# Characterization of the basis of lipoprotein [a] lysine-binding heterogeneity

**Jiazhi Xia, Lorraine F. May, and Marlys L. Koschinsky[1]**

Department of Biochemistry, Queen's University, Kingston, Ontario Canada K7L 3N6

**Abstract** **Although elevated plasma concentrations of lipoprotein [a] (Lp[a]) are considered to be a risk factor for atherosclerosis, the mechanisms by which Lp[a] mediates its pathogenic effects have not been conclusively determined. The apolipoprotein [a] (apo[a]) component of Lp[a] confers unique structural properties to this lipoprotein, including the ability to bind to lysine residues in biological substrates. It has been shown, however, that only a fraction of plasma Lp[a] (Lp[a]-Lys$^+$) binds to lysine-Sepharose in vitro. The nature of the non-lysine-binding Lp[a] fraction in plasma (Lp[a]-Lys$^-$) is currently unknown. In the present study, the Lp[a]-Lys$^+$ fraction was determined in the plasma of six unrelated individuals; the Lp[a]-Lys$^+$ fraction in these plasma samples ranged from ~37 to ~48%. Interestingly, purification of the Lp[a] by density gradient ultracentrifugation followed by gel filtration and ion-exchange chromatography resulted in progressive increases in the Lp[a]-Lys$^+$ fraction. Addition of either purified low density lipoprotein (LDL) or fibronectin to the purified Lp[a] at a 1:1 molar ratio reduced the Lp[a]-Lys$^+$ fraction (maximal decrease of 34 and 20%, respectively) whereas addition of both fibronectin and LDL to the purified Lp[a] resulted in a further decrease (45% maximally) in this fraction. Similar results were obtained by using a recombinant expression system for apo[a]: addition of a 4-fold molar excess of either LDL or fibronectin to conditioned medium containing metabolically labeled recombinant apo[a] reduced the Lys$^+$ fraction by 49 and 23%, respectively. Taken together, our data suggest that the lysine-binding heterogeneity of plasma Lp[a] is not primarily an intrinsic property of the lipoprotein, but rather results in large part from its ability to noncovalently associate with abundant plasma components such as LDL and fibronectin. These interactions appear to mask the lysine-binding site in apo[a] kringle IV type 10, which mediates the interaction of Lp[a] with lysine-Sepharose. The contribution of these interactions to the function of Lp[a] in vivo remains to be investigated.**—Xia, J., L. F. May, and M. L. Koschinsky. **Characterization of the basis of lipoprotein [a] lysine-binding heterogeneity. *J. Lipid Res.* 2000.** 41: **1578–1584.**

**Supplementary key words** apolipoprotein [a] • low density lipoprotein • kringles • fibronectin • ε-aminocaproic acid • atherosclerosis

Numerous studies have demonstrated that elevated plasma levels of lipoprotein [a] (Lp[a]) (greater than a risk threshold of ~25–30 mg/dl) are associated with the development of coronary heart disease (1, 2). Plasma Lp[a] concentrations vary more than 1,000-fold in the population, ranging from less than 0.1 to greater than 100 mg/dl. Lp[a] contains a low density lipoprotein (LDL)-like moiety but is clearly distinguishable from LDL by the presence of apolipoprotein [a] (apo[a]), which is covalently linked to apolipoprotein B-100 (apoB) by a single disulfide bridge (3, 4). Human apo[a] contains multiple repeated copies of a sequence closely resembling plasminogen kringle IV, followed by sequences that 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 varying numbers of copies, which constitutes the basis of Lp[a] isoform size heterogeneity (6, 7). Of the kringle IV sequences in apo[a], the sequence of apo[a] kringle IV type 10 ($KIV_{10}$) most closely resembles that of plasminogen kringle IV. Like plasminogen kringle IV, apo[a] $KIV_{10}$ contains a lysine-binding site (LBS) (8), which has been postulated to mediate the interaction of Lp[a] with biological substrates such as fibrin (9, 10). Weaker LBS have also been identified in apo[a] kringle IV types 5–8; these LBS mediate the initial noncovalent interaction between apo[a] and apoB-100 and appear to be masked in the context of the Lp[a] particle (11).

Particular interest has arisen regarding the heterogeneity of Lp[a] with respect to its ability to bind to lysine. Initial studies by Armstrong and coworkers (12) showed that plasma Lp[a] from individuals can be fractionated into species that either bind (Lp[a]-Lys$^+$) or fail to bind

Abbreviations: ε-ACA, ε-aminocaproic acid; apo[a], apolipoprotein [a]; apoB, apolipoprotein B-100; CM, conditioned medium; CNBr, cyanogen bromide; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; Fn, fibronectin; $KIV_{10}$, apo[a] kringle IV type 10; LBS, lysine-binding site(s); LDL, low density lipoprotein; Lp[a], lipoprotein [a]; Lp[a]-Lys$^+$, lysine-binding Lp[a] fraction; Lp[a]-Lys$^-$, non-lysine-binding Lp[a] fraction; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; r-apo[a], recombinant apo[a]; tPA, tissue-type plasminogen activator.

[1] To whom correspondence should be addressed.

(Lp[a]-Lys$^-$) to lysine-Sepharose. It was subsequently demonstrated that the proportions of the Lp[a]-Lys$^+$/Lp[a]-Lys$^-$ fractions are donor dependent and apo[a] isoform-size independent (13). This study also showed that the Lp[a]-Lys$^-$ fraction was incapable of inhibiting tissue-type plasminogen activator (tPA)-mediated plasminogen activation in vitro, and did not bind to cyanogen bromide (CNBr)-digested fibrinogen (13). However, the biochemical basis that underlies the inability of a fraction of plasma Lp[a] to bind to lysine-Sepharose is not known. It has been reported that a Trp→Arg substitution in human apo[a] kringle IV type 10 abolishes lysine binding of the corresponding Lp[a] species (14). However, because the frequency of the mutant allele in the population appears to be low, it is unlikely that this polymorphism plays a major role in the determination of Lp[a] lysine-binding heterogeneity.

In the present study, we have examined the lysine-binding properties of Lp[a] isolated from several unrelated individuals. We have compared the lysine-binding ability of the Lp[a] as a function of its degree of purification, and found that the lysine-binding ability of the Lp[a] in all cases increased as a function of Lp[a] purity. We were also able to demonstrate that the ability of the purified Lp[a] to bind to lysine-Sepharose could be decreased by the addition of LDL or fibronectin. Similar results were obtained with a recombinant form of apo[a] (r-apo[a]); the addition of either purified LDL or fibronectin to the r-apo[a] significantly decreased its ability to bind to lysine-Sepharose. Taken together, our data suggest that plasma proteins such as LDL and fibronectin can interact noncovalently with Lp[a], thereby altering the lysine-binding properties of Lp[a] in vivo.

## EXPERIMENTAL PROCEDURES

### Isolation and characterization of Lp[a]

After obtaining informed consent, blood was collected from healthy volunteers (according to the ethical guidelines established by Queen's University, Kingston, Ontario, Canada) into tubes containing ethylenediaminetetraacetic acid (EDTA) (final concentration, 1 mM). Plasma was then obtained by centrifugation of whole blood at 2,000 *g* for 20 min at 4°C. Phenylmethylsulfonyl fluoride (PMSF) was added to the plasma to 1 mM (final concentration) to prevent endogenous proteolysis. Both Lp[a] concentrations (15) and apo[a] isoform sizes (16) were determined as previously described for the six donor plasma samples.

Lp[a] purification was performed as previously described (17), with some modifications. Plasma (25 ml) was adjusted to a density of 1.006 g/ml with solid sodium bromide and centrifuged at 90,000 *g* for 20 h at 8°C. After centrifugation, the top 3 ml containing very low density lipoprotein (VLDL) was removed with a syringe, and the infranatant was adjusted to d = 1.21 g/ml with sodium bromide. Samples were then centrifuged at 90,000 *g* for an additional 20 h at 8°C. The top 5 ml was removed and dialyzed extensively against 20 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 0.01% $NaN_3$ (buffer A). The dialyzed sample (designated lipid flotation fraction) was then subjected to gel-filtration chromatography over Sepharose CL-4B (Pharmacia, Piscataway, NJ) (2.5 × 110 cm column) in buffer A containing 0.1% (v/v) Tween 20 and 0.1 M proline. Seven-milliliter column fractions were collected and the Lp[a] in each fraction was quantitated by enzyme-linked immunosorbent assay (ELISA) as previously described (15). On the basis of the ELISA data, Lp[a]-containing fractions were pooled, diluted 3-fold with distilled water, and loaded onto a DEAE-Sepharose Fast Flow (Pharmacia) ion-exchange column (2.5 × 2.5 cm). Bound Lp[a] was eluted with a linear gradient of NaCl (50 to 300 mM in 20 mM Tris-HCl, pH 7.4). The Lp[a] in each column fraction was quantitiated by ELISA and Lp[a]-containing fractions were pooled (15). For all samples, Lp[a] consistently eluted from this column at approximately 180 mM NaCl. Lp[a] concentrations in plasma, the lipid flotation fraction, and pooled DEAE-Sepharose column fractions were measured by ELISA as previously described (15); the respective Lp[a]-containing fractions from the purification scheme were used for lysine-Sepharose binding analysis (see below). The integrity of the purified Lp[a] preparations was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of ~5 μg of the samples, using a 2.5–15% gradient gel under nonreducing conditions followed by silver staining; purified human LDL (isolated as described below) was used as a size standard. The average percent recovery of purified Lp[a] from plasma was approximately 20%. The loss in yield was almost entirely attributable to losses incurred after density gradient ultracentrifugation of the plasma samples.

### Isolation and measurement of LDL

LDL was prepared from the plasma of a normolipidemic volunteer by sequential flotation within the density range 1.006 < d < 1.05 g/ml as previously described (18). Briefly, plasma was centrifuged at 436,000 *g* for 2 h at 8°C. The d < 1.006 g/ml fraction was removed with a syringe; the infranatant was adjusted to d < 1.05 g/ml with solid sodium bromide and centrifuged for an additional 2 h under the conditions described above. The d < 1.05 g/ml was isolated, and centrifuged at d = 1.05 g/ml for a final 2 h. LDL isolated using this procedure was found to contain no contaminating Lp[a], as determined by Western blot analysis and ELISA. The LDL concentration in the final preparation was measured using a modified Bradford assay (Bio-Rad, Hercules, CA).

LDL-apoB was measured in all human plasma samples as previously described (19). LDL was isolated by ultracentrifugation as previously described and apoB in this fraction was measured in duplicate, using an immunoturbidometric assay (Boehringer Mannheim Biochemicals, Indianapolis, IN) on a COAS MIRA-S (Roche Diagnostics, Nutley, NJ) automated analyzer.

### Isolation of fibronectin

Fibronectin was isolated as previously described (20), using fresh-frozen plasma obtained from the Blood Bank at Kingston General Hospital (Kingston, Ontario, Canada). Briefly, plasma was clarified by centrifugation at 2,000 *g* for 20 min at 4°C. The clarified plasma was then diluted with an equal volume of 50 mM Tris-HCl, pH 7.5, and loaded onto a gelatin-Sepharose (Pharmacia) column (2.5 × 6.0 cm). The column was washed with 50 mM Tris-HCl, pH 7.5, and then washed with this buffer containing 1 M urea until the absorbance at 280 nm of the effluent returned to baseline. The fibronectin was eluted from the column in 50 mM Tris-HCl, pH 7.5, containing 4 M urea. The protein-containing fractions were pooled, dialyzed against 50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, and concentrated in a dialysis bag immersed in PEG 20,000 (Fluka, Buchs, Switzerland). The fibronectin concentration was determined by measurement of absorbance at 280 nm [$\varepsilon_{0.1\%}(280)$ = 1.28]. Purity of the preparation was determined by SDS-PAGE followed by silver staining.

The presence of fibronectin in the lipid flotation fraction and in purified Lp[a] (i.e., after DEAE-Sepharose chromatography) was assessed by Western blot analysis using a polyclonal anti-

human fibronectin antibody (Calbiochem, La Jolla, CA) and purified human fibronectin as a marker.

### Measurement of the lysine-Sepharose binding affinity of Lp[a]

The ability of Lp[a] contained in plasma and in the lipid flotation fraction (see above) to bind to lysine-Sepharose matrix was compared with that of purified Lp[a] obtained from the DEAE-Sepharose column. In all cases, 20 μg of Lp[a] (as determined by ELISA) was diluted to 7 ml in buffer A (see above). The samples were applied to 2 ml of lysine-Sepharose CL-4B (Pharmacia) in a 1.1 × 10 cm column. The column was sealed on both ends and rocked gently for 2 h at 4°C to allow batch adsorption of Lp[a] to the resin. The slurry was then allowed to pack in the column and the flowthrough (A) was collected. The column was then washed with 25 ml of buffer A. The resultant flowthrough (B) was then collected and combined with flowthrough A; the combined fractions were designated as the unbound or Lp[a]-Lys$^-$ fraction. Specifically bound Lp[a] was then eluted with 12.5 ml of buffer A containing 0.2 M ε-aminocaproic acid (ε-ACA). The eluted material was designated as the Lp[a]-Lys$^+$ fraction. The Lp[a] content in both Lys$^-$ and Lys$^+$ fractions was determined by ELISA and expressed in terms of the percentage of Lp[a] that was capable of binding to lysine-Sepharose.

In some experiments, purified Lp[a] (20 μg) from either of two donors was diluted to 7 ml in buffer A, and was then mixed with purified LDL or fibronectin (1:1 molar ratio of either) or a combination of the three (1:1:1 molar ratio). Mixtures were incubated with lysine-Sepharose and the Lp[a]-Lys$^+$ fraction was determined as described above.

### Expression and metabolic labeling of recombinant apo[a]

Human embryonic kidney (293) cells were cultured in 100-mm dishes in minimal essential medium (MEM; GIBCO-BRL, Rockville, MD) supplemented with 5% fetal calf serum. Cells were transiently transfected by calcium phosphate precipitation (21), using 10 μg of the plasmid encoding either the 17KΔCys derivative (ref. 22; lacking the free cysteine in apo[a] kringle IV type 9 that mediates covalent association of apo[a] and apoB-100) or the 17K derivative (ref. 23; containing the free cysteine in apo[a] kringle IV type 9 that is involved in disulfide linkage with apoB) per 100-mm culture dish. The precipitate was left on the cells for 7 h, after which time the transfected cells were washed thoroughly with phosphate-buffered saline (PBS) and fresh MEM containing 5% fetal calf serum was added. After overnight recovery, cells were preincubated for 1 h in Met/Cys-depleted MEM (GIBCO-BRL) without fetal calf serum. [$^{35}$S]Cysteine (ICN, Irvine, CA) was then added (40 μCi/ml of medium). Conditioned medium (CM) containing labeled r-apo[a] was harvested after a 3.5-h labeling period and was clarified by brief centrifugation. The r-apo[a] in the CM was quantified by ELISA (15).

### Chromatographic analysis of the binding of labeled r-apo[a] to lysine-Sepharose columns

CM containing [$^{35}$S]Cys-labeled 17KΔCys (1 ml) was applied to a 1-ml lysine-Sepharose (Pharmacia) column. The column was washed with 7 column volumes of PBS, followed by 5 column volumes of PBS containing 0.2 M ε-ACA. One-milliliter column fractions were collected and immunoprecipitated with 4 μg of the apo[a]-specific monoclonal antibody 2G7 as previously described (11). Immunoprecipitates were analyzed by SDS-PAGE on a 6% polyacrylamide gel; the gel was treated with Enlightning (DuPont, Wilmington, DE), dried under vacuum, and exposed to Hyperfilm (Amersham, Arlington Heights, IL).

In other experiments, 1 ml of CM containing [$^{35}$S]Cys-labeled 17KΔCys was incubated at 37°C for 2 h with a 4-fold M excess of either purified LDL or purified fibronectin. Reaction mixtures were subjected to lysine-Sepharose chromatography and column fractions were analyzed as described above. In control experiments, a 10-fold molar excess of bovine serum albumin (Sigma, St. Louis, MO) was incubated with r-apo[a] prior to lysine-Sepharose chromatography.

In some experiments, 1 ml of CM containing [$^{35}$S]Cys-labeled 17K r-apo[a] was incubated with a 4-fold molar excess of LDL; reaction mixtures were subjected to lysine-Sepharose chromatography and column fractions were analyzed as described above.

In all cases, fluorograms were scanned with a Hewlett-Packard (Palo Alto, CA) ScanJet 3c flatbed scanner and analyzed with Corel PhotoPaint (version 5.0, Corel, Ottawa, Canada) and SigmaGel (version 1.0; Jandel Scientific, San Rafael, CA) software in order to determine the percentage of r-apo[a] that bound to lysine-Sepharose.

## RESULTS

### Effect of the purification of Lp[a] on its ability to bind to lysine-Sepharose

Lp[a] was purified from the plasma of six unrelated subjects for the present study. Plasma Lp[a] levels in these

TABLE 1. Lysine-binding fraction of Lp[a] during purification

| Donor | Isoform(s) | LDL-apoB (Plasma) | Lp[a] (Plasma) | Lp[a]-Lys$^+$ | | |
|---|---|---|---|---|---|---|
| | | | | Plasma | Flotation | Pure |
| | | *mg/dl* | | | % | |
| 1 | 15 | 98 | 57.3 ± 6.4 | 47.9 ± 0.3 | 55.4 ± 0.3 | 92.7 ± 0.1 |
| 2 | 17 | 76 | 33.4 ± 5.6 | 38.0 ± 0.1 | 41.6 ± 2.1 | 87.1 ± 2.8 |
| 3 | 16 | 57 | 73.2 ± 5.1 | 46.8 ± 0.3 | 56.3 ± 5.9 | 88.5 ± 0.6 |
| 4 | 23, 22 | 50 | 32.0 ± 2.6 | 37.4 ± 0.9 | 67.7 ± 2.8 | 93.4 ± 3.1 |
| 5 | 22, 17 | 96 | 17.8 ± 2.5 | 46.9 ± 0.2 | 60.1 ± 1.2 | 93.7 ± 0.3 |
| 6 | 16 | 45 | 106 ± 3 | 46.7 ± 4.2 | 49.2 ± 2.2 | 80.3 ± 2.1 |
| Summary mean ± SD | | | | 44.0 ± 4.9 | 50.1 ± 13.0 | 89.3 ± 5.2[a] |

The apo[a] isoforms and Lp[a] levels were measured as previously described. Numbers for isoforms represent the total number of kringle IV repeats for each detectable isoform. The LDL-apoB levels were determined as described in text. The Lp[a]-Lys$^+$ fraction for plasma and for each subsequent purification step was determined by ELISA measurement of lysine-Sepharose-binding and nonbinding fractions and expressed as a percent. Values are the standard deviation calculated from a representative experiment in which measurements were performed in triplicate. Also indicated are the summary means and standard deviations for the Lp[a]-Lys$^+$ fraction at each purification stage.

[a] Significantly different from Lp[a]-Lys$^+$ fraction in plasma ($P < 0.0005$) as determined by a paired $t$-test.

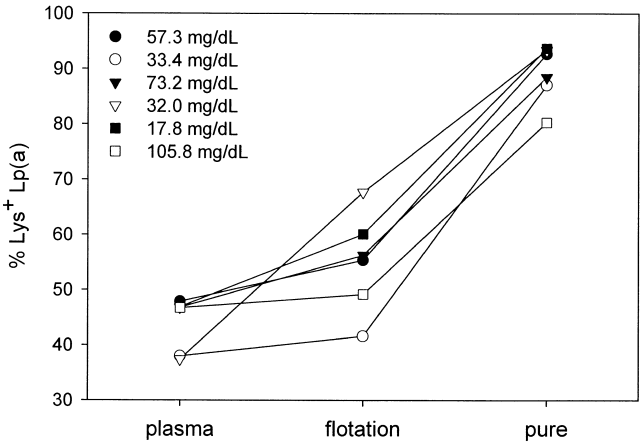


**Fig. 1.** The effect of Lp[a] purification on its ability to bind to lysine-Sepharose. The Lp[a]-Lys$^+$ binding fraction from six unrelated individuals was determined in plasma, in the lipid flotation fraction, and after gel exclusion and ion-exchange chromatography. The percentage of Lp[a]-Lys$^+$ (i.e., that which bound specifically to lysine-Sepharose) was determined at each stage by ELISA as described in text. Plasma Lp[a] concentrations are given for each individual as well as apo(a) isoform sizes.

individuals were determined by ELISA as previously described (15). As summarized in **Table 1**, Lp[a] levels varied in these subjects from ~18 to ~106 mg/dl. In addition, we determined apo[a] isoform sizes (see Table 1), which ranged from 15 to 23 kringle IV repeats. We measured, by ELISA, the ability of Lp[a] to bind to lysine-Sepharose during different stages of the purification process (see Table 1). In all cases, compared with the Lp[a]-Lys$^+$ fraction observed in the plasma samples (ranging from approximately 37 to 48%), the Lp[a]-Lys$^+$ fraction observed after lipoprotein flotation increased; the increase ranged from 5 to 81%. In addition, in all cases, there was a further substantial increase (ranging from 38 to 109%) in the Lp[a]-Lys$^+$ fraction in the final purified Lp[a] samples (see Table 1 and **Fig. 1**).

We hypothesized that the increase in lysine-binding ability of Lp[a] observed as a function of its state of purification may be attributable to noncovalent interactions between Lp[a] and other plasma constituents. Two such candidate plasma components to which Lp[a] has been shown to bind and that are present in plasma in sufficient abundance are fibronectin and LDL. LDL would of course be present in the lipoprotein flotation fraction. In our purification scheme, LDL is separated from Lp[a] by ion-exchange chromatography (**Fig. 2**), being detected in peak I of the DEAE-Sepharose elution profile (data not shown). We were also able to detect fibronectin in the lipid flotation fraction by Western blot analysis (**Fig. 3**). Fibronectin was not detectable by this analysis in the pure Lp[a] fraction obtained after DEAE-Sepharose chromatography (Fig. 3), nor was it present in peak I of the DEAE column profile (data not shown). This suggests that the fibronectin was largely removed during the gel-filtration step.

### Effect of the addition of exogenous LDL and fibronectin to purified Lp[a] on its ability to bind to lysine-Sepharose

To assess whether the presence of LDL and fibronectin in the plasma and lipoprotein flotation fractions could account for the reduced ability of Lp[a] in these fractions to bind to lysine-Sepharose, we determined the ability of purified Lp[a] to bind to this resin in the presence of an equimolar amount of either purified LDL or purified fibronectin. The results, which are summarized in **Table 2**, clearly demonstrate that the addition of either LDL or fibronectin to Lp[a] markedly decreases its ability to bind to lysine-Sepharose. Interestingly, a combination of these proteins with purified Lp[a] in a 1:1:1 molar ratio further decreases the binding of Lp[a] to lysine-Sepharose.

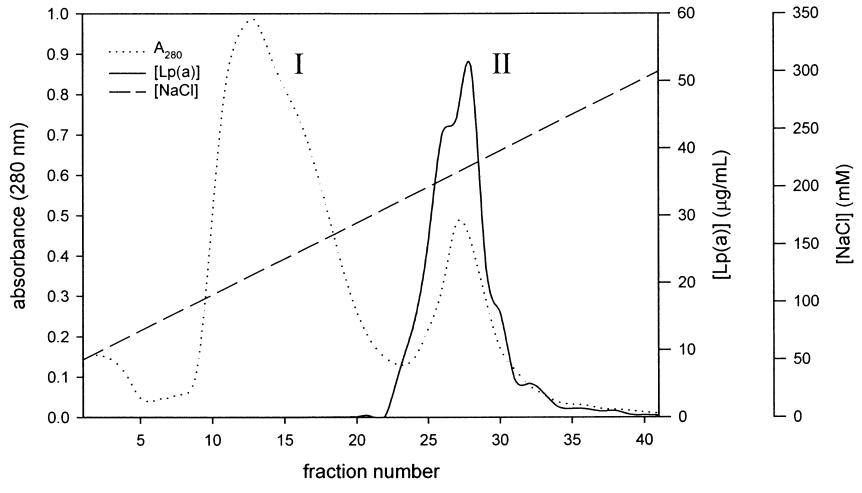


**Fig. 2.** Column profile of Lp[a] chromatographed over DEAE-Sepharose. Lp[a]-containing fractions from the gel-filtration column were loaded onto a DEAE-Sepharose Fast Flow ion-exchange column (2.5 × 2.5 cm). Protein content in the fractions was measured by absorbance at 280 nm (dotted line). Bound Lp[a] was eluted with a linear gradient of NaCl (50 to 300 mM) (dashed line). Lp[a]-containing fractions were identified by ELISA (solid line) and these fractions were pooled and dialyzed against PBS. The presence of LDL but not fibronectin in peak I was determined by SDS-PAGE and Western blotting (data not shown).

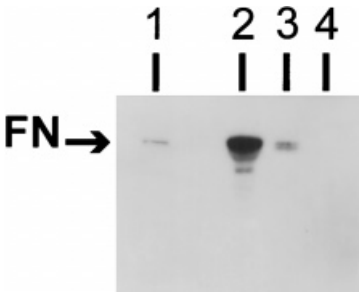


**Fig. 3.** Demonstration of fibronectin in the lipid flotation fraction. Plasma was subjected to ultracentrifugation as described in Experimental Procedures. The Lp[a] concentration in the lipid flotation fraction was determined by ELISA. Purified fibronectin (52 ng) (lane 1), lipid flotation samples containing 240 ng of Lp[a] (lane 2) or 60 ng of Lp[a] (lane 3), and 60 ng of purified Lp[a] (after ion-exchange column chromatography) (lane 4) were electrophoresed on a 4–15% gradient gel and transferred to a polyvinylidene difluoride (PVDF) membrane. The blot was probed with a polyclonal sheep anti-human fibronectin antibody and immunoreactive bands were visualized by chemiluminescence.

### Effect of LDL and fibronectin on the binding of r-apo[a] to lysine-Sepharose

We determined the effect of LDL and fibronectin on the ability of metabolically labeled 17KΔCys to bind to lysine-Sepharose columns. As expected on the basis of our previous studies (11), we found that 100% of the r-apo[a] bound to the lysine-Sepharose column as shown in **Fig. 4A**. However, on the addition of a 4-fold molar excess of LDL, the amount of r-apo[a] bound to the lysine-Sepharose column was reduced to ~51% (Fig. 4B). Incubation of r-apo[a] with a 4-fold molar excess of fibronectin resulted in ~77% of the r-apo[a] bound to the column (Fig. 4C). The addition of a 10-fold molar excess of bovine serum albumin had no effect on the ability of the 17KΔCys r-apo[a] to bind to lysine-Sepharose in the absence of LDL or fibronectin (data not shown).

Experiments were also performed in which metabolically labeled 17K was incubated with an ~4-fold molar excess of LDL. In these experiments, a population of covalent r-Lp[a] particles (representing ~50% of the r-apo[a]) was formed; approximately 50% of the covalent r-Lp[a] particles were capable of binding to the lysine-Sepharose column (data not shown).

TABLE 2. Effect of LDL and fibronectin on lysine-binding of Lp[a]

| | Lp[a]-Lys$^+$ | | | |
|---|---|---|---|---|
| Donor | Pure | 1:1 Lp[a]:LDL | 1:1 Lp[a]:Fn | 1:1:1 Lp[a]:LDL:Fn |
| | | % | | |
| 1 | 92.7 ± 0.1 | 61.3 ± 0.7 | 74.6 ± 1.3 | 51.4 ± 1.2 |
| 6 | 80.3 ± 2.1 | 74.1 ± 0.3 | 72.7 ± 0.7 | 64.4 ± 0.9 |

Purified LDL or fibronectin (Fn) were added to purified Lp[a] from donors 1 and 6 and the percentage of Lp[a] binding to lysine-Sepharose (i.e., Lp[a]-Lys$^+$) was determined as described in text.

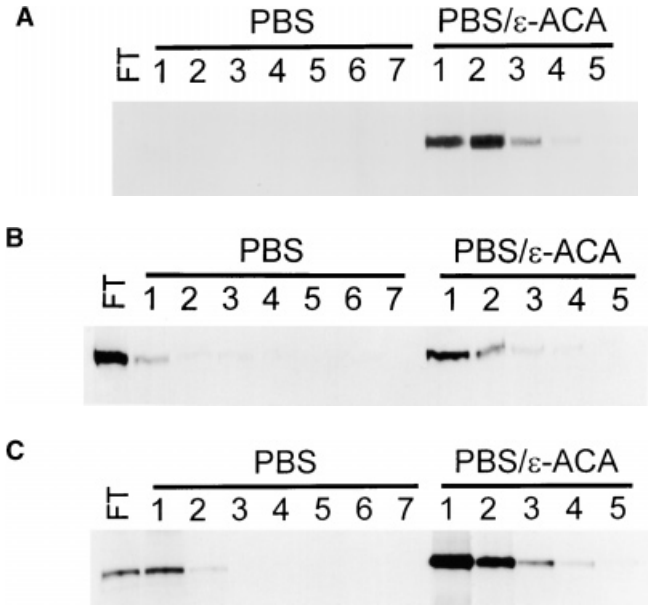


**Fig. 4.** Effect of LDL and fibronectin on the ability of r-apo[a] to bind to lysine-Sepharose. 293 cells were transiently transfected with an expression plasmid encoding the 17KΔCys r-apo[a] derivative. Cells were metabolically labeled posttransfection and CM was harvested 3.5 h postlabeling. CM (1 ml) was chromatographed over lysine-Sepharose with no additions (A) or in the presence of a 4-fold molar excess of LDL (B) or fibronectin (C). Columns were washed with 7 column volumes of PBS; specifically bound material was eluted with 5 column volumes of PBS containing 0.2 M ε-ACA. Each column fraction including the flowthrough (lane FT), PBS wash fractions (lanes 1–7), and the PBS/ε-ACA elution fractions (lanes 1–5) was immunoprecipitated with the apo[a]-specific monoclonal antibody 2G7. Immunoprecipitates were subjected to SDS-PAGE on 6% gels and exposed to film. The percentage of r-apo(a) bound to the column (i.e., Lys$^+$ apo[a]) was determined by densitometric measurement of the amount of apo[a] present in the elution fractions, divided by the total amount of apo[a] present in the FT, wash, and elution fractions.

## DISCUSSION

Although elevated plasma concentrations of Lp[a] have been correlated with an increased risk for atherosclerotic disorders, the mechanisms by which Lp[a] mediates its pathogenic effects remain unclear at present. Lp[a] studies are complicated by the structural heterogeneity that characterizes this lipoprotein. In addition to isoform size heterogeneity, it has been reported that plasma Lp[a] is heterogeneous with respect to its ability to bind to lysine-Sepharose (12, 13). In this regard, both binding and nonbinding species have been identified. Leerink and colleagues (13) have reported that the size of the lysine-binding Lp[a] fraction (i.e., Lp[a]-Lys$^+$) is donor dependent, but is independent of apo[a] isoform size. Although the functional significance of these two Lp[a] populations in vivo remains unclear, in vitro studies have suggested that compared with the Lp[a]-Lys$^+$ fraction, the Lp[a]-Lys$^-$ fraction does not inhibit plasminogen activation and does not bind to CNBr-digested fibrinogen (13). Clearly, large prospective studies are required to assess the

significance of the Lp[a]-Lys$^+$/Lys$^-$ fractions in the determination of vascular risk attributable to Lp[a].

The molecular basis of lysine-binding heterogeneity of Lp[a] is currently not understood and therefore formed the basis of our present investigation. Initially, we analyzed the lysine-binding properties of plasma Lp[a] in six unrelated individuals. The plasma Lp[a]-Lys$^+$ fraction did not appear to correlate with apo[a] isoform sizes of the donors (see Table 1), which is in keeping with the findings of Leerink and co-workers (13). In our study, on subsequent purification of the plasma Lp[a] by ultracentrifugation and column chromatography, the Lp[a]-Lys$^+$ fraction was found to increase with each purification step. We interpreted this to suggest that proteins present in plasma and retained in subsequent purification steps can interact with Lp[a] and interfere with its lysine-binding properties. The study by Leerink and co-workers (13) reported a broad range for the Lp[a]-Lys$^+$ fraction (17 to 91% in eight donors). The Lp[a]-Lys$^+$ fraction measured in a density fraction $1.055 < d < 1.15$ g/ml by Armstrong et al. (12) ranged from 40 to 81% in five donors. We found that the Lp[a]-Lys$^+$ fraction in six donors ranged from ~37 to ~48% in plasma, from ~42 to ~68% in the lipid flotation fraction, and from ~80 to ~94% in the purified Lp[a] preparation (Table 1). We did not study the lysine-binding properties of Lp[a] isolated in a comparable density range by density gradient ultracentrifugation, thereby complicating direct comparisons of the data in our study with that in the aforementioned studies.

It should be pointed out that a variable fraction of our purified Lp[a] species (from ~6 to ~20%; Table 1) did not bind to lysine-Sepharose, despite its apparent purity as assessed by SDS-PAGE followed by silver staining (data not shown). Thus, some minor variation in lysine-binding capacity, inherent in the Lp[a] molecule, may exist. This may reflect differences in glycosylation or in the degree of oxidation of the apo[a] moiety; oxidation of Lp[a] in vitro has been shown to significantly decrease its ability to bind to lysine-Sepharose (24). Alternatively, purified Lp[a] may self-associate to some extent, thereby masking its lysine-binding capability.

Because the lysine-binding properties of Lp[a] appear to be conferred by apo $KIV_{10}$ (11), we speculate that proteins may interact with plasma Lp[a] in such a way as to mask the LBS present in this kringle. On the basis of previous studies, we considered the possibility that either fibronectin or LDL, both of which are abundant in human plasma, could function in this capacity. Both our group (20) as well as Salonen and co-workers (25) have documented the interaction of apo[a]/Lp[a] with fibronectin. Although this interaction is not lysine dependent, incubation of Lp[a] and fibronectin in a 1:1 molar ratio decreases the lysine-binding ability of purified Lp[a] (see Table 2); similar results were observed when using a 5:1 molar ratio of fibronectin to Lp[a] (data not shown). To determine whether this behavior could be attributed to the apo[a] portion of Lp[a], we utilized a previously characterized recombinant expression system for apo[a]. We utilized the 17KΔCys derivative such that we could avoid the formation of covalent apo[a] homodimers in solution, which would have complicated our analysis. Similar to what we observed when purified Lp[a] was mixed with fibronectin, when we incubated CM containing labeled r-apo[a] with purified human fibronectin and chromatographed the mixture over lysine-Sepharose, the fraction of apo[a] that bound to this resin was decreased (Fig. 4). This suggests that the fibronectin can interact noncovalently with apo[a], thereby masking the LBS in $KIV_{10}$. The mechanism of this masking effect is unclear, however, because we have demonstrated that the $KIV_{10}$ sequence is not required for fibronectin-apo[a] interactions (M. L. Koschinsky, unpublished results). We would speculate that the binding of apo[a] to fibronectin results in steric blocking of LBS in $KIV_{10}$.

The interaction of Lp[a] with apoB-containing particles including LDL has been extensively characterized by Trieu and co-workers (26). The Lp[a]-LDL interaction can be inhibited in vitro by the addition of proline and involves the one or more of the kringle IV domains in apo[a]. In the Lp[a] purification procedure utilized in our study, we employed density gradient ultracentrifugation to obtain a crude lipoprotein fraction, and then subjected this material to size-exclusion chromatography followed by ion-exchange chromatography. In the size-exclusion step, we resuspended the lipoprotein fraction in a buffer containing 0.1 M proline to promote dissociation of the Lp[a]-LDL complex and developed the column in this buffer (see Experimental Procedures). However, we found that after this step, there still remained contaminating LDL that was separated from Lp[a] by chromatography over DEAE-Sepharose utilizing a gradient of NaCl concentration. Although the precise sequence in apo[a] that mediates the Lp[a]:LDL interaction is unclear at present, it is possible that $KIV_{10}$ may play a role in the context of the Lp[a] particle. As such, apo[a] in the context of Lp[a]-LDL complexes may lose the ability to bind to lysine-Sepharose. Evidence of this is provided by the results of experiments in which we found that the addition of LDL to purified Lp[a] in a 1:1 molar ratio reduced the lysine-binding ability of the Lp[a] (Table 2); addition of LDL in a 5:1 molar ratio with Lp[a] yielded similar results (data not shown). Comparable results were also observed when using an r-apo[a] expression system to form noncovalent r-apo[a]-LDL complexes (Fig. 4).

Given the amounts of LDL and fibronectin in plasma (>1 and ~0.5 μM, respectively) compared with the molar concentration of Lp[a] (~0.3 μM for an individual with Lp[a] at 30 mg/dl and a 17K-sized apo[a] isoform), as well as the respective dissociation constants for these binding interactions [10 nM for LDL-Lp[a] binding (26) and 1 μM for apo[a]-fibronectin binding (M. L. Koschinsky, unpublished data)], it is likely that at least some of the Lp[a] in plasma is complexed to either LDL or fibronectin or both in vivo. In addition, Lp[a] has been shown to bind to other abundant plasma proteins including plasminogen (27). It is likely the totality of the interactions between apo[a] and other plasma proteins that dictates the size of the Lp[a]-Lys$^+$ fraction in plasma. Why this fraction varies

among individuals remains to be investigated. To address this question, future studies in a larger population should include the examination of a possible relationship between the size of the Lp[a]-Lys$^+$ fraction and plasma levels of plasminogen, LDL-apoB, LDL-cholesterol, fibronectin, and fibrinogen. In addition, because studies to date have focused on individuals with Lp[a] levels greater than 30 mg/dl (12, 13), it would be of interest to quantify the Lp[a]-Lys$^+$ in individuals with plasma concentrations less than 10 mg/dl. On the basis of our findings, we would predict that the Lp[a]-Lys$^+$ fraction may be low due to the larger molar excess of plasma proteins such as fibronectin in these individuals.

In conclusion, we have provided evidence that the lysine-binding heterogeneity of plasma Lp[a] is for the most part not an intrinsic property of the lipoprotein, but rather is a function of the binding of Lp[a] to other plasma proteins such as LDL and fibronectin. The significance of lysine-binding and non-lysine-binding Lp[a] populations in the plasma is unclear, however, because the relative contribution of Lp[a]-Lys$^+$ versus Lp[a]-Lys$^-$ to the atherosclerotic process has not been assessed. Indeed, mechanisms of action for Lp[a] pathogenicity involving both lysine- and non-lysine-binding functions of Lp[a] have been proposed (1, 2). Clearly, however, the ability of Lp[a] to bind to LDL and/or fibronectin in plasma may provide an additional means by which Lp[a] can be delivered to the arterial wall in a piggy-back fashion.

The authors wish to thank Dr. Murray Huff (Robarts Research Institute, London, Ontario, Canada) for LDL-apoB determinations. This work was supported by a grant from the Heart and Stroke Foundation of Ontario (#T3395).

*Manuscript received 4 January 2000 and in revised form 12 May 2000.*

## REFERENCES

1. Koschinsky, M. L., and S. M. Marcovina. 1997. Lipoprotein(a): structural implications for pathophysiology. *Int. J. Clin. Lab. Res.* **27:** 14–23.
2. Marcovina, S. M., and M. L. Koschinsky. 1998. Lipoprotein(a) as a risk factor for coronary artery disease. *Am J. Cardiol.* **82:** 57U–66U.
3. Koschinsky, M. L., G. P. Côté, B. Gabel, and Y. Y. van der Hoek. 1993. Identification of the cysteine residue in apolipoprotein(a) that mediates extracellular coupling with apolipoprotein B-100. *J. Biol. Chem.* **268:** 19819–19825.
4. Brunner, C., H-G. Kraft, G. Utermann, and H-J. Muller. 1993. Cys4057 of apolipoprotein(a) is essential for lipoprotein(a) assembly. *Proc. Natl. Acad. Sci. USA.* **90:** 11643–11647.
5. McLean, J. W., J. E. Tomlinson, W. J. Kuang, D. L. Eaton, E. Y. Chen, G. M. Fless, A. M. Scanu, and R. M. Lawn. 1987. cDNA sequence of human apolipoprotein(a) is homologous to plasminogen. *Nature.* **330:** 132–137.
6. Lackner, C., J. C. Cohen, and H. H. Hobbs. 1993. Molecular definition of the extreme size polymorphism in apolipoprotein(a). *Hum. Mol. Genet.* **2:** 933–940.
7. Van der Hoek, Y. Y., M. E. Wittekoek, U. Beisiegel, J. J. P. Kastelein, and M. L. Koschinsky. 1993. The apolipoprotein(a) kringle IV repeats which differ from the major repeat kringle are present in variably sized isoforms. *Hum. Mol. Genet.* **2:** 361–366.
8. Sangrar, W., S. M. Marcovina, and M. L. Koschinsky. 1994. Expression and characterization of apolipoprotein(a) kringle IV types 1, 2, and 10 in mammalian cells. *Protein Eng.* **7:** 723–731.
9. Harpel, P. C., B. R. Gordon, and T. S. Parker. 1989. Plasmin catalyzes binding of lipoprotein(a) to immobilized fibrinogen and fibrin. *Proc. Natl. Acad. Sci. USA.* **86:** 3847–3851.
10. Loscalzo, J., M. Weinfeld, G. M. Fless, and A. M. Scanu. 1990. Lipoprotein(a), fibrin binding and plasminogen activation. *Arterioscler. Thromb. Vasc. Biol.* **16:** 1559–1567.
11. Gabel, B. R., L. F. May, S. M. Marcovina, and M. L. Koschinsky. 1996. Lipoprotein(a) assembly. Quantitative assessment of the role of apo(a) kringle IV types 2–10 in particle formation. *Arterioscler. Thromb. Vasc. Biol.* **16:** 1559–1567.
12. Armstrong, V. W., B. Harrach, H. Robenek, M. Helmhold, A. K. Walli, and D. Seidel. 1990. Heterogeneity of human lipoprotein [a]: cytochemical and biochemical studies on the interaction of two Lp[a] species with the LDL receptor. *J. Lipid Res.* **31:** 429–441.
13. Leerink, C. B., P. F. C. C. M. Duif, J. A. Gimpel, W. Kortlandt, B. N. Bouma, and H. J. M. van Rijn. 1992. Lysine-binding heterogeneity of Lp(a): consequences for fibrin binding and inhibition of plasminogen activation. *Thromb. Haemost.* **68:** 185–188.
14. Scanu, A. M., D. Pfaffinger, J. C. Lee, and J. Hinman. 1994. A single point mutation (Trp726Arg) in human apo(a) kringle 4–37 associated with a lysine binding defect in Lp(a). *Biochim. Biophys. Acta.* **1227:** 41–45.
15. Wong, W. L. T., D. L. Eaton, A. Berloui, B. Fendly, and P. E. Hass. 1990. A monoclonal-antibody-based enzyme-linked immunosorbent assay of lipoprotein(a). *Clin. Chem.* **36:** 192–197.
16. Marcovina, S. M., Z. H. Zhang, V. P. Gaur, and J. J. Albers. 1993. Identification of 34 apolipoprotein(a) isoforms: differential expression of apolipoprotein(a) alleles between American Blacks and Whites. *Biochem. Biophys. Res. Commun.* **191:** 1192–1196.
17. Marcovina, S. M., J. J. Albers, B. Gabel, M. L. Koschinsky, and V. P. Gaur. 1995. Effect of the number of apolipoprotein(a) kringle 4 domains on immunochemical measurements of lipoprotein(a). *Clin. Chem.* **41:** 246–255.
18. Havel, R. J., H. A. Eder, and J. D. Bragdon. 1955. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* **34:** 1345–1353.
19. Huff, M. W., D. E. Telford, K. Woodcroft, and W. L. P. Strong. 1985. Mevinolin and cholestyramine inhibit the direct synthesis of low density lipoprotein apolipoprotein B in miniature pigs. *J. Lipid Res.* **26:** 1175–1186.
20. van der Hoek, Y. Y., W. Sangrar, G. P. Cote, J. J. P. Kastelein, and M. L. Koschinsky. 1994. Binding of recombinant apolipoprotein(a) to extracellular matrix proteins. *Arterioscler. Thromb.* **14:** 1792–1798.
21. Graham, F. L., and A. J. van der Eb. 1973. A new technique for the assay of infectivity of human adenovirus IV DNA. *Virology.* **52:** 456–467.
22. Koschinsky, M. L., S. M. Marcovina, L. F. May, and B. R. Gabel. 1997. Analysis of the mechanism of lipoprotein(a) assembly. *Clin. Genet.* **52:** 338–346.
23. Koschinsky, M. L., J. E. Tomlinson, T. F. Zioncheck, K. Schwartz, D. L. Eaton, and R. M. Lawn. 1991. Apolipoprotein(a): expression and characterization of a recombinant form of the protein in mammalian cells. *Biochemistry.* **30:** 5044–5051.
24. Harpel, P. C., A. Hermann, and N. Azrolan. 1995. Reduction and oxidation of lipoprotein(a) have opposite effects on thromboatherogenic effects dependent on lysine-binding. *In* Atherosclerosis. X. F. P. Woodward, J. Davignon, and A. Sniderman, editors. Elsevier Science B. V., Amsterdam. 888–890.
25. Salonen, E-M., M. Jauhiainen, L. Zardi, A. Vaheri, and C. Ehnholm. 1989. Lipoprotein(a) binds to fibronectin and has serine protease activity capable of cleaving it. *EMBO J.* **8:** 4035–4040.
26. Trieu, V. N., T. F. Zioncheck, R. M. Lawn, and W. J. McConathy. 1991. Interaction of apolipoprotein(a) with apolipoprotein B-containing lipoproteins. *J. Biol. Chem.* **266:** 5480–5485.
27. Sangrar, W., B. R. Gabel, M. B. Boffa, J. B. Walker, M A. Hancock, S. M. Marcovina, A. J. G. Horrevoets, M. E. Nesheim, and M. L Koschinsky. 1997. The solution phase interaction between apolipoprotein(a) and plasminogen inhibits the binding of plasminogen to a plasmin-modified fibrinogen surface. *Biochemistry.* **36:** 10353–10363.