Rhesus Monkey Lipoprotein(a) Binds to Lysine Sepharose and U937 Monocytoid Cells Less Efficiently than Human Lipoprotein(a)

Evidence for the Dominant Role of Kringle 4_{37}

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Abstract

Rhesus lipoprotein(a) (Lp[a]) binds less efficiently than human Lp(a) to lysine-Sepharose and to cultured U937 cells. Studies using elastase-derived plasminogen fragments indicated that neither kringle 5 nor the protease domain of Lp(a) are required in these interactions pointing at an involvement of the K4 region. Comparative structural analyses of both the human and simian apo(a) K4 domain, together with molecular modeling studies, supported the conclusion that $K4_{37}$ plays a dominant role in the lysine binding function of apo(a) and that the presence of arginine 72 rather than tryptophan in this kringle can account for the functional deficiency observed with rhesus Lp(a). These in vitro results suggest that rhesus Lp(a) may be less thrombogenic than human Lp(a). (*J. Clin. Invest.* 1993. 91:283–291.) Key words: rhesus monkey lipoprotein(a) • lysine-Sepharose binding of lipoprotein(a) • K4 domains of apolipoprotein(a) • mutation of $K4_{37}$ of apolipoprotein(a) • thrombogenicity of lipoprotein(a)

Introduction

In vitro and ex vivo studies have shown that lipoprotein(a) (Lp[a])[1] purified from human plasma has the capacity to interfere with the processes leading to plasmin generation including competition for the binding of plasminogen to cells. These and other recently reviewed studies (1–4) have suggested that Lp(a) may have a thrombogenic action. Plasminogen has five kringles. These disulfide-looped structures of ~90 amino acids bind lysine with varying affinities. Based upon x ray crystallography (5), nuclear magnetic resonance (6), group specific modifications (7–10) and mutational analyses, seven specific amino acids have been shown to form the lysine binding pocket of plasminogen fourth kringle (K4). apo(a), the unique apolipoprotein of Lp(a), has numerous kringles which are similar to the K4 of plasminogen (82–87% amino acid sequence identity) and a single homologue of the fifth plasminogen kringle (11). As human Lp(a) binds to lysine-Sepharose and its binding to cells is, in part, inhibited by lysine, one or more of its K4 is a functional lysine binding site. Of the K4 repeats of human Lp(a), $K4_{37}$ is the most likely to exhibit lysine binding function. It has all six of the seven amino acids, and a very conservative Lys for Arg substitution at the seventh position. Several other K4 have five of these residues with conversative substitutions at the other positions (3) and could have lysine-binding activity. Indeed, all identified effects of Lp(a) on the plasminogen system can be attributed to the lysine-binding functions of the apo(a) kringles (1–4).

Previous work from this laboratory has shown that Lp(a) is a major component of rhesus monkey plasma and like its human counterpart is heterogenous in size and density (12). Recent studies have also demonstrated that the high degree of apo(a) size polymorphism is a function of the number of K4 tandem repeats (13). However, there are important structural differences between rhesus and human apo(a). These differences include: (*a*) the substitution at position 72 in the $K4_{37}$ homologue of rhesus apo(a) with an Arg instead of a Trp, which is one of the seven key amino acids forming the lysine binding site in plasminogen K4; (*b*) the absence of a K5 homologue in rhesus apo(a). Thus, potential functional differences might exist between the Lp(a) from the two species. To test this hypothesis we have compared purified preparations of human and rhesus Lp(a) for their ability to bind to lysine-Sepharose and to plasminogen receptors on U-937 monocytoid cells as a model cell type. The results show that rhesus Lp(a) has a significantly decreased capacity to bind in both systems. Based upon molecular modeling, it is suggested that a single amino acid substitution of position 72 in the lysine binding region of the rhesus $K4_{37}$ may account for these functional differences.

Methods

Of the 13 rhesus monkeys studied, 10 were from a pedigree having members with either normo- or hypercholesterolemia depending on the absence or presence of a genetically determined LDL receptor deficiency (14–17). Half of the 10 animals were LDL receptor deficient. With the exception of two animals, all had high plasma Lp(a) levels varying between 7 and 60 mg/dl (in terms of protein), a concentration which appeared unrelated to LDL receptor deficiency as we reported previously (16). Five animals exhibited a single band apo(a) phenotype (1.13/1.13) on reducing SDS-polyacrylamide gels; the other five had a two-band apo(a) phenotype. A summary of these findings is given in Table I. Besides the animals of the pedigree listed in Table I, four unrelated female normocholesterolemic rhesus monkeys (two males and two females) with normal LDL receptor activity were studied. All of these animals were housed at the Southwest Foundation for Biomedical Research in San Antonio, TX. The studies were approved

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1. *Abbreviations used in this paper:* EACA, ϵ-aminocaproic acid; K4, fourth kringle; Lp(a), lipoprotein(a); PK4, plasminogen K4.

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Table I. Source of Plasma from Rhesus Monkey Pedigree Used in Lp(a) Isolation

Animal	Sex	LDL-receptor deficiency	Total cholesterol	LDL-cholesterol	HDL-cholesterol	Triglycerides	Lp(a)	Apo(a) phenotype*
				mg/dl				
FH pedigree								
1000	M	Y	272	186	79	38	60	1.13/1.13
7069	F	Y	228	135	78	75	21	0.64/1.13
7436	F		160	74	70	82	30	0.8/1.13
7643	M	Y	241	154	78	45	50	1.13/1.13
7700	M		112	55	45	58	12	1.13/1.13
8204	M		112	47	58	36	49	1.13/1.13
8256	M	Y	205	113	82	49	19	1.13/1.13
8796	F	Y	311	206	90	76	23	1.80/1.13
8806	M		127	56	65	32	22	0.8/1.13
Unrelated animals‡								
6230	M		142	48	85	45	7	1.0/1.41
6232	M		127	72	45	51	14	1.41/1.41
7890	F		128	67	52	46	2	0.8/0.8
7893	F		179	99	74	28	7	0.80/1.13

* The phenotypes are described as mobilities of each isoform on SDS-PAGE relative to the B isoform = 100. ‡ Used as breeders.

by the Institutional Review Board (monkey protocol No. 53011; human protocol No. 5833).

At the time of the study the monkeys were fed a Purina chow cholesterol-free diet and were fasted overnight before collecting 20 ml of venous blood in tubes containing 0.01% EDTA. The plasma was separated from cells at 3,000 rpm for 15 min in a refrigerated centrifuge and mailed by overnight delivery in wet ice via Federal Express from the Southwest Foundation for Biomedical Research in San Antonio to Chicago where the plasma samples were individually processed for the isolation of Lp(a). The isolation procedure was an adaptation of that previously described for human Lp(a) (18) except that the lysine-Sepharose step was omitted upon establishing its lack of usefulness in preliminary studies. In brief, the plasma was spun at 48,000 rpm in a 50.2 Ti rotor at 10°C for 20 h at *d* 1.045 g/ml. After removal of the top layer, the undernatant was adjusted to *d* 1.070 g/ml with NaBr, and spun for 20 h at 48,000 rpm at 10°C in a 50.2 Ti rotor (Beckman Instruments, Inc., Fullerton, CA). The top layer containing mainly Lp(a) and some LDL and HDL was removed and dialyzed extensively against 10 mM Tris, 0.01% EDTA, 0.01% sodium azide, pH 7.4. Because of overlapping density distributions, Lp(a) could not be separated from LDL by density gradient centrifugation (0–12% NaBr) in the SW-40 rotor (Beckman Instruments, Inc.). For this reason we turned to fast performance liquid chromatography and injected this fraction into a MonoQ column (Pharmacia Fine Chemicals, Piscataway, NJ) and eluted the absorbed components with a 20-min linear gradient consisting of 10 mM Tris, pH 7.4, and 10 mM Tris, NaCl, pH 7.4, at a flow rate of 1 ml/min with continuous monitoring at 280 nm. As seen in Fig. 1, LDL eluted earlier (together with HDL) at 0.18 M NaCl whereas Lp(a) eluted with 0.21 M NaCl from the Mono-Q column (Pharmacia Fine Chemicals). Fractions of 0.5 ml were collected and checked for the presence of Lp(a) by SDS-PAGE on 2–16% polyacrylamide gel. The fractions containing pure Lp(a), were pooled and dialyzed against 0.15 M NaCl, pH 7.4, 0.01% EDTA and 0.01% sodium azide.

The isolation of Lp(a) and LDL from human plasma was carried out as described before (19, 20). Protein concentrations were determined by the Lowry method as modified by Markwell et al. (21) using BSA as a standard. The pure fractions were stored at 4°C in air-tight microtubes (Sarstedt, Inc., Princeton, NJ) before use.

The binding studies with Lp(a) in whole plasma to lysine-Sepharose were carried out in Chicago by an adaptation of the original method of Deutsch and Mertz (22). Human or rhesus plasma (1 ml), dialyzed against the equilibrating buffer, was passed over 5 ml (6.4 cm × 0.79 cm^2) lysine-Sepharose columns equilibrated with 0.1 M phosphate, 0.01% Na_2 EDTA and 0.01% NaN_3, pH 7.4, which had a conduc-

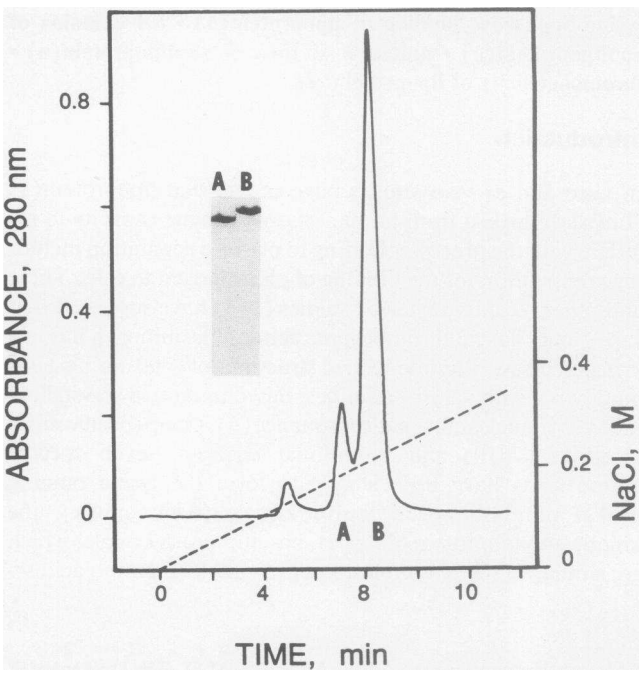


Figure 1. Purification of rhesus Lp(a) by fast performance liquid chromatography using a Mono-Q column (Pharmacia Fine Chemicals). Less than 5 mg of the lipoprotein mixture contained in the *d* 1.045 to *d* 1.070 g/ml interval was applied to the MonoQ column in 10 mM Tris, 0.01% Na_2 EDTA and 0.01% NaN_3, pH 7.4 at 8°C. Lp(a) was eluted with a 20-min linear gradient consisting of 10 mM Tris, pH 7.4, and 10 mM Tris, 0.5 M NaCl, pH 7.4, LDL, (peak A) eluted at 0.18 M NaCl, and Lp(a) (peak B) eluted at 0.21 M NaCl. SDS-PAGE (2–16% gradient gel) of LDL (peak A) and Lp(a) (peak B), 15 μg each, were stained with Coomassie brilliant blue and are shown in the insert.

Table II. Binding to Lysine-Sepharose of Plasma and Unlabeled Lp(a) from Rhesus Monkeys and Human Subjects

Source	Rhesus monkeys	Human subjects
	Percent bound	
Plasma*	1.5±0.8 (3.1–0.7)‡	78.9±5.1 (88.8–72.6)
	$n = 6$	$n = 11$
Lp(a)§	8.4±10.6 (24.9–0)‖	77.1±1.5 (78.5–75.0)
	$n = 10$	$n = 3$

* The percentage of bound Lp(a) was calculated from the immunodetectable Lp(a) in the bound and unbound fractions. ‡ Mean±SD (range). § The percentage of bound Lp(a) was calculated from the areas of the bound and unbound Lp(a) obtained by monitoring the lysine-Sepharose eluan at 280 nm. ‖ Some of the studies were carried out on mixtures of Lp(a) phenotypes.

tivity of 13 mS. The columns were washed with 6 bed vol of the same buffer and 1 bed vol of 0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.2. Bound Lp(a) was eluted with 200 mM ϵ-aminocaproic acid (EACA) in 0.1 M phosphate buffer. The concentration of Lp(a) in the bound and unbound fractions was determined with an ELISA for Lp(a) that is not affected by the presence of plasminogen (23).

Binding of purified, unlabeled human, or rhesus Lp(a) to lysine-Sepharose was carried out on a 1-ml column (5 cm × 0.20 cm^2) equilibrated with 10 mM phosphate, 0.15 M NaCl, 0.01% Na_2 EDTA, 0.01% NaN_3, pH 7.4 (conductivity, 15 mS). A vol of 200 μl Lp(a) (1 mg/ml protein) was applied at a flow rate of 10 ml/h. The column was monitored at 280 nm using an ultraviolet monitor (model 150; Altex Scientific, Inc., Berkeley, CA) equipped with a chart recorder (model BD41; Kipp & Zonen, Bohemia, NY). The column was washed with the same buffer until the absorbance reached baseline values. Bound Lp(a) was eluted with 200 mM EACA in PBS. Areas corresponding to bound and unbound Lp(a) on the chart paper were determined with a computerized digitizer. Aliquots of both human and rhesus Lp(a) were used in Chicago and also mailed by overnight delivery in wet ice by Federal Express to the Scripps Research Institute.

At Scripps, radiolabeling of Lp(a) was carried out using the ICl procedure as previously described (24). $Na^{125}I$ (2 mCi) was added to 20 μl of ICl prepared by diluting the stock ICl preparation (Kodak) 1:1,500 in 1 N HCl, 1 M NaCl, and incubating for 1 min at 4°C. Then 1 ml Lp(a), 10–50 μg/ml, was diluted with 330 μl 2 M glycine, 0.2 M EACA, pH 10.3, and added to the labeled reagent. After incubation for 10 min at 4°C, the radiolabeled Lp(a) was separated from free ^{125}I and EACA on a PD 10 column (Pharmacia) and dialyzed extensively into 0.15 M NaCl, 0.01% NaN_3, 0.01% Na_2 EDTA at 4°C. The radiolabeled Lp(a) preparations had specific activities of 0.2–0.6 μCi/μg and were >98% precipitable in 13% TCA.

In assays where the binding of ^{125}I-Lp(a) was measured in the presence of lysine-Sepharose beads, the latter were packed by centrifugation at 2,500 *g* for 3 min to permit their consistent addition to the assay system. 94 μl of the packed beads, ^{125}I-Lp(a) at a final concentration of 10 nM, and buffer, with or without 200 mM EACA were mixed to a final vol of 250 μl. The buffer used was the same as that used for the studies with unlabeled Lp(a) with a conductivity of 15 mS with and 1.9 mS without NaCl. After incubation for 10 min at 22°C, the reaction was terminated by centrifugation in a microfuge (Beckman Instruments, Inc., Fullerton, CA) for 3 min, and duplicate aliquots of the supernatants were removed and counted. Under the assay conditions, 15–30% of human ^{125}I-Lp(a) was typically bound to the lysine-Sepharose beads. When the unbound material was reapplied to the beads, 15–30% bound again, indicating the percent binding was a function of the assay conditions and not of the capacity of ^{125}I-Lp(a) preparations to interact with lysine Sepharose. Of the total binding, >95% was inhibitable by 200 mM EACA. Percent bound was calculated by the formula:

$$1 - \frac{\text{cpm exptl} - \text{cpm in presence of EACA}}{\text{cpm buffer} - \text{cpm in presence of EACA}} \times 100$$

Plasminogen was isolated from fresh human plasma by lysine-Sepharose chromatography as previously described (25). Elastase degradation products of plasminogen were prepared also as previously described (26). Contamination of each elastase degradation product preparation with the other elastase degradation product derivatives or with plasminogen was estimated by SDS-PAGE and was determined to be <1%.

Modeling of human and rhesus kringles. Given the one-to-one correspondence of residues of human and rhesus monkey kringle 37's with the crystallographically determined structure of the EACA-hu-

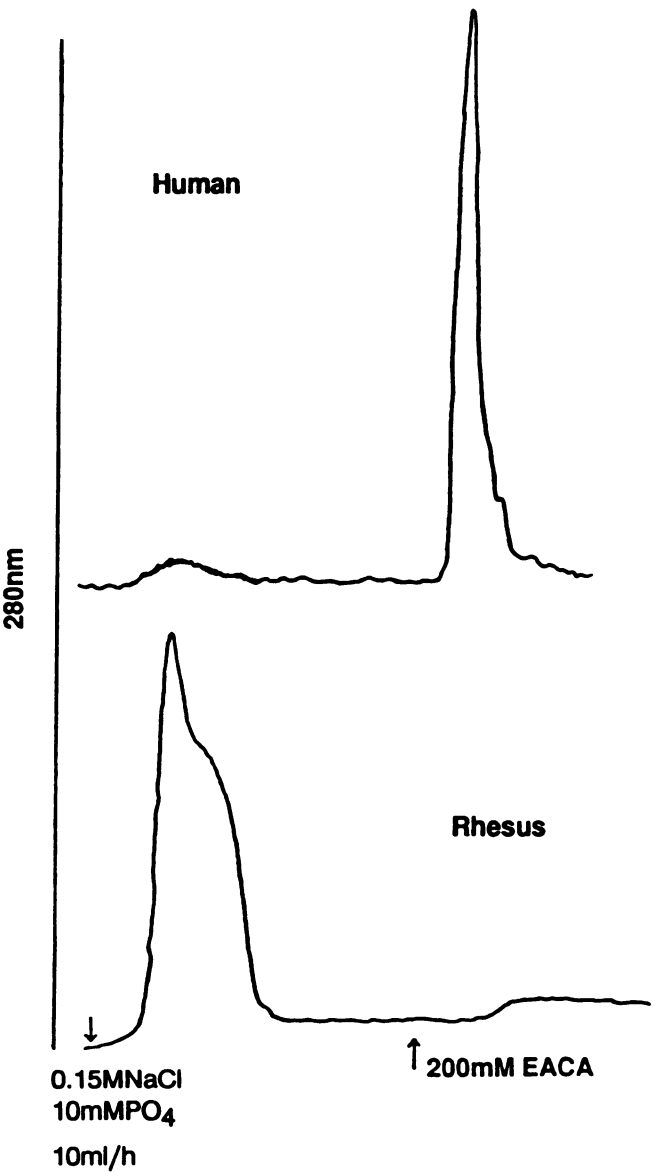


Figure 2. Binding of human and rhesus plasma Lp(a) to lysine-Sepharose. (*Upper panel*) Human Lp(a); (*lower panel*) rhesus Lp(a). Approximately 200 μg Lp(a) was applied to a 1-ml Lys-Sepharose column in 10 mM phosphate, 0.15 M NaCl, 0.01% Na_2 EDTA, 0.01% NaN_3, pH 7.4 at a flow rate of 10 ml/h. Bound Lp(a) was eluted with 200 mM EACA dissolved in the above buffer.

man plasminogen kringle 4 (PK4) complex determined by Tulinsky's group (5, 27), modeling by homology was possible without insertions or deletions. Backbone coordinates for each of the modeled structures were assigned directly from the crystal structure of PK4. Coordinates for sidechain atoms in the models were derived from standard bond lengths and angles and from corresponding atoms of PK4, where possible, using the computer program HOMOLOGY (Biosym Technologies, Inc., San Diego, CA). Each of the initially modeled structures was subjected to energy refinement using the computer program DISCOVER (Biosym Technologies, Inc.), wherein backbone atoms were held fixed and sidechain atoms were appropriately charged (Glu, Asp, Lys, and Arg) and movable. In the case of human $K4_{37}$, the refined structure was superimposed on PK4 and the coordinates of the EACA ligand were added to the $K4_{37}$ structure. The structure of the complex was again energy refined holding backbone atoms fixed. Although energy refinement with fully charged sidechains in the absence of water tends to overemphasize salt bridges, no gross structural distortions were possible with the backbones fixed.

Results

Lysine-Sepharose binding of human and rhesus Lp(a). When plasma from six different rhesus monkeys was passed over lysine-Sepharose, the binding of rhesus Lp(a) was very poor, in that only 1.5% of Lp(a) was found in the fraction eluted with 200 mM EACA (Table II). In contrast, 78.9% of human Lp(a) bound to lysine-Sepharose when plasma was applied to the column under identical conditions. Similar species differences were observed when isolated rhesus and human Lp(a) was passed over lysine-Sepharose (see example in Fig. 2). When five different preparations of rhesus monkey Lp(a) were subjected to lysine-Sepharose chromatography, only 8.4% of Lp(a) bound to the support in contrast to the 77.1% observed with human Lp(a) (Table II). No significant differences were observed between single band and double band phenotypes. It is appropriate here to note that the elution pattern for rhesus Lp(a) in Fig. 2 is suggestive of some degree of binding to the lysine column. However, this pattern can also reflect a natural broadening of the peak independent of lysine binding. In support of the latter interpretation are the results on our K42 recombinant studies showing a comparable pattern of elution that was unaffected by the presence of EACA (28).

Independent verification of the low affinity of rhesus Lp(a) for lysine was obtained using ^{125}I-Lp(a). This radiolabeled ligand was added to the lysine-Sepharose beads at a 10-nM concentration, and the extent of binding was measured after a 10-min incubation at 22°C. As summarized in Table III, binding of three separate rhesus Lp(a) preparations to the lysine-Sepharose beads was significantly reduced as compared to the human Lp(a) preparation used. At physiologic NaCl concentrations, with the extent of human Lp(a) binding defined as 100%, the binding of rhesus Lp(a) was 20% or less. Indeed, no binding of Lp(a) pooled from monkeys 8256/7700 was detected. Thus, under this condition of ionic strength, the lysine binding function of rhesus Lp(a) was significantly reduced. At lower ionic strength (omission of NaCl from the phosphate buffer), some binding of the rhesus Lp(a) preparations was observed. Compared to human Lp(a), 37–54% as much rhesus Lp(a) bound

Table III. Binding of Radiolabeled Human and Rhesus Lp(a) to Lysine-Sepharose

Lp(a)		NaCl 0.15 M	NaCl Absent
		Percent bound	
Human		100.0	100.0
Rhesus	No. 7643	12.7	44.6
	No. 8806/7436	20.0	37.6
	No. 8256/7700	0.0	53.8

^{125}I-Lp(a) preparations at 10-nM concentration in 0.01 M phosphate buffer, pH 7.4, with or without 0.15 M NaCl, were added to lysine-Sepharose beads. Incubation was for 10 min at 22°C. Percent bound is expressed as a percentage of the human Lp(a) bound (assigned a value of 100%) in the same experiment. In the experiments shown, at the 10-nM input concentration, 18.0% and 18.6% of the human Lp(a) bound to the lysine-Sepharose beads in the presence or absence of NaCl, respectively.

Table IV. Binding of ^{125}I-Lp(a) to U937 Cells

^{125}I-Ligands		Cell binding: Buffer, Molecules 10^4/cell	Cell binding: EACA, Molecules $\times 10^4$/cell	Cell binding: EACA, Percent inhibition
Lp(a) Human		3.0	1.4	53
Lp(a) Rhesus	No. 7643	2.2	2.2	0
	No. 8806/7436	1.1	1.0	9

^{125}I-Lp(a) preparations at 10 nM were incubated with U937 cells (4.1 $\times$ 10^6/ml) in the presence or absence of EACA (0.2 M) for 120 min at 37°C. Plasminogen binding was 95% inhibitable by EACA in the same experiment.

Table V. Effects of Plasminogen Derivatives on the Binding of ^{125}I-Lp(a) and ^{125}I-Plasminogen to Cells

Competitor	^{125}I-Lp(a) Binding: Bound*	^{125}I-Lp(a) Binding: Inhibition‡	^{125}I-Plasminogen binding: Bound§	^{125}I-Plasminogen binding: Percent inhibition‡
None	3.7	—	2.0	—
EACA, 0.2 M	1.2	68	0.0	100
LDL, 125 μg/ml	2.4	35	1.9	5
Plasminogen, 20 μM	1.6	57	0.0	100
EDPII, 20 μM (Containing K4)	2.5	32	1.0	50
EDPIII, 20 μM (Containing K5)	3.6	3	1.1	45

^{125}I-Lp(a) or plasminogen (10 nM each) were incubated with U937 cells (4.1 $\times$ 10^6/ml) in the presence of the indicated competitor for 120 min at 37°C. Similar results were obtained using at least two different preparations of EDP fragments. Nonspecific binding of Lp(a) is similar to the sum of EACA + LDL (76%). * Molecules $\times$ 10^3/cell. ‡ Calculated relative to the ^{125}I-ligand without inhibitor. § Molecules $\times$ 10^5/cell.

to the lysine-Sepharose under this condition. The interaction still represented specific binding as it was inhibited by EACA. Our finding that conditions of low ionic strength favor the binding of Lp(a) to lysine-Sepharose confirms a recent report by Helmhold et al. (29). However, the differences in behavior between rhesus and human Lp(a) with regard to lysine binding cannot be attributed solely to ionic strength effects, since rhesus Lp(a) bound less efficiently to lysine-Sepharose at both extremes of ionic strength.

Thus, the results derived from the use of nonlabeled and radiolabeled Lp(a) were consistent and taken together suggest that rhesus Lp(a) binds less avidly to lysine-Sepharose than human Lp(a). These data also indicate that iodination does not alter the lysine-binding functions of human and rhesus Lp(a).

Binding of human and rhesus ^{125}I-Lp(a) to U937 cells. As shown previously (24), U937 monocytoid cells are capable of binding human Lp(a) both via the plasminogen receptors and the low affinity lipoprotein receptors. Under the culture and assay conditions used (i.e., cells grown in fetal calf serum and binding analyses performed at 37°C in the absence of divalent cations) we have not detected significant binding of human Lp(a) to either the LDL receptor or the scavenger receptor. As summarized in Table IV, 53% of the Lp(a) binding to U937 cells was inhibited by EACA. Under the same conditions we observed 30–66% less binding of rhesus Lp(a) from monkeys 8806 and 7436 as compared to human Lp(a). Moreover, virtually none of the binding of the rhesus Lp(a) preparations to the cells was inhibited by EACA. In a separate analysis, similar results were observed with the ^{125}I-Lp(a) preparation from monkeys 8256/7700; 63% fewer molecules of this Lp(a) were bound to the cells compared to the human Lp(a) analyzed in parallel. None of the observed binding was inhibited by EACA. Thus, under the conditions of the cell-binding analyses, rhesus Lp(a) exhibited no interaction with plasminogen-binding sites on the cells.

Effect of human plasminogen derivatives on the binding of human Lp(a) to cells. Plasminogen derivatives containing either K4 or K5 inhibit binding of the parent plasminogen molecule to cells (25). Thus, the failure of rhesus Lp(a) to bind to plasminogen receptors on cells suggested that either: (*a*) amino acid substitutions in the binding site of rhesus $K4_{37}$ or other K_4 domains which mediate recognition of lysine by the intact Lp(a) particle, or (*b*) the absence of the K5 domain in rhesus Lp(a) renders rhesus Lp(a) nonfunctional. To distinguish between these two possibilities, we assessed the capacity of human plasminogen fragments containing either K4 or K5 to inhibit the binding of human Lp(a) to U397 cells. In parallel experiments we examined the effects of these derivatives on plasminogen binding to the cells. As shown in Table V, EDPII, which contains K4 of plasminogen, inhibited plasminogen binding to cells. This result is consistent with previous observations indicating that EDPII inhibits plasminogen binding to the cells (25). Inhibition of the cellular binding of Lp(a) by EDPII is also consistent with a role of the K4 lysine binding site in the interaction of Lp(a) with cells as previously demonstrated using an immunochemical approach (30). The relatively lower potency of EDP II on the binding of Lp(a) as compared to plasminogen presumably reflects the failure of EDP II to influence the lipoprotein-mediated component of Lp(a) binding to cells. In separate experiments, we showed that EDPII does not influence the binding of ^{125}I-LDL to U937 cells. The effects of EDPIII on plasminogen and Lp(a) binding to the cells were strikingly different. EDPIII, which contains K5 and the latent protease domain of plasminogen is an inhibitor of plasminogen binding to cells as previously reported (25). Despite its effective inhibition of plasminogen binding to cells EDP III had no effect on the cellular binding of Lp(a). Similar results were obtained with two separate preparations of EDPII and EDPIII; namely, EDPIII failed to inhibit Lp(a) binding, whereas EDPII was inhibitory.

These data suggest that the binding of human Lp(a) to

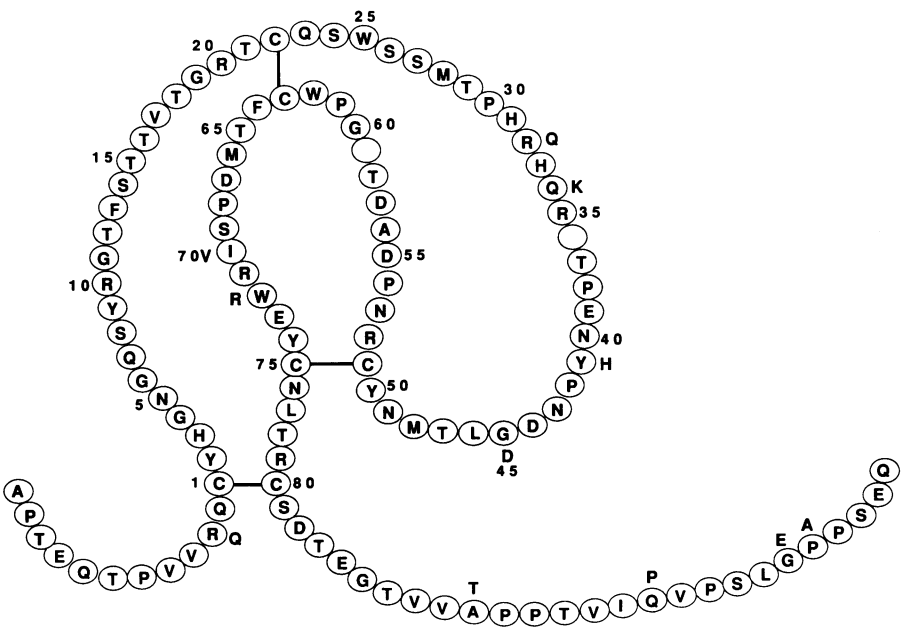
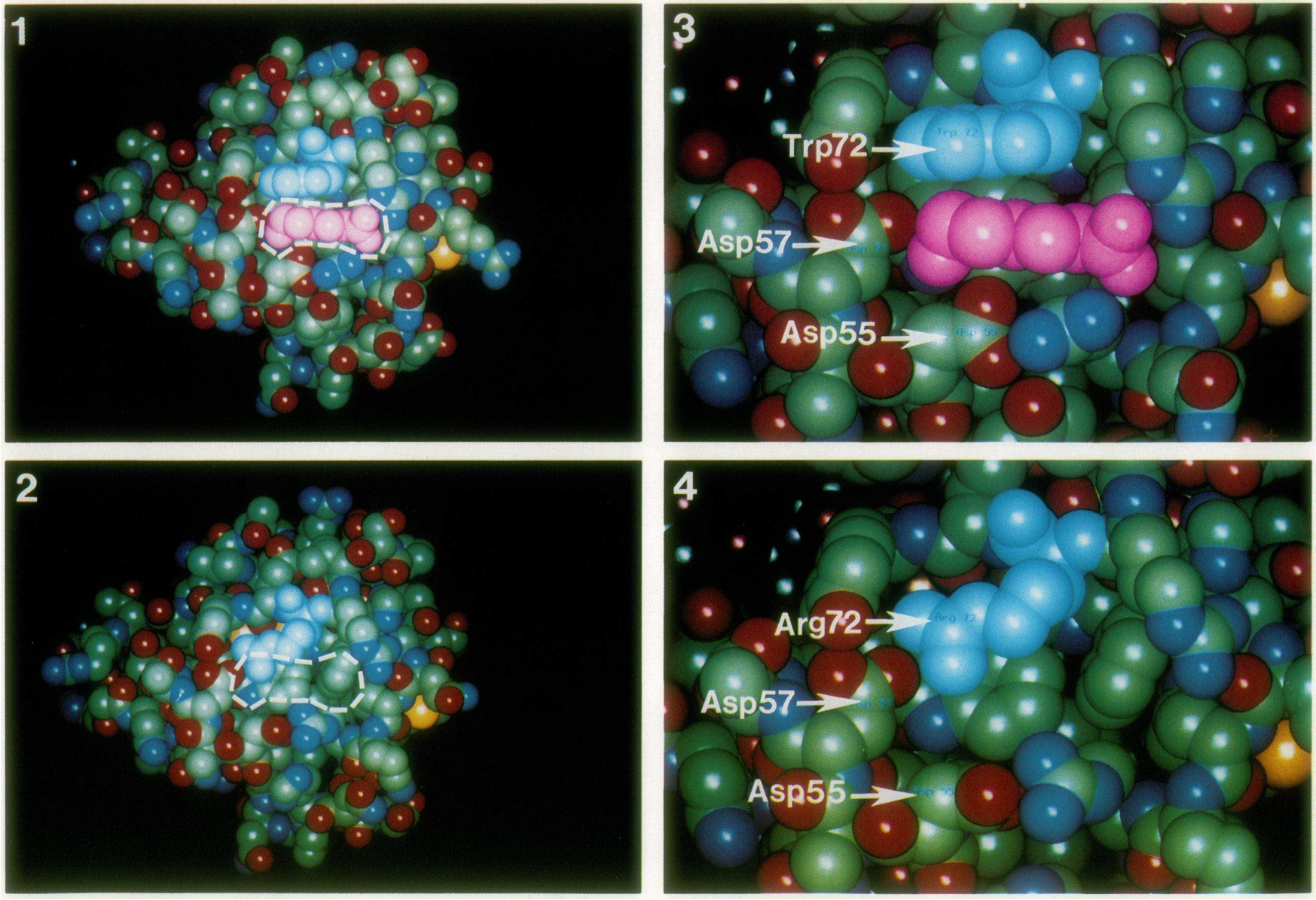


Figure 3. Comparative structural scheme of human and rhesus $K4_{37}$. Amino acid substitutions are shown in positions 32, 34, 41, 45, 70, and 72. The rhesus monkey data are from (6). Positions 36 and 59 are left unfilled to permit comparison of human and rhesus K_4 with human plasminogen K_4. The numbering follows the convention of plasminogen K5.


1
2
3
Trp72
Asp57
Asp55
4
Arg72
Asp57
Asp55

plasminogen-binding sites on the cell membrane depends upon neither K5 nor the protease domain but rather on the K4 region. Therefore, the failure of rhesus Lp(a) to bind to cells cannot be explained by the absence of K5 but probably by structural changes in its K4 domains.

Discussion

Rhesus apo(a) while exhibiting structural features in common with human apo(a) differs from it in several respects (13): (*a*) in the catalytic triad (His, Asp, Ser) His is replaced by Cys and Ser by Asn; (*b*) the nine amino acids deleted in the protease region of human apo(a) relative to human plasminogen are present in rhesus apo(a); (*c*) the K5-like domain which is present in human apo(a) is absent in rhesus apo(a) making the rhesus K4-like sequence contiguous to the protease region; (*d*) the 5′-untranslated and prepeptide encoding regions of rhesus apo(a) appear to be divergent from those in human apo(a); (*e*) with the exception of $K4_{37}$, all rhesus K4 homologues identified thus far, have lost the key aspartic acid residue 57 which is required for lysine analogue binding in plasminogen K1 and K4; (*f*) rhesus $K4_{37}$ contains the key aspartic acid in position 57 but has arginine instead of tryptophan 72, another key amino acid involved in lysine binding (Fig. 3).

From the above information we may postulate that either the loss or substitutions of critical amino acids in the lysine binding pocket of the apo(a) kringles accounts for the reduced ability of rhesus apo(a) to bind to either lysine Sepharose or U937 cells. The possibility that the absence of K5 (6) contributes to this is ruled out since K5 does not have sufficient affinity to mediate EDPIII binding to lysine-Sepharose (26) and by the results using elastase degradation products of human plasminogen (summarized in Table V) showing that the plasminogen receptor-dependent interaction of human apo(a) involves neither K5 nor the protease region.

Our current observations implicate the participation of $K4_{37}$ in binding of apo(a) to both lysine and to plasminogen receptors. This suggestion is supported by our recent studies showing that the $K_{4\text{-}2}$ domain that is repeated more than 20 times in human apo(a) and rhesus apo(a), when expressed in *Escherichia coli*, exhibits no lysine binding capacity (28) probably due to the substitution of valine for aspartic acid in position 57 since the other prominent amino acids that are important in lysine binding are not modified. Further support for the key role of $K4_{37}$ in lysine-binding function was derived from modeling studies. Seven key amino acid residues form the lysine binding pocket within K4 of plasminogen: Lys 35, Asp 55, Asp 57, Trp 62, Phe 64, Arg 71, and Trp 72. Kringle 37 ($K4_{37}$) of apo(a) has the same amino acids at these key positions and a conservative Arg for Lys substitutions at position 35 (see below), although several other kringles have one or two conservative substitutions at these positions. Thus, it is likely that the lysine binding functions of human apo(a) depend upon $K4_{37}$ with potential contributions from other kringles. The failure of rhesus Lp(a) to bind lysine suggests that its $K4_{37}$ equivalent is nonfunctional. At the seven key amino acid positions, human and rhesus $K4_{37}$ differ in a single residue, namely, Trp 72 in human apo(a) is an Arg in rhesus apo(a) (see Fig. 3). Models were constructed for the two kringles based upon backbone coordinates derived from the crystal structure of human plasminogen K4 (5, 31). The residues of human and rhesus $K4_{37}$ were substituted within this basic framework, and the structures were allowed to reconform by energy minimization. The structures generated are shown in Fig. 4: panels 1 and 2 represent the molecular models for the entire kringle domain of human and rhesus $K4_{37}$, respectively, and panels 3 and 4 are close-ups of the lysine-binding pockets within these structures. In comparing human apo(a) $K4_{37}$ to plasminogen K4, these structures were found to be extremely similar, supporting the prediction of a functional lysine binding site in human $K4_{37}$. Although subtle differences between the structures of these sites within the two kringles in the absence of a lysine ligand were noted, particularly with respect to the positioning of Trp 72, the lysine-binding pockets became virtually identical upon insertion of EACA into the two structures. The molecular modeling showed that in plasminogen K4 and human apo(a) $K4_{37}$, Trp 72 forms a "side wall" of the lysine-binding site. In contrast in rhesus $K4_{37}$, the side chain of Arg 72 occupies the lysine-binding site. This configuration precludes insertion of EACA into the structure of rhesus $K4_{37}$. Thus, the molecular models predict that human $K4_{37}$ would function as a lysine-binding site, whereas rhesus $K4_{37}$ would function poorly if at all.

Kringle 1 of prothrombin also has Arg at position 72 and lacks lysine-binding function. The potential impact of this substitution on the lysine-binding properties of prothrombin was originally noted by Tulinsky et al. (32), although other amino acid differences between the prothrombin and plasminogen kringles could also contribute to the functional differences. The great similarity in the structures of $K4_{37}$ of rhesus and human apo(a) (92% identity at the primary sequence level) suggests that the substitution at this single position 72 is probably sufficient to determine the lysine binding function of kringles. Several kringles, of human apo(a) other than $K4_{37}$, have as many as five of the seven residues that form the lysine binding pocket of plasminogen K4. All of the same combinations of substitutions at these positions are also found in rhesus apo(a). As rhesus Lp(a) exhibits minimal lysine binding function, it appears that the other kringles of human apo(a) contribute minimally to the lysine binding function of Lp(a). While the molecular modeling is consistent with the key role of $K4_{37}$ in the lysine-binding activity of human apo(a), direct proof of

Figure 4. Space filling models of human and rhesus monkey apo(a) kringle 4_{37}. The models were constructed based upon atomic coordinates derived from the crystal structure of kringle 4 of human plasminogen as described in the methods. Panels 1 (human) and 2 (rhesus) are models of the full kringles, and panels 3 and 4 are close-ups of the vicinity of the functional lysine binding site in the case of human $K4_{37}$ (panel 3) and the dysfunctional lysine binding site in rhesus $K4_{37}$ (panel 4). In all panels, the atoms in the space filling models are colored by atom type (green, carbon; red, oxygen; blue nitrogen; yellow, sulphur) except for the residue at position 72. The residue at position 72 (Trp, human; Arg, rhesus) is colored cyan. The EACA ligand in human $K4_{37}$ is colored magenta. The dotted line in panel 2 is the position at which the EACA ligand would be placed if it could fit into the rhesus structure. In addition to residue 72, the Asp residues at positions 55 and 57, which are critical components of the lysine-binding site, are also identified.

this conclusion will require expression of the individual kringles and assessment of their function. This approach has been implemented by our group (28).

The involvement of K4 repeats in lysine and cell binding and the notion that the number of K4 domains determines the molecular size of each apo(a) phenotype raise the question as to whether the effects observed were related to apo(a) polymorphism or apo(a) phenotype. The relatively low number of apo(a) phenotypes at our disposal does not allow us to answer this question unequivocally although there appeared to be no significant difference between single band and double band apo(a) phenotypes in terms of binding efficiency to either lysine-Sepharose or U937 cells. In this regard it should be noted that at present we lack knowledge on the structure of the NH_2-terminal region of rhesus apo(a) which from the reports in the literature may be divergent from that of human apo(a) (13).

Our current results may also have important biological implications since rhesus Lp(a), contrary to human Lp(a), has a limited antifibrinolytic effect in vitro suggesting that it may also be comparatively less thrombogenic. This conclusion is supported by the limited ability of rhesus Lp(a) to bind to fibrin in vitro (Loscalzo, J., unpublished observations) and by the studies by Nachman et al. (33) showing no co-localization of Lp(a) and fibrinogen in atherosclerotic lesions of cynamologous macaques fed a high fat atherogenic diet. This contrasts with the extensive association between these two components reported by Beisiegel et al. (34) and Smith and Cochran (35) in human lesions. We have also noted an absence of co-localization between Lp(a) and fibrin in the atherosclerotic plaques of a deceased female member of our rhesus monkey pedigree (36). The animal had high plasma levels of Lp(a) and an LDL receptor deficiency that was likely responsible for the sustained hypercholesterolemia observed in this animal fed a cholesterol-free Purina chow diet. From the evolutionary standpoint it is difficult to understand why difference in prothrombotic action should exist between rhesus and human Lp(a). In this respect, it is of interest to point out that Rouy et al. (37) have recently reported that the Lp(a) of the hedgehog, an animal low in the evolutionary scale, exhibits in vitro prothrombic effects that are similar to those reported for human Lp(a) (1–4). At this time the structure of hedgehog apo(a) is unknown.

On a more general level, the current studies suggest that functional mutations can occur at the level of individual kringles and raise the possibility that the type of mutation observed in $K4_{37}$ of the rhesus monkey may also occur in man. If this were to be the case, apo(a) phenotypes with the same size and thus the same number of K4 may be functionally different in terms of their thrombogenic potential. This type of functional polymorphism adds a new dimension to the established size polymorphism of apo(a) and invites studies on the development of techniques aimed at identifying mutations at the level of individual kringles.

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