280 nm (slit width of 2.5 nm) and an emission wavelength of 340 nm (slit width of 5.0 nm). A cutoff filter at 290 nm was placed in the emission beam. The kringle was titrated with L-lysine or lysine analogues; the titrant solutions [in 20 mM Tris-HCl (pH 7.9) and 0.1% (v/v) Tween 20] contained 4 μM KIV_{10} protein to correct for dilution effects. The titrations were continued until no further change in the fluorescence signal was observed. The fluorescence data were fit to the following equation:

$$I = I_0 + \Delta I_{max}[\text{ligand}]/K_D + [\text{ligand}] \qquad (1)$$

where I is the intensity, I_0 is the initial intensity, ΔI_{max} is the maximum change in relative fluorescence intensity, and K_D is the equilibrium binding constant.

Molecular Modeling of Baboon KIV_{10}. The structure of baboon KIV_{10} was modeled on the basis of the coordinates of human KIV_{10} containing bound ϵ-ACA (Protein Data Bank entry 3KIV) (*21*). Modeling, structural analyses, and energy minimization were carried out using Sybyl, version 5.3 (Tripos Inc., St. Louis, MO). Diagrams were generated using MOLSCRIPT (*22*).

RESULTS

Partial Sequence of Baboon Apo(a) cDNA. Oligonucleotide primers were designed corresponding to regions of identity between the published rhesus monkey apo(a) cDNA and human apo(a) cDNA and were used to amplify a portion of the baboon apo(a) cDNA from a liver cDNA library prepared from mRNA from a single baboon. The amplified region contained 864 bp of open reading frame corresponding to baboon apo(a) KIV_9 through the first two-thirds of the protease domain. The sequence is 92% identical with that of rhesus apo(a) and 85% identical with that of human apo(a). No sequence was identified corresponding to apo(a) KV (Figure 1). The presence of a free Cys in baboon KIV_9 was noted, as well as the substitution of at least one of the three members of the catalytic triad in the protease-like region (Figure 1). The tPA cleavage site in the deduced baboon protease-like domain is present (Arg at position 19 of the protease-like domain), unlike rhesus and human apo(a) which have a Ser residue in this position. The sequenced region encompasses two of the three residues corresponding to the His-Asp-Ser catalytic triad of plasminogen. Of these two, the His is replaced with an Arg in baboon apo(a) (Figure 1).

Of particular interest is the sequence encoding the lysine-binding pocket of baboon apo(a) KIV_{10}. In contrast to rhesus monkey KIV_{10}, the corresponding sequence in the baboon contains a predicted amino acid sequence that corresponds

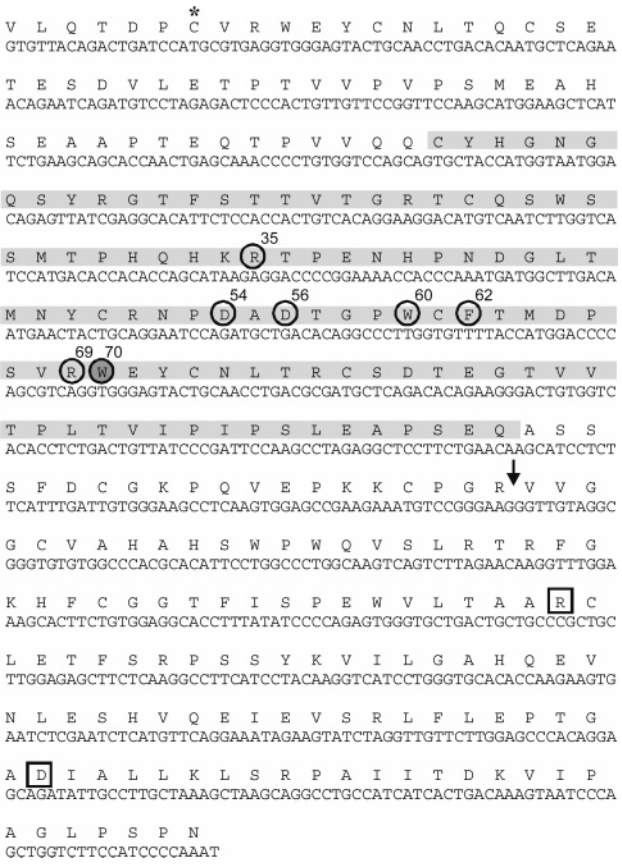

FIGURE 1: Partial nucleotide and predicted amino acid sequence of baboon apo(a) from KIV_9 (residue 61, where residue 1 is the first Cys in the kringle) through the first approximately two-thirds of the protease region. The seventh, unpaired, Cys in KIV_9 is denoted with an asterisk. The KIV_{10} sequence is shaded. Amino acids comprising the lysine-binding site in KIV_{10} are circled and numbered, with the shaded circle indicating the site of the Trp^{70} → Arg polymorphism. The arrow indicates the site analogous to the tPA cleavage site in plasminogen, and the boxes represent two of the amino acids corresponding to the catalytic triad in plasminogen.

to the consensus LBS present in human apo(a) KIV_{10} (Figure 1). While rhesus monkey apo(a) KIV_{10} has been reported to contain an Arg at amino acid position 70 (where position 1 refers to the first Cys in the kringle), the baboon cDNA encoded a sequence with a Trp at this position that is believed to be necessary for functional lysine binding (*21*).

Identification of a Trp → Arg Polymorphism at Amino Acid Position 70 in Baboon Apo(a) KIV_{10}. A small population study was undertaken in which 10 unrelated baboons were assessed for the identity of the amino acid at position 70 of the KIV_{10} sequence. At the genomic level, the kringle-encoding sequences are generally arranged such that two exons encode each kringle (*23*). One of the seven amino acids implicated in lysine binding is encoded by exon 1 (Arg^{35}), and the remaining six (Asp^{55}, Asp^{57}, Trp^{62}, Phe^{64}, Trp^{70}, and Arg^{71}) are encoded by exon 2. PCR analysis of each baboon apo(a) KIV_{10} exon (strategy shown in Figure 2A) was carried out a minimum of four times to be certain that both alleles were represented in the final sequence analysis. Exon 1 sequences were identical for all baboons, and Arg^{35} encoded in this region was completely conserved. In the second exon, seven of ten baboons were homozygous for the consensus lysine-binding amino acids (Trp^{70} as well as the other five amino acids comprising the LBS was present on both alleles);

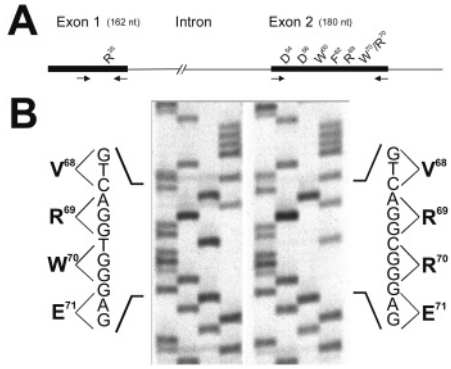

FIGURE 2: Nucleotide sequence analysis of KIV_{10} in 10 unrelated baboons. (A) Strategy for PCR analysis of the apo(a) KIV_{10} sequence using baboon genomic DNA as the template. Coding regions encompassing LBS residues, as indicated, were amplified using the indicated primers. Sequences of primers are given in Table 1. (B) Representative sequencing reactions of PCR products from genomic DNA demonstrating the T → C nucleotide substitution present in three baboon alleles which results in a Trp^{70} → Arg substitution in baboon apo(a) KIV_{10}.

three baboons were heterozygous for a Trp → Arg substitution at position 70, resulting from a T → C nucleotide polymorphism (Figure 2B). No homozygous potential lysine binding-deficient genotypes (i.e., Arg^{70} encoded by both alleles) were observed.

Lysine and Fibrin Binding Properties of Baboon Lp(a). Lp(a) was prepared from each of the 10 baboons using density ultracentrifugation and was subsequently chromatographed over a lysine−Sepharose column to assess its ability to bind to lysine. No Lp(a) species from any of the baboons showed detectable affinity for lysine−Sepharose under the conditions we used. This finding is exemplified by the results for two of the baboons (Figure 3), both of which are heterozygous for the Trp^{70} → Arg polymorphism and possess two electrophoretically observable Lp(a) isoforms. Neither Lp(a) isoform from either animal bound specifically to lysine−Sepharose [i.e., no Lp(a) was eluted by the addition of ϵ-ACA] (Figure 3).

To assess the ability of baboon Lp(a) to bind to partially degraded fibrin, we immobilized fibrinogen in microtiter wells, treated with plasmin to expose carboxyl-terminal lysines on the surface, and incubated the wells with dilutions of either human or baboon plasma lipoprotein fractions. Binding to the plasmin-modified surface was assessed by an ELISA using an anti-human apo(a) monoclonal antibody that reacts comparably with baboon and human Lp(a). Saturable binding to the plasmin-modified surface was observed using the human Lp(a)-containing fraction, while a very low level of binding was seen using the baboon Lp(a)-containing fraction (Figure 4). For both human and baboon Lp(a), parallel incubations containing 0.2 M ϵ-ACA completely abolished the binding.

Expression, Purification, and Characterization of Baboon KIV_{10}. To gain further insight into its lysine binding properties, baboon apo(a) KIV_{10} was expressed in *E. coli*. Similar to what has been previously reported for the expression of human KIV_{10} (*19*), the majority of the recombinant protein was present in inclusion bodies. The protein was purified from the bacterial pellet by denaturation and refolding, followed by chromatography over a nickel−Sepharose column; size exclusion chromatography was

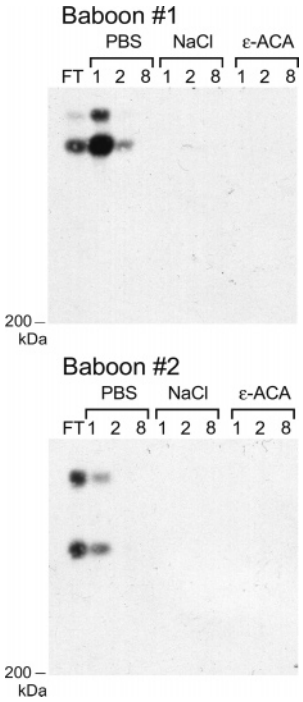

FIGURE 3: Binding of baboon Lp(a) to lysine−Sepharose. Dialyzed total lipoprotein fractions isolated from the plasma of two different baboons were loaded onto lysine−Sepharose columns. Both baboons are heterozygous for the Trp → Arg polymorphism at position 70 in KIV_{10} and possess two observable apo(a) isoforms. Columns were washed with 10 column volumes of PBS, followed by PBS containing 0.5 M NaCl. Specifically bound Lp(a) was eluted in a PBS/NaCl mixture containing 0.2 M ϵ-ACA. Lp(a) in the indicated column fractions was detected by Western blot analysis using an anti-human apo(a) polyclonal antibody.

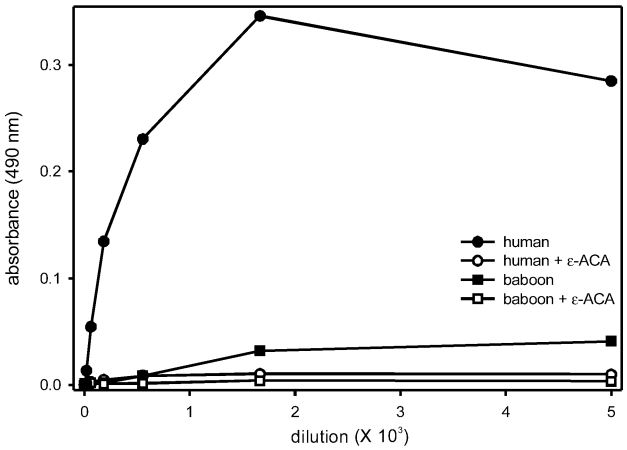

FIGURE 4: Binding of baboon and human Lp(a) to a plasmin-modified fibrinogen surface. Human fibrinogen was immobilized in microtiter wells and was subsequently treated with plasmin. The wells were then incubated with various dilutions of total lipoprotein fractions from a normolipidemic human volunteer (●) or a baboon homozygous for the allele encoding the Trp at position 70 of KIV_{10} (■). Parallel incubations of lipoprotein fractions were carried out in the presence of 0.2 M ϵ-ACA [(○) human and (□) baboon]. In all cases, bound Lp(a) was detected by an ELISA using an anti-human monoclonal antibody and a horseradish peroxidase-conjugated secondary antibody.

subsequently used to remove high-molecular weight species from the partially purified protein. Following extensive refolding, the integrity of the purified protein was assessed by SDS−PAGE using a 15% polyacrylamide gel followed by silver staining. Similar to what we have reported for

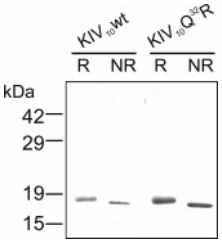

FIGURE 5: Expression of baboon apo(a) KIV_{10} in *E. coli*. Sequences encoding the wild-type baboon KIV_{10} (KIV_{10}wt) or a mutant version containing a Gln → Arg substitution at position 32 ($KIV_{10}Q^{32}R$) were cloned into pET16b for expression in *E. coli*. The recombinant proteins were purified by affinity chromatography over Ni^{2+}−Sepharose, followed by gel filtration chromatography. The purified proteins (250 ng of each) were analyzed by SDS−PAGE under reducing (R) or nonreducing (NR) conditions followed by silver staining. The positions of molecular mass standards are shown at the left of the gel.

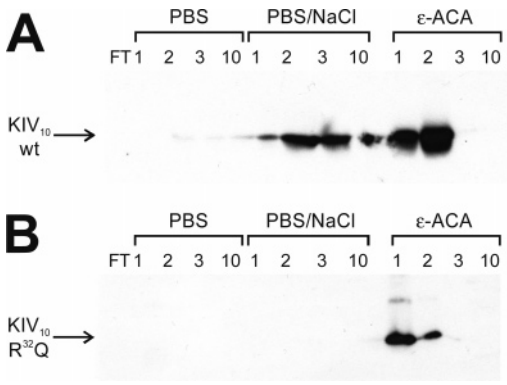

FIGURE 6: Binding of purified baboon KIV_{10} to lysine−Sepharose. Purified baboon KIV_{10} was loaded onto a lysine−Sepharose column. Columns were washed with 10 column volumes of PBS, followed by PBS containing 0.5 M NaCl. Specifically bound KIV_{10} was eluted using a PBS/NaCl mixture containing 0.2 M ϵ-ACA. Kringle IV type 10 in the indicated column fractions was detected by Western blot analysis using an anti-human apo(a) polyclonal antibody. Data for the wild-type baboon kringle are shown in panel A, while data for the Gln^{32} → Arg mutant are shown in panel B.

human KIV_{10}, the baboon KIV_{10} migrated as a single band under both nonreducing and reducing conditions, and had an apparent molecular mass under reducing conditions of ~17 kDa (Figure 5).

We initially investigated the lysine binding capabilities of baboon KIV_{10} by studying its ability to bind to lysine−Sepharose. Purified protein was loaded onto a lysine−Sepharose column in PBS, and unbound protein was removed by washing with PBS. Weakly bound protein was removed by washing with PBS containing 0.5 M NaCl. Specifically bound protein was eluted using 0.2 M ϵ-ACA. The presence of protein in the respective fractions was visualized by SDS−PAGE followed by Western blotting (Figure 6). Approximately half of the baboon KIV_{10} was removed from the column in the NaCl wash, while the remainder was eluted by the addition of ϵ-ACA. This is in contrast to what we have reported for the human KIV_{10} which could only be eluted from a lysine−Sepharose column by the addition of ϵ-ACA.

To characterize further the affinity and specificity of the LBS of baboon KIV_{10}, its equilibrium binding to lysine and lysine analogues was studied by measurements of intrinsic fluorescence. Specific binding of each ligand was associated with a saturable increase in the intrinsic fluorescence of the kringle. The respective dissociation constants (K_D) were calculated by subjecting the fluorescence data to nonlinear regression analysis using eq 1 (see Experimental Procedures). Titration curves were generated for the binding of baboon KIV_{10} to the following ligands: L-lysine, ϵ-ACA, N^{α}-acetyl-L-lysine, and N^{ϵ}-acetyl-L-lysine (Table 2). In all cases, the binding affinities of the baboon KIV_{10} species for the ligands were comparable to that which we have previously reported for human KIV_{10} (i.e., a <2-fold difference) (see Table 2). Representative titration data for baboon KIV_{10} binding to L-lysine and ϵ-ACA are shown in Figure 7.

Table 2: Comparison of the Affinities of Baboon and Human Apo(a) KIV_{10} for Lysine and Lysine Analogues[a]

	K_D (μM)		
ligand	baboon KIV_{10}wt	baboon $KIV_{10}Q^{32}R$	human apo(a) KIV_{10}
L-lysine	85.9	115	172
ϵ-ACA	22.7	54.4	33.2
N^{α}-acetyl-L-lysine	27.0	38.1	51.7
N^{ϵ}-acetyl-L-lysine	NC[b]	NC[b]	NC[b]

[a] Purified baboon apo(a) KIV_{10} was titrated with the indicated amino acids; the intrinsic fluorescence of the kringle (λ_{ex} = 280 nm; λ_{em} = 340 nm) was measured after each addition of ligand. Equilibrium dissociation constants (K_D) were obtained by nonlinear regression of the fluorescence data to eq 1. The stoichiometries of the interactions were found to be approximately 1:1 for each ligand. Data for human apo(a) KIV_{10} are taken from Rahman *et al.* (*34*) and are included for the purposes of comparison. [b] No change in fluorescence observed.

It is curious that baboon KIV_{10}, unlike the human kringle, can be partially eluted from the lysine−Sepharose column by 0.5 M NaCl despite the very similar affinity for lysine in solution of the two kringles. Molecular modeling of baboon KIV_{10} reveals that one of the five amino acid substitutions in this kringle, relative to the human sequence, may account for these observations (see Figure 8). In the human kringle, there is an Arg at position 32 that repels the Arg at position 69, thus favoring the location of the side chain of the latter in the lysine-binding pocket where it contacts the carboxylate group of the ligand. There is a Gln at position 32 in the baboon kringle that, under conditions of high salt as observed during the 0.5 M NaCl wash of the lysine−Sepharose column, may form a hydrogen bond with the side chain of Arg^{69}, thus swinging it out of the lysine-binding pocket. Under the low-salt conditions in which our lysine analogue binding assays were performed (Table 2), this hydrogen bond would be less favorable due to increased solvation of these polar side chains.

To directly assess this possibility, we expressed a mutant variant of baboon KIV_{10} in which Gln^{32} has been substituted with Arg as in the human kringle. Like the wild-type baboon kringle, the mutant variant was very similar in its binding affinity for lysine and lysine analogues in solution (Table 2 and Figure 7). However, like the human KIV_{10}, the mutant baboon kringle could not be eluted from lysine−Sepharose in the presence of 0.5 M NaCl (Figure 6B). We consistently noted that the maximal change in fluorescence upon ligand binding was greater for the wild type than for the mutant kringle (Figure 7 and data not shown). This may also be explained by our molecular model (Figure 8; see above): the location of the Arg^{69} side chain outside of the lysine-binding pocket in the wild-type baboon kringle suggests that a greater conformational change may be required to accom-

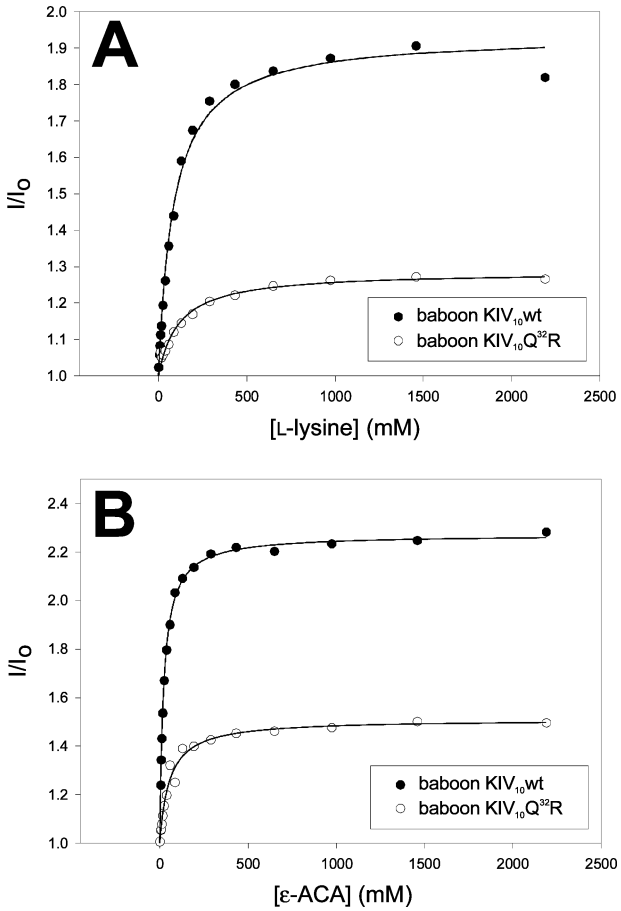

FIGURE 7: Analysis of binding of baboon KIV_{10} to lysine and lysine analogues as measured by intrinsic fluorescence. Purified baboon KIV_{10} [either the wild type (wt) or $Q^{32}R$ variant] was titrated with L-lysine (A) or ϵ-ACA (B) and the intrinsic fluorescence of the kringle measured ($\lambda_{ex} = 280$ nm; $\lambda_{em} = 340$ nm). The circles (filled for KIV_{10}wt and empty for $KIV_{10}Q^{32}R$) represent the fluorescence intensity, expressed relative to that observed prior to addition of ligand (I/I_o). The lines are regression lines based on nonlinear regression of the fluorescence data to eq 1.

modate a ligand in the pocket, thus accounting for the greater change in intrinsic fluorescence.

DISCUSSION

In addition to humans, Lp(a) appears to be present only in Old World monkeys (e.g., chimpanzees, rhesus monkeys, and baboons) and the hedgehog. While this peculiar species distribution has hampered the development of relevant animal models for Lp(a), it is useful for studying the properties of Lp(a) from other species to provide clues about the role of this lipoprotein in atherothrombotic disease. In this study, analysis of baboon liver apo(a) cDNA sequence in the region encoding KIV_9 through the protease-like domain reveals a high degree of sequence similarity with the published rhesus monkey apo(a) cDNA sequence; amino acids of known function are specifically conserved such as the unpaired Cys in KIV_9, as are substitutions in amino acids comprising the catalytic triad in the protease-like domain that would render the respective molecules catalytically inactive (*6*). In contrast to the reported rhesus monkey apo(a) sequence which contains a $Trp^{70} \rightarrow$ Arg substitution in KIV_{10} (*6*), the baboon cDNA sequence corresponding to apo(a) KIV_{10} encodes a Trp at this position. On the basis of our understanding of

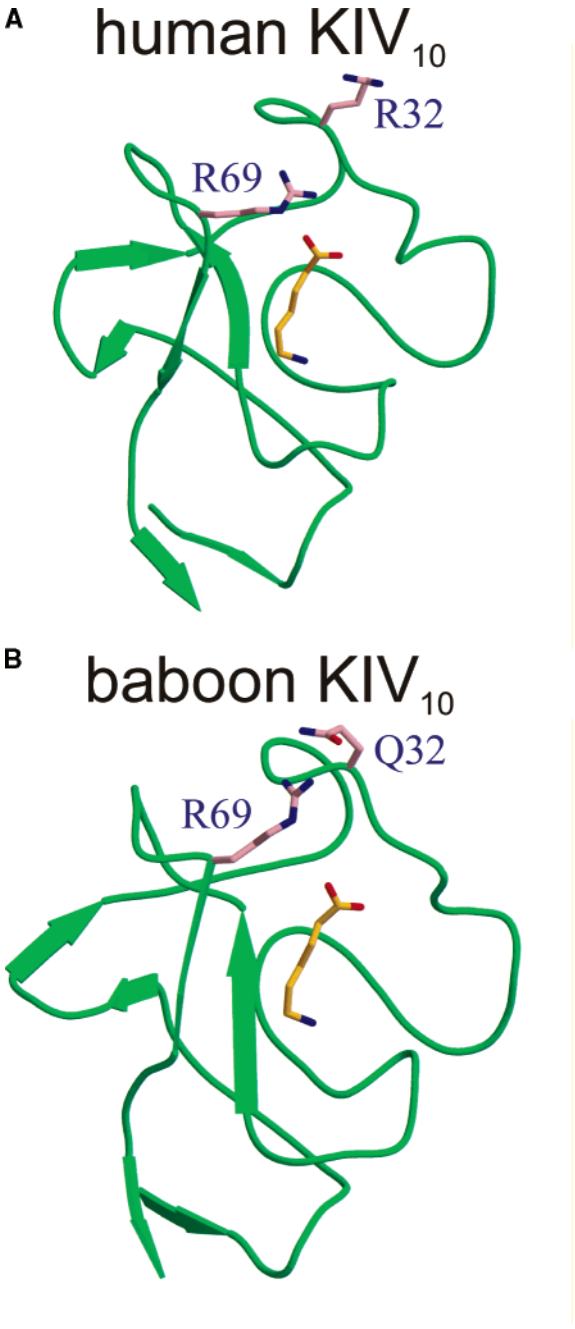

FIGURE 8: Model of baboon KIV_{10} structure. (A) Ribbon representation of the human apo(a) KIV_{10} crystal structure (green) together with the bound lysine analogue ϵ-ACA (yellow). In this structure, Arg^{69} rotates downward to interact with the carboxylate group of the lysine analogue since Arg^{69} is repelled by Arg^{32}. (B) Ribbon representation of a model of the baboon KIV_{10} structure (green) together with the bound lysine analogue ϵ-ACA (yellow) based on the coordinates of the ϵ-ACA-bound human KIV_{10} structure (*21*). In this structure, repulsion of Arg^{69} is relieved by the presence of Gln^{32} and Gln^{32} may in fact hydrogen bond with Arg^{69}, thus interfering with the interaction of Arg^{69} with the ligand.

the structure of the lysine-binding pocket (*21*), this should render baboon Lp(a) more similar to human Lp(a) than to rhesus Lp(a) in terms of its predicted lysine binding ability. The $Trp^{70} \rightarrow$ Arg substitution in rhesus monkey apo(a) KIV_{10} has been correlated with the inability of rhesus Lp(a) to bind to lysine (*15*), although this conclusion was based on the apo(a) sequence of only one monkey (*6*). Indeed, we have evidence to suggest that a polymorphism occurs in rhesus

KIV_{10} similar to that which we have reported for baboon apo(a), although the majority of rhesus alleles in our analysis encoded an Arg at position 70 (A. R. B. and M. L. K., unpublished data). In humans, an analogous $Trp^{70} \rightarrow$ Arg mutation has been reported that only occurs in approximately 2% of the human population; Lp(a) from individuals whose apo(a) contains this mutation lacks the ability to bind lysine−Sepharose (*24*).

Subsequent analysis of the apo(a) KIV_{10} sequence from 10 unrelated baboons led us to conclude that as is the case in humans, there is a Trp → Arg polymorphism at amino acid position 70 in baboon KIV_{10}. Of the 10 baboons that were analyzed, three were heterozygous for the $Trp^{70} \rightarrow$ Arg phenotype and the remaining seven were homozygous for the Trp at this position. No baboon apo(a) species homozygous for Arg at position 70 were identified in our study. With the exception of the Trp → Arg polymorphism, all other amino acids critical for lysine binding are conserved in baboon KIV_{10}.

A partial cDNA sequence from a single chimpanzee has revealed a nucleotide substitution in apo(a) KIV_{10} that results in an $Asp^{54} \rightarrow$ Asn substitution within the LBS of this kringle (*16*). This substitution appears to correlate with an impairment of the lysine binding ability of the corresponding chimpanzee Lp(a). It is not known whether the allele encoding Asn at position 54 in KIV_{10} is present in all chimpanzees, or whether it represents a polymorphism in this species similar to the Trp → Arg polymorphism present in rhesus monkey, baboon, and human apo(a). The remainder of the residues required for the lysine-binding pocket in the chimpanzee apo(a) KIV_{10} appear to be conserved, including Trp^{70}.

In the comparison of Lp(a) from the chimpanzee and the rhesus monkey, it has been suggested that the inability of Lp(a) from these species to bind to lysine is due to substitutions of critical amino acids in the lysine-binding site in apo(a) KIV_{10}. In our study, we similarly observed that baboon Lp(a) is incapable of binding specifically to lysine−Sepharose, yet no obvious perturbations of the LBS were present in the majority of the baboon apo(a) KIV_{10} sequences that were examined. Moreover, computer modeling of KIV_{10} from baboon (containing Trp at position 70) and human apo(a) revealed no significant structural differences in the LBS (Figure 8 and data not shown). Our data clearly demonstrate that baboon apo(a) KIV_{10} possesses a functional LBS that has an affinity for lysine and lysine analogues that is comparable to that of human apo(a) (Figures 6 and 7 and Table 2). We therefore conclude that this strong LBS in baboon apo(a) KIV_{10} is somehow masked in the context of the baboon Lp(a) particle.

There is a large variation in Lp(a) lysine binding ability within human populations (*25*), which is not related to the isoform size of the apo(a) and cannot be explained by the presence of mutations in apo(a) KIV_{10} (*24*). Perhaps not unexpectedly, non-lysine-binding Lp(a) species have been shown to be incapable of binding to fibrinogen, which involves lysine-dependent binding interactions (*26*). A more recent report from our group demonstrates that the fraction of plasma-derived Lp(a) that can bind lysine can be enhanced by improved purification of Lp(a) (*27*). On the basis of these data, we proposed that plasma constituents may bind to Lp(a) in such a way as to impair its ability to bind to lysine; in other words, the donor-dependent lysine binding heterogeneity of Lp(a) is not necessarily an intrinsic property of the Lp(a) itself. In our study, we hypothesized that a similar effect may have led to the absence of a correlation between the integrity of the baboon KIV_{10} LBS and the ability of the corresponding baboon plasma Lp(a) to bind to lysine−Sepharose. To address this hypothesis, the baboon plasma lipoprotein fraction containing Lp(a) was applied to an anti-apo(a) affinity column; this partially purified Lp(a) failed to exhibit any lysine binding (A. R. B. and M. L. K., unpublished data). On this basis, it appears that the nature of the impairment of baboon Lp(a) binding to lysine−Sepharose is unlikely to be explained by the presence of plasma contaminants masking the LBS of baboon apo(a) KIV_{10}.

We recently made the intriguing observation that the presence of KV is required for the ability of human recombinant apo(a) to inhibit plasminogen activation in the presence of tPA and fibrin (*28*). Our kinetic analyses under these conditions are consistent with a model in which apo(a) binds to the ternary plasminogen−tPA−fibrin catalytic complex, thus reducing its turnover number. Although fibrin binding by apo(a) is likely a key component of this model, mutation of the LBS in human KIV_{10} only partially weakens the ability of apo(a) to inhibit plasminogen activation. Thus, KV appears to allow the apo(a) to adopt a particular inhibition competent conformation. By analogy, baboon apo(a) that lacks KV may adopt a conformation that masks the LBS in KIV_{10}. This notion is supported by preliminary analysis of the lysine binding properties of Lp(a) formed using human recombinant apo(a) lacking a KV domain; this species exhibits significantly weakened lysine−Sepharose binding compared to Lp(a) containing wild-type recombinant apo(a) (A. R. B. and M. L. K., unpublished data). Therefore, although the binding of baboon apo(a) KIV_{10} and the binding of human apo(a) KIV_{10} are comparable, it appears that the inability of KIV_{10} to contribute to the lysine binding capability of baboon Lp(a) may reflect the structural inaccessibility of this kringle due to the lack of the KV sequence. This inaccessibility could be related to steric hindrance of the KIV_{10} or binding of an intraparticle ligand by KIV_{10}. We predict that rhesus Lp(a) containing apo(a) in which there is a functional LBS in KIV_{10} (i.e., a Trp at position 70) would be similarly unable to bind to lysine because of the absence of apo(a) KV in this species.

There are a few alternative explanations for the lack of lysine−Sepharose binding of baboon Lp(a) besides our hypothesis of altered apo(a) structure. Baboon KIV_{10} may be binding to an intraparticle ligand and thus is unavailable for binding to lysine−Sepharose [although the binding to the intraparticle ligand again may arise from the particular conformation of baboon apo(a) that results from the lack of KV]. We cannot exclude the possibility that KV in human apo(a) contains a weak LBS that acts cooperatively with the strong LBS in KIV_{10} to mediate lysine−Sepharose binding; the lack of KV in baboon apo(a) and thus the absence of this kind of multivalent binding may account for the very weak lysine binding ability of baboon Lp(a). Plasminogen kringle 5 has been demonstrated to possess an LBS that is weaker than the one in plasminogen kringle 4 (*29*−*31*). Both plasminogen kringle 5 and human apo(a) KV contain substitutions at the amino acid positions corresponding to

Arg^{35}, Phe^{62}, Arg^{69}, and Trp^{70} (*4*, *32*). An additional explanation for the weak lysine binding ability of baboon Lp(a) could be differences in glycosylation of baboon versus human apo(a). There is a potential N-linked site at Asn^{74} of baboon KIV_{10} (Figure 1), but this site is also present in human KIV_{10} (*4*). At this time, we cannot rule out differences in O-linked glycosylation, although the O-linked sites are principally located in the flexible interkringle regions (*33*).

What does comparative evolution suggest about the function of Lp(a)? It appears that all species characterized to date contain an inactive protease domain; interestingly, the mechanisms that result in inactivation are variable and range from mutations in the catalytic triad and/or activation site through deletions altering the conformation of the active site (*10*) to the complete lack of this domain as was reported in the hedgehog (*14*). Additionally, it appears that for Old World monkeys, the lack of functional lysine binding has also been achieved through different mechanisms, including the lack of a KV domain in rhesus and baboon Lp(a) as well as mutation of the KIV_{10} LBS in the presence of a KV domain as has been reported for the chimpanzee (*16*). Clearly, further studies are required to understand the significance of these observations, especially in light of the fact that human Lp(a) is a risk factor for atherothrombotic disease and retains the ability to bind biological substrates through lysine-dependent interactions.

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