Evidence that the Fibrinogen Binding Domain of Apo(a) Is Outside the Lysine Binding Site of Kringle IV-10

A Study Involving Naturally Occurring Lysine Binding Defective Lipoprotein(a) Phenotypes

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Abstract

It is now established that the lysine binding site (LBS) of apo(a) kringle IV-10, and particularly Trp72, plays a dominant role in the binding of lipoprotein(a) [Lp(a)] to lysine. To determine the role of the LBS in the binding of Lp(a) to fibrinogen, we examined the binding to plasmin-modified (PM) fibrinogen of human and rhesus monkey Lp(a) species classified as either Lys^+ or Lys^- based on their capacity to bind lysine Sepharose and to have Trp or Arg, respectively, in position 72 of the LBS of kringle IV-10. We also examined the free apo(a)s obtained by subjecting their corresponding parent Lp(a)s to a mild reductive procedure developed in our laboratory. Our results show that both Lys^+ and Lys^- Lp(a)s and their derived apo(a)s, bound to PM-fibrinogen with similar affinities (K_ds: 33–100 nM), whereas the B_{max} values were threefold higher for apo(a)s. Both the lysine analog ϵ-aminocaproic acid and L-proline inhibited the binding of Lp(a) and apo(a) to PM fibrinogen. We conclude that the LBS of kringle IV-10 is not involved in this process and that apo(a) binds to PM-fibrinogen via a lysine-proline–sensitive domain located outside the LBS and largely masked by the interaction of apo(a) with apoB100. The significant difference in the PM fibrinogen binding capacity also suggests that apo(a) may have a comparatively higher athero-thrombogenic potential than parent Lp(a). (*J. Clin. Invest.* 1996. 98:185–191.) Key words: lipoprotein(a) • apo(a) kringle IV-10 • lysine binding site • fibrinogen binding • L-proline

Introduction

Lipoprotein(a) [Lp(a)][1] is a lipoprotein particle having as a protein moiety apoB100 linked by a disulfide bridge to apo(a), a multikringle glycoprotein with a high degree of structural similarity to plasminogen (1). Like plasminogen, apo(a) binds

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1. *Abbreviations used in this paper:* EACA, ε-aminocaproic acid; LBS, lysine binding site; Lp(a), lipoprotein(a); Lys^+, lysine binding positive; Lys^-, lysine binding negative; PM, plasmin-modified.

to lysine-Sepharose (2). Previous work from this laboratory on rhesus monkeys (3) and humans (4) has provided evidence that this binding depends on a functional lysine binding site (LBS) located in apo(a) kringle IV-10. This evidence has been corroborated by studies dealing with apo(a) recombinants (5–7) and also wild-type and mutant (Trp72→Arg) kringle IV-10 expressed in our laboratory in *Escherichia coli* and CHO cells (8). Using a newly developed procedure for the selective mild reduction of Lp(a) to yield free apo(a) (9), we studied the interaction between apo(a) and apoB100 and identified a Lys-Pro–sensitive domain in apo(a), which is distant from the LBS of kringle IV-10 and important for reassembly with apoB100. A second lysine binding domain has also been proposed from the results of reassembly studies of the apo(a) recombinants and apoB100 (5, 10, 11). Lp(a) also binds to fibrin(ogen) both intact and modified by the action of plasmin (PM-fibrinogen) (12, 13), however, the apo(a) site for this binding has not yet been clearly defined except for recent studies, which have suggested the participation in this binding of the carboxyl-terminal domain obtained by subjecting apo(a) to proteolysis with thermolysin (14). Moreover, apo(a) kringle IV-10 expressed in *E. coli* has been reported to bind to PM-fibrinogen (7, 8). In contrast, no binding to intact fibrinogen was reported for kringle IV-10 expressed in 293 human kidney embryonic cells (6).

In the current work, we approached the problem of apo(a) binding to fibrinogen by examining whether the LBS of apo(a) kringle IV-10 is involved in fibrinogen binding and whether a contribution to this binding may come from the Lys-Pro–sensitive domain, which we recently identified in apo(a) released from its attachment to apoB100 of Lp(a) under mild reductive conditions (9). As a representative model for binding, we used human PM-fibrinogen based on the observation of Harpel et al. (12) that the proteolytic cleavage of fibrinogen by plasmin increases the capacity of Lp(a) to bind to this substrate. To achieve our goals we made use of (*a*) wild-type human Lp(a) with different apo(a) size isoforms capable of binding to lysine Sepharose (Lys^+) and having Trp72 in the LBS domain of apo(a) kringle IV-10; (*b*) mutant human Lp(a), lysine binding defective (Lys^-), having Arg72 instead of Trp in the LBS of apo(a) kringle IV-10; (*c*) rhesus monkey Lp(a) phenotypically Lys^- (3) with Arg72 in the LBS of apo(a) kringle IV-10 and lacking kringle V (15). We also assessed whether the PM-fibrinogen binding capacity of apo(a) may be affected by its linkage to apoB100 in Lp(a). Moreover, we compared free apo(a) and Glu-plasminogen to determine whether these two proteins may bind to PM-fibrinogen by a common mechanism.

The results of our current studies show that the PM-fibrinogen binding of apo(a) is markedly higher than that of Lp(a) and occurs in a domain which is outside the LBS of apo(a) kringle IV-10 and is likely the same domain involved in the interaction between apo(a) and apoB100 in the process of Lp(a) assembly.

Methods

Chemicals and reagents. ε-aminocaproic acid (EACA), PMSF, EDTA, L-proline, dithioerythritol, PBS, human fibrinogen, and goat anti–rabbit IgG alkaline phosphatase conjugate were obtained from Sigma Chemical Co., St. Louis, MO; Kallikrein inactivator from Calbiochem Corp., San Diego, CA; human plasmin from Enzyme Research Laboratories, South Bend, IN. Glu-plasminogen and rabbit anti-plasminogen kringle 4 antibody were a generous gift from Dr. E. Plow (Cleveland Clinic Foundation, Cleveland, OH). Rabbit affinity-purified antibodies to apo(a), Lp(a), and LDL were prepared as previously described (16). Anti-Lp(a) was devoid of immunoreactivity to LDL and plasminogen, and anti-LDL was unreactive to apo(a).

Human subjects. Information on the eight subjects studied is provided in Table I. Six of them were Lys$^+$, had a wide variation in plasma Lp(a) levels, had two expressed apo(a) alleles and were wild-type in terms of having Trp72 in apo(a) kringle IV-10. Two were Lys$^-$, had the Trp72→Arg mutation and were described before (4). All of them were in good health and had no personal history of atherosclerotic cardiovascular disease. They gave a written informed consent for their participation in the study. The blood was obtained by venipuncture in the Blood Bank of the University of Chicago.

Preparation of human wild-type and mutant Lp(a). The plasma was adjusted with 0.15% EDTA, 0.01% NaN$_3$, 10,000 U/liter Kallikrein inactivator, and 1 mM PMSF. Lys$^+$ Lp(a) was isolated by sequential ultracentrifugation and lysine-Sepharose chromatography as previously described (17). For the isolation of Lys$^-$ Lp(a), the plasma was brought to *d* 1.050 g/ml with solid NaBr, and L-proline at a final concentration of 0.1 M was added to prevent the interaction of LDL with the small amounts of Lp(a) present in these subjects (9). The solution was ultracentrifuged at 302,120 *g* for 20 h at 15°C, the floating fraction was removed, and the density of the bottom fraction adjusted to 1.10 g/ml and recentrifuged under the same conditions. Subsequently, the floating fraction was dialyzed against 10 mM Tris-HCl, pH 7.5, and subjected to FPLC ion-exchange chromatography as described previously (3). The purity of the product was assessed by mobility on precast 1% agarose gels (Ciba-Corning, Palo Alto, CA) and Western blots of SDS-PAGE, using anti-Lp(a) and anti-apoB. The purified Lp(a) were filter sterilized and stored at 4°C. An aliquot of both blood and plasma from each subject was used for apo(a) genotyping and phenotyping, respectively. In four of the subjects, K.W., B.K.,

Table I. Characteristics of Lys$^+$ and Lys$^-$ Subjects

Subject	Age	Sex*	Race‡	Lp(a) Protein	Lp(a) Lys binding	Phenotype	Genotype	Trp→Arg mutants
	yr			mg/dl		kD§	kb‖	
K. B.	41	M	A	32.30	+	289; 488	58; 113	Absent
K. W.	47	M	A	0.24	+	460; 500	90; 130	''
B. K.	57	F	C	43.20	+	333; 341	69; 74	''
L.H.	58	M	A	7.28	+	480; 500	115; 130	''
A. D.	74	F	C	0.18	+	450; 490	80; 115	''
M. T.	19	F	C	25.94	+	340; 500	75; 115	''
B. M.	34	F	C	0.18	−	480	111	Present
T. T.	34	M	C	0.16	−	279	56	''

*M, male; F, female; ‡A, African-American; C, Caucasian; §The molecular weights of each of the two phenotypes from K.B. and one of the phenotypes from B.K. (341 kD) were measured in the analytical ultracentrifuge (17) and included the weight of the carbohydrates. The size of the phenotypes from the remaining subjects was estimated on Western blots of reduced SDS-PAGE. ‖Allele size was estimated by PFGE in 1% agarose gels using the mobilities of lambda DNA standards in a 48.5-kb ladder encompassing a size range of 0.05–1 mb.

L.H., and A.D., the two apo(a) phenotypes were close in size (Table I); thus Lp(a) species with a single phenotype could not be separated by the procedure used. On the other hand, in the case of K.B. and M.T., the high differential in apo(a) size and thus Lp(a) density permitted the separation of two Lp(a)s, one containing the 289-kD and the other the 488-kD apo(a) isoform from K.B. Lp(a) and one containing the 340-kD and the other the 500-kD apo(a) isoform from M.T. Lp(a). The technique used was an adaptation of that described by Fless et al. (18).

Preparation of rhesus monkey Lp(a). The four rhesus monkeys studied were from the same pedigree previously described (3). All had high plasma Lp(a) protein levels varying between 20 and 40 mg/dl, exhibited a single apo(a) phenotype, and were housed at the Southwest Foundation for Biomedical Research in San Antonio, TX. The monkeys were fasted overnight before collecting 20 ml of venous blood in tubes containing 0.01% EDTA, and the plasma prepared by low speed ultracentrifugation (3). Subsequently, the plasma was shipped to Chicago overnight in ice. The isolation of Lp(a) followed the procedure essentially as described previously (3) with the following modifications: The plasma was spun at 302,120 *g* at 10°C for 20 h at *d* 1.050 g/ml. After removal of the top layer, the infranatant was adjusted to *d* 1.070 g/ml with solid NaBr and spun under the same conditions. The top layer containing mainly Lp(a) was removed, dialyzed against 10 mM Tris-HCl, 0.01% EDTA, 0.01% NaN$_3$, and subjected to FPLC ion-exchange chromatography as fully described by Scanu et al. (3). The isolated Lp(a) was filter sterilized and stored refrigerated in 33 mM phosphate buffer containing 2 mM PMSF, 0.15% EDTA, and 0.01% NaN$_3$, pH 7.5, under nitrogen.

Isolation of apo(a) from Lp(a). Apo(a) was obtained from Lp(a) by the technique described by Edelstein et al. (9). This technique makes use of a mild reductive procedure to cleave the disulfide bond linking apo(a) to apoB100 without affecting the stability and function of apo(a). Briefly, we incubated Lp(a) with 2 mM dithioerythritol in the presence of 0.1 M EACA for 1 h under argon gas at room temperature. Subsequently, the incubated mixture was dialyzed and ultracentrifuged at 412,160 *g* at 10°C overnight in a 30% sucrose solution containing 0.1 M EACA using a TLA 100.3 titanium rotor in a table top TL100 ultracentrifuge. After centrifugation, the bottom 1.0-ml fraction containing the free apo(a) was dialyzed against the appropriate buffer or alternatively stored in the sucrose solution at −80°C until use. The purity of the free apo(a) was examined by Western blots of SDS-PAGE gels using anti-Lp(a) and anti-LDL as probes. The free apo(a) bound to lysine-Sepharose and was eluted from it with EACA.

Fibrinogen binding assay. The fibrinogen was immobilized on microtiter plates and either used as such in the assay or treated with plasmin following the method described by Harpel et al. (12). Various concentrations of Lp(a), free apo(a), or Glu-plasminogen were added to the wells in TBS buffer (50 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 0.1% BSA) with or without 0.2 M EACA or 0.2 M L-proline and incubated overnight at 22°C. For some experiments, a constant amount (10 nM) of Lp(a) and apo(a) was incubated with PM-fibrinogen in the presence of various concentrations of EACA. After washing with TBST buffer (TBS supplemented with 0.01% Tween-20), the detecting antibody, either rabbit anti-apo(a) (1:2,000) or rabbit anti-plasminogen kringle 4 (1:100) was added and incubated for 1 h at 22°C. At this time, the wells were washed with TBST and the goat anti–rabbit IgG conjugated to alkaline phosphatase (1:2,000) was added for 1 h at 22°C. After washing, *p*-nitrophenyl phosphate (1 mg/ml in diethanolamine buffer, pH 9.8) was added, and the color development was followed at 405 nm on a microplate reader (Biomek 100; Beckman Instruments Inc., Fullerton, CA). Analysis of binding data was performed on the assumption of single-site binding. Dissociation constants (K_d) were derived from the slope of the linearized expression of the Langmuir equation (19):

$$[Fn \bullet X] = [Fn_0] \frac{K[X]}{(1 + K[X])} \tag{1}$$

where [Fn$_0$] represents the total number of fibrinogen binding sites, [Fn•X] the number of molecules of Lp(a) or apo(a) adsorbed on fibrinogen, [X] concentration of Lp(a) or apo(a), and K the association constant.

Quantification of Lp(a) and apo(a). Lp(a) protein was quantitated by a sandwich ELISA essentially as previously described (16) except that anti-Lp(a) IgG was used as the capture antibody and anti-LDL IgG conjugated to alkaline phosphatase as the detection antibody. For the ELISA quantitation of apo(a), anti-apo(a) IgG conjugated to alkaline phosphatase was used as the detection antibody.

Phenotyping and genotyping of apo(a). Apo(a) phenotyping was performed on either plasma, isolated apo(a), or Lp(a) samples in the presence of 2-mercaptoethanol by SDS-PAGE followed by immunoblotting using anti-Lp(a) (9). The mobility of the individual apo(a) bands was compared with isolated apo(a) isoforms of known molecular weights (17). For apo(a) genotyping, DNA plugs were prepared from blood mononuclear cells and subjected to digestion with restriction enzymes. The obtained DNA fragments were fractionated by pulsed-field gel electrophoresis and identified with an apo(a)-specific probe essentially as described earlier (20).

Results

Comparative binding of human Lys$^+$ Lp(a) and derived apo(a) to fibrinogen and PM-fibrinogen. These assays were carried out in the early phase of our studies based on the report by Harpel et al. (12) that the binding of Lp(a) to intact fibrinogen is approximately one-third of that observed with PM-fibrinogen. Our results confirmed those observations. Moreover, we performed control experiments where Lp(a) and apo(a) were incubated with plasmin-treated immobilized albumin. We found that neither Lp(a) nor apo(a) bound to this substrate (data not shown), a result in agreement with the data of Harpel et al. (12). Based on these findings, we used only PM-fibrinogen in all subsequent studies.

Binding of human Lys$^+$ Lp(a) and derived apo(a) to PM-fibrinogen. As shown by the representative binding isotherms in Fig. 1 (*left* and *right panels*), the total binding of Lp(a) and apo(a) to PM-fibrinogen was concentration-dependent and approached saturation at a protein concentration of 120 nM. In both cases the apparent K_d was in the range of 35–87 nM (Table II); however, the binding of apo(a) was about threefold higher than that of Lp(a). Since the binding of Lp(a) and apo(a) to PM-fibrinogen was previously shown to be lysine-dependent (12, 13), we also performed the binding assays in the presence of the lysine analogue, EACA. Under our experimental conditions, EACA at a concentration of 0.2 M caused a marked decrease of the binding of Lp(a) and apo(a) to PM-fibrinogen, 70% and 90%, respectively (Fig. 1, *top panels*). Moreover, we carried out binding experiments in the presence of L-proline based on the observation of Trieu et al. (11) that this amino acid can inhibit the interaction of apo(a) with apoB100-containing lipoproteins. Under the conditions of our assays, L-proline at a concentration of 0.2 M inhibited the binding of Lp(a) and apo(a), 50 and 70%, respectively (Fig. 1, *top panels*). When both proline and EACA, each at 0.2 M, were added together to the reaction system, their inhibitory effect on the binding of Lp(a) and apo(a) to PM-fibrinogen was in each case equal to that of 0.2 M EACA (data not shown), indicating that EACA is a more potent inhibitor than proline and that these reagents may have an inhibitory component in common. To determine the specific lysine-mediated (EACA-inhibitable) and proline-mediated binding of Lp(a) and apo(a)
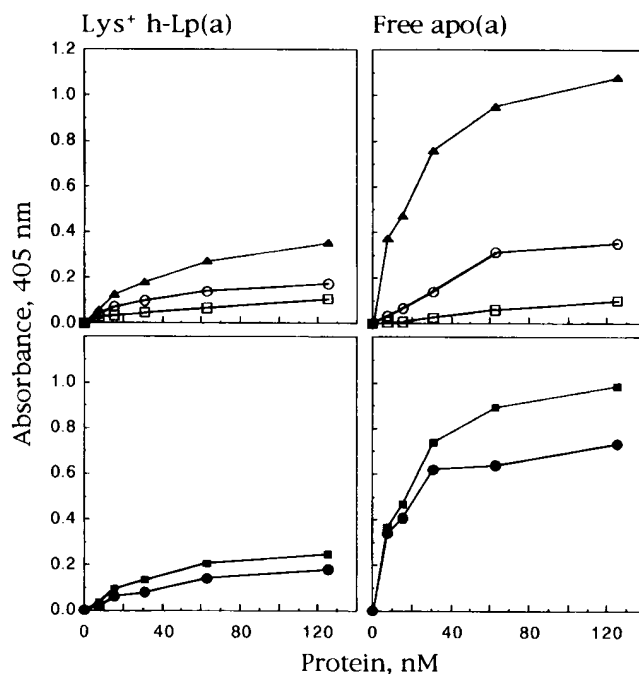
Figure 1. Binding of human Lys$^+$ Lp(a) and derived apo(a) to PM-fibrinogen. The concentrations of Lys$^+$ Lp(a) and derived apo(a) were expressed in terms of apo(a) molarity and ranged between 0 and 125 nM. The proteins were incubated with immobilized PM-fibrinogen overnight at 22°C either in the presence or in the absence of 0.2 M EACA and 0.2 M L-proline, and the amount bound was detected with anti-apo(a) antibody as described in Methods. *Left panels*: human Lys$^+$ Lp(a) [Lys$^+$ h-Lp(a)]. *Right panels*: free apo(a). *Top panels*: total binding (▲), binding in the presence of 0.2 M EACA (□), 0.2 M proline (○). *Lower panels*: EACA-inhibitable, or lysine-mediated binding (■), proline-mediated binding (●) obtained by subtracting the binding in the presence of either EACA or proline from the total binding. The data are the means of two determinations for a representative experiment conducted with Lp(a) and apo(a) isolated from a single donor.

to PM-fibrinogen, each data point on the binding curve obtained in the presence of either 0.2 M EACA or 0.2 M proline was subtracted from the respective values of the total binding curve (Fig. 1, *bottom panels*). The results show that the lysine- and proline-mediated binding of free apo(a) was about fourfold higher than that of Lp(a). In contrast, the binding affinities of Lp(a) and apo(a) for PM-fibrinogen were comparable (see K_d values in Table II). The results of these studies were not affected by apo(a) size polymorphism.

To determine whether the difference between Lp(a) and apo(a) binding to PM-fibrinogen was related to the long time of incubation (overnight), the binding experiments were carried out for 2 and 4 h at 22°C. In all cases there was an important difference in the B_{max} values between apo(a) and Lp(a), whereas the apparent K_ds were in the same order of magnitude (data not shown).

Binding of human Lys$^−$ Lp(a) and derived apo(a) to PM-fibrinogen. To determine whether the LBS of apo(a) kringle IV-10 plays a role in the binding of Lp(a) and apo(a) to PM-fibrinogen, we studied two subjects previously shown (4) to be Lys$^−$ and to have a Trp72→Arg mutation in the LBS of apo(a) kringle IV-10 (see subjects B.M. and T.T. in Table I). As

Sample	Total	Lys-mediated*	Pro-mediated‡
		K^d, nM§	
Lys$^+$ human			
Lp(a)	$35.1\pm17.1(8)$	$43.5\pm30.0(8)$	$71.7\pm32.8(2)$
apo(a)	$87.2\pm27.3(8)$	$85.5\pm29.1(8)$	$35.3\pm12.1(4)$
Lys$^-$ human			
Lp(a)	$99.9\pm18.3(4)$	$93.8\pm49.2(4)$	$85.3\pm19.2(2)$
apo(a)	$33.3\pm8.3(2)$	$34.3\pm15.2(2)$	$35.4\pm12.3(2)$
Rhesus monkey			
Lp(a)	$54.8\pm5.1(6)$	$51.1\pm11.2(6)$	$43.0\pm8.7(4)$
apo(a)	$77.8\pm34.6(6)$	$45.1\pm17.8(2)$	$49.7\pm29.6(4)$

*Lys-mediated binding was obtained by subtracting the binding in the presence of 0.2 M EACA from the total binding. ‡Pro-mediated binding was obtained by subtracting the binding in the presence of 0.2 M proline from the total binding. §Dissociation constants (K_d) are the mean$\pm$SD of the data obtained for Lp(a) and apo(a) isolated from several donors described in Table I (human) and Methods (rhesus monkey). The values in parentheses represent the number of independent experiments.

shown in Fig. 2, Lys$^-$ Lp(a) and derived apo(a) bound to PM-fibrinogen in a manner similar to that of the Lys$^+$ products both in terms of total and lysine- and proline-mediated binding, except that both EACA and proline inhibited the binding to the same extent. Similarly, there was an important differ-
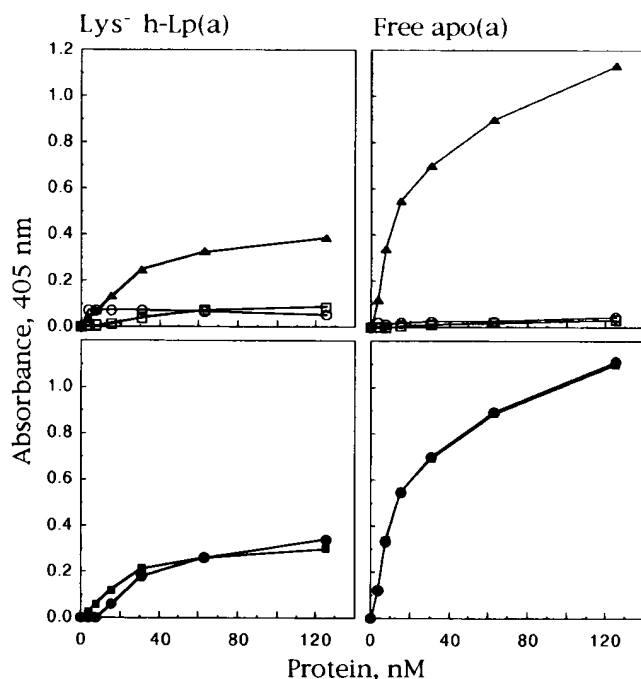
ence in the B_{max} values between apo(a) and Lp(a) whereas the apparent K_ds were comparable (Table II).

The time of incubation with PM-fibrinogen (2 h, 4 h, or overnight at 22°C) had no effect on the difference in the B_{max} values between Lys$^-$ Lp(a) and derived apo(a), as in the case of their Lys$^+$ human counterparts. In the same vein, there was no effect on the apparent K_ds (data not shown).

Binding of rhesus Lp(a) and derived apo(a) to PM-fibrinogen. Rhesus Lp(a) and derived apo(a) exhibited the same binding behavior to PM-fibrinogen as the corresponding human products (Fig. 3, left and right panels). The total binding of free apo(a) was 4-fold higher than that of Lp(a). Of note, the binding of both Lp(a) and apo(a) was almost completely abolished by the presence of 0.2 M EACA. Proline, 0.2 M, completely inhibited the binding of Lp(a), and decreased the binding of apo(a) by 75%. Moreover, the lysine- and proline-mediated binding of apo(a) was much greater than that of Lp(a), 3.8- and 3-fold, respectively. The apparent K_ds for the total, lysine- and proline-mediated binding of both Lp(a) and apo(a) were in the same order of magnitude (Table II).

Inhibitory effect of EACA on the binding of Lp(a) and apo(a) to PM-fibrinogen. To further define the potential functional relationships between Lys$^+$ and Lys$^-$ species in terms of their PM-fibrinogen binding capacities, experiments were performed in the presence of various concentrations of EACA (Fig. 4). To this end, 10 nM human Lys$^+$ Lp(a), Lys$^-$ Lp(a), rhesus Lp(a) and corresponding apo(a) derivatives were incubated with PM-fibrinogen in the presence of 0–100 mM EACA. As shown in Fig. 4, *top*, EACA specifically decreased the binding of Lp(a) to PM-fibrinogen in a concentration-dependent manner. The IC$_{50}$ was 5 mM for human Lys$^+$ Lp(a),

Figure 2. Binding of human Lys$^-$ Lp(a) and derived apo(a) to PM-fibrinogen. Conditions for the binding assays were as described in the legend to Fig. 1. *Left panels*: human Lys$^-$ Lp(a) [Lys$^-$ h-Lp(a)]. *Right panels*: free apo(a). *Top panels*: total binding (▲), binding in the presence of 0.2 M EACA (□), 0.2 M proline (○). *Bottom panels*: EACA-inhibitable, or lysine-mediated binding (■), proline-mediated binding (●).
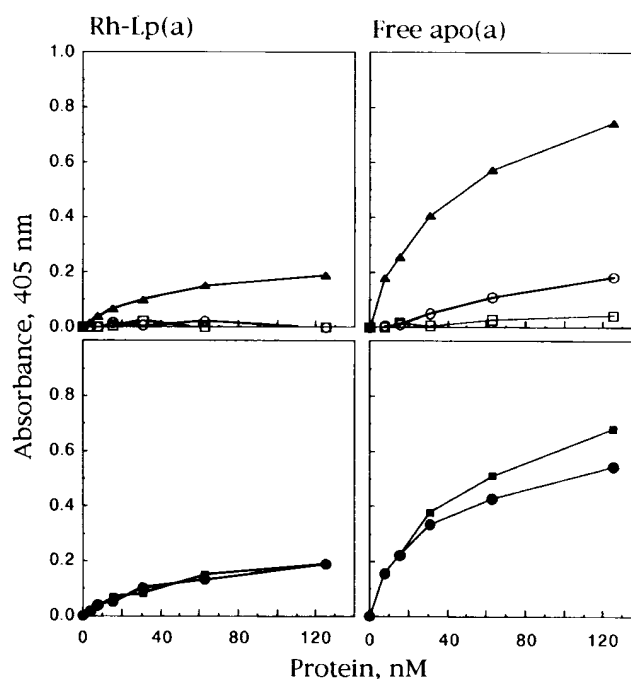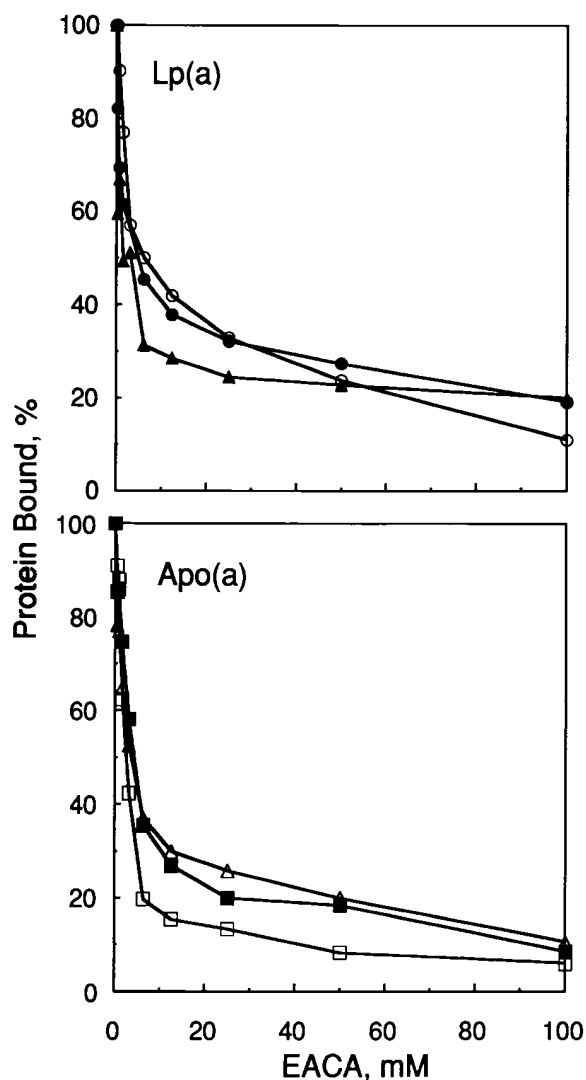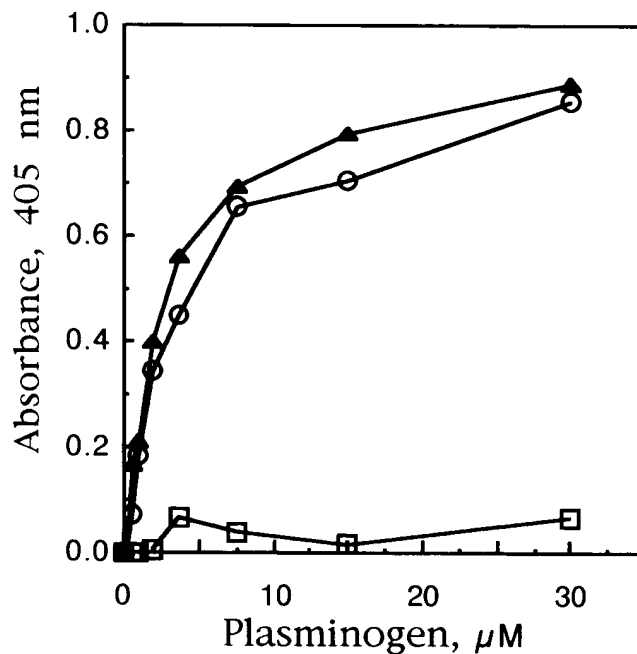

Figure 3. Binding of rhesus monkey Lp(a) and derived apo(a) to PM-fibrinogen. Conditions for the binding assays were as described in the legend to Fig. 1. *Left panels*: rhesus Lp(a) [Rh-Lp(a)]. *Right panels*: free apo(a). *Top panels*: total binding (▲), binding in the presence of 0.2 M EACA (□), 0.2 M proline (○). *Bottom panels*: EACA-inhibitable, or lysine-mediated binding (■), proline-mediated binding (●).

Figure 5. Binding of Glu-plasminogen to PM-fibrinogen. Glu-plasminogen at the indicated concentrations (0–30 μM) was incubated with immobilized PM-fibrinogen in either the absence ($\blacktriangle$) or in the presence of 0.2 M EACA ($\square$) and 0.2 M proline ($\bigcirc$). The data are the means of two determinations for a given experiment.

Figure 4. Inhibitory effect of EACA on the binding of Lp(a) and apo(a) to PM-fibrinogen. 10 nM human Lys^+ Lp(a), human Lys^- Lp(a), rhesus Lp(a), or corresponding apo(a) derivatives were incubated with PM-fibrinogen in the presence of various concentrations of EACA (0–100 mM). The Lp(a) concentration is expressed in terms of apo(a) molarity. The amount of the protein bound to PM-fibrinogen was determined as described in Methods and expressed as percentage of the protein which bound in the absence of the inhibitor. The data are the means of two determinations for a representative experiment conducted with Lp(a) and apo(a) isolated from a single donor. *Top*: binding of human Lys^+ Lp(a) ($\bullet$), human Lys^- Lp(a) ($\bigcirc$), and rhesus Lp(a) ($\blacktriangle$). The IC_{50} (the concentration of EACA that produced 50% inhibition of the binding) was 5 mM for human Lys^+ Lp(a), 6 mM for human Lys^- Lp(a), and 3.7 mM for rhesus Lp(a). *Bottom*: binding of human Lys^+ apo(a) ($\blacksquare$), human Lys^- apo(a) ($\square$), and rhesus apo(a) ($\triangle$). The IC_{50} was 4.3 mM for human Lys^+ apo(a), 2.5 mM for human Lys^- apo(a), and 3.7 mM for rhesus apo(a).

6 mM for human Lys^- Lp(a) and 3.7 mM for rhesus Lp(a). The average IC_{50} values calculated for Lp(a) isolated from several individual donors were in the same range: 9.2 ± 3.6 mM ($n = 3$) for human Lys^+ Lp(a), 7.4 ± 1.9 mM ($n = 2$) for human Lys^- Lp(a), and 5.6 ± 2.7 mM ($n = 3$) for rhesus Lp(a). Moreover, as shown in Fig. 4, *bottom*, EACA had a similar inhibitory effect

on the binding of human Lys^+ apo(a), human Lys^- apo(a), and rhesus apo(a) to PM-fibrinogen: IC_{50} of 4.3 mM for human Lys^+ apo(a), 2.5 mM for human Lys^- apo(a), and 3.7 mM for rhesus apo(a). The average IC_{50} values determined for apo(a) from several individual donors were 5.2 ± 2.1 mM ($n = 3$) for human Lys^+ apo(a), 5.1 ± 3.6 mM ($n = 2$) for human Lys^- apo(a) and 3.3 ± 1.1 mM ($n = 3$) for rhesus apo(a).

Binding of Glu-plasminogen to PM-fibrinogen. The observation that the binding of Lp(a) and apo(a) to fibrinogen can be significantly inhibited by proline, prompted us to determine whether this also applies to plasminogen whose binding to fibrinogen has been shown to be hampered by EACA (12, 21). In the present study we confirmed this finding (Fig. 5). However, contrary to the Lp(a) system, 0.2 M proline, which inhibited the PM-fibrinogen binding of Lp(a) and apo(a), had no effect on the binding of Glu-plasminogen to PM-fibrinogen (Fig. 5).

Discussion

In a previous study we showed that apo(a) released from apoB100 by subjecting Lp(a) to mild reductive conditions unmasks a domain which is critical for the process of Lp(a) reassembly and is sensitive to both EACA and proline (9). We now show that this "lysine-proline (Lys-Pro)-sensitive domain" is also involved in fibrinogen binding based on the following observations: (*a*) free apo(a), derived from either Lys^+ or Lys^- Lp(a), binds to both apoB100 and PM-fibrinogen; (*b*) both processes are inhibited by EACA and proline although to a different extent; (*c*) as in the case of Lp(a) reassembly (9) the binding of apo(a) to PM-fibrinogen is affected by neither a functional LBS in kringle IV-10 nor by the presence of kringle V. On the strength of our own results (9) and the reassembly

studies by Ernst et al. (5) and Frank et al. (10), we have suggested that the Lys-Pro binding domain of apo(a) spans between kringle IV-4 and IV-9. This suggestion receives additional support from our current results on free apo(a) and those on apo(a) recombinant by Harpel et al. (22) indicating that kringle IV-2 is not involved in the fibrinogen binding, since this process is not significantly influenced by the size of apo(a), which depends on the number of kringle IV-2. Other investigators studying the whole Lp(a) particle have reported an effect of apo(a) size on fibrin(ogen) binding (18, 23, 24). We cannot reconcile these results except for invoking differences in the apo(a) phenotypes studied and assay conditions. In the latter respect, our current results show that the measurement of the fibrinogen binding capacity of whole Lp(a) provides just a fraction of the binding exhibited by whole free apo(a) and thus an incomplete estimate of the total binding efficiency. We would like to point out that the differences in PM-fibrinogen binding between Lp(a) and apo(a) were in terms of B_{max} and not K_d values, indicating that both masked and unmasked sites were components of the Lys-Pro binding domain only differing in their accessibility to this substrate.

Our results, when combined with previous ones on lysine binding (5, 9), clearly indicate that apo(a) has two lysine-dependent binding sites. The one represented by the LBS of kringle IV-10 is open and is mainly responsible for the binding of Lp(a) to a lysine-Sepharose column, a conclusion supported by the observations that human or rhesus monkey Lp(a) species having Arg instead of Trp in position 72, are Lys⁻ (3, 4). The other site, represented by the Lys-Pro–sensitive domain, has the capacity to bind to both PM-fibrinogen and lysine-Sepharose and is $\sim 70\%$ masked by the attachment of apo(a) to the apoB100 of Lp(a). The degree of masking appears to account for the extent of apo(a) binding to PM-fibrinogen in that such a masking may determine the number of sites on this domain available to ligand interaction. It is of interest to note that the Lys-Pro domain, when totally unmasked, binds to immobilized lysine less efficiently than the LBS of kringle IV-10 (C. Edelstein, unpublished observations). This relatively low degree of binding helps explain why Lp(a) particles with a functionally defective LBS in kringle IV-10 do not bind to lysine-Sepharose in spite of a partially unmasked Lys-Pro–sensitive domain (3, 4, 9).

We (8) and LoGrasso et al. (7) previously reported that recombinant apo(a) kringle IV-10 has a capacity to bind to immobilized PM-fibrinogen. However, the affinity of this binding was much lower than that of whole apo(a): micromolar vs. nanomolar range. This information taken together with the current findings that Lys⁻ Lp(a) and the derived apo(a) exhibit affinities comparable to those of Lys⁺ Lp(a) counterparts, does not support a critical role of kringle IV-10 in the binding of apo(a) to fibrinogen. This conclusion is in keeping with the findings of Rouy et al. (13), who studied the fibrin binding of Lp(a) and a recombinant form of apo(a).

With reference to the Lys-Pro domain, it is important to note that the K_d of the proline-mediated binding to PM-fibrinogen was comparable to that of lysine-mediated binding (35–90 nM). Proline has been shown previously to have an inhibitory effect on the binding of apo(a) to apoB100 in Lp(a) (11). Thus, the Lys-Pro domain appears to have a site where both electrostatic and hydrophobic forces in either a distinct or a cooperative way play a role in the interaction of apo(a) with fibrinogen and apoB100. From the published sequence of apo(a), kringles IV-5 and IV-9 contain a potential EACA-sensitive lysine binding pocket with two anionic (Asp55, Glu57) and two cationic (Arg35, Arg71) amino acids located at opposite ends of a hydrophobic trough (Trp62, Tyr64, Trp72). In contrast, kringle IV-4, by having Leu57 instead of Glu in the anionic site, could favor the formation of a sufficiently larger hydrophobic pocket to accommodate proline. Moreover, a role in proline-mediated binding can also be played by the linkers between kringles IV-4 and IV-9, which, based on amino acid secondary structures and energy minimization criteria, may bring these kringles in close juxtaposition to each other, thus promoting cooperative interactions among them (C. Edelstein, unpublished observations). It is of interest to note that the binding of Glu-plasminogen to PM-fibrinogen was not inhibited by proline, indicating a difference in binding mechanism between plasminogen and apo(a), which may be anticipated from the known structural differences between these two proteins (25).

From all these observations it is apparent that lysine binding is not synonymous with fibrinogen binding. Thus, if we assume that fibrinogen binding relates to the athero-thrombogenic potential of Lp(a) (26), Lys⁻ subjects may still be at cardiovascular risk both via Lp(a) and free apo(a). It should be noted that in the circulation, free apo(a) only represents a very small proportion of the whole apo(a) pool, which is mostly present in Lp(a) (27). On the other hand, at the lesion sites in the arterial wall, a significant accumulation of apo(a) can occur (28–30) due to either in situ release from Lp(a) and/or preferential proteolysis of apoB (31). Based on the results of the current studies, free apo(a) would be better suited than Lp(a) for complexation with fibrin(ogen) and contribute to the formation of the atherosclerotic plaque. If this were to be the case, therapeutic attempts to reduce the levels of plasma Lp(a) by favoring the generation of free apo(a) would not be beneficial. Our results may also help explain the failure of some studies to demonstrate a binding of Lp(a) to fibrinogen (32). We may speculate that those studies may have dealt with Lp(a) particles with a totally masked Lys-Pro domain and, thus, incompetent to interact with fibrin(ogen). Alternatively, or in addition, those negative results may have been caused by the choice of substrate. As shown by Harpel et al. (12) and by our current studies, the use of fibrinogen instead of PM-fibrinogen would make the system less amenable to Lp(a) binding. As a corollary, the assessment of the actual fibrin(ogen) binding potential of Lp(a) may have to include the determination of free apo(a).

Acknowledgments

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