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The Solution Phase Interaction between Apolipoprotein(a) and Plasminogen Inhibits the Binding of Plasminogen to a Plasmin-Modified Fibrinogen Surface†

Waheed Sangrar,‡ Brent R. Gabel,‡ Michael B. Boffa,‡ John B. Walker,‡ Mark A. Hancock,‡ Santica M. Marcovina,§ Anton J. G. Horrevoets,‡,|| Michael E. Nesheim,‡ and Marlys L. Koschinsky*,‡

Department of Biochemistry, Queen's University, Kingston, Ontario, Canada K7L 3N6, and Department of Medicine, Northwest Lipid Research Laboratories, University of Washington, 2121 North 35th Street, Seattle, Washington 98103

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ABSTRACT: In the present study, we assessed the binding of recombinant forms of apolipoprotein(a) [r-apo(a)] to plasminogen. Apo(a)−plasminogen interactions were demonstrated to be lysine-dependent, as they were abolished by the addition of ϵ-aminocaproic acid. Binding of r-apo(a) and plasma-derived Lp(a) to Glu-plasminogen was assessed in solution using a mutant form of recombinant plasminogen [Plg(S741C)] labeled at the active site with 5′-(iodoacetamido)fluorescein. High-affinity binding of apo(a) to plasminogen was observed with the 17-kringle r-apo(a) (K_d = 20.1 ± 3.3 nM) as well as with plasma-derived Lp(a) (K_d = 5.58 ± 0.08 nM). Binding studies using various truncated and mutant forms of r-apo(a) demonstrated that sequences within apo(a) kringle IV types 2−9 and the strong lysine binding site (LBS) in apo(a) kringle IV type 10 are not required for high-affinity binding to plasminogen. In all cases, the binding stoichiometry for the apo(a)−plasminogen interaction was determined to be 1:1. Binding data obtained using a 17-kringle r-apo(a) derivative lacking the protease-like domain (17KΔP; K_d = 3158 ± 138 nM) indicate that sequences within the protease-like domain of apo(a) mediate its interaction with LBS in plasminogen. We determined that r-apo(a) and plasminogen bind to distinct sites on plasmin-modified fibrinogen with the concentration of plasminogen binding sites exceeding the concentration of r-apo(a) sites by a factor of 10. Furthermore, r-apo(a) is capable of inhibiting the binding of plasminogen to plasmin-modified fibrinogen surfaces, an effect which we show is attributable to the formation of a solution phase apo(a)/plasminogen complex which exhibits a greatly reduced affinity for plasminogen binding sites on plasmin-modified fibrinogen. The results of this study provide new insights into the mechanism by which apo(a) and Lp(a) may inhibit fibrinolysis, thus contributing to the atherothrombotic risk associated with this lipoprotein.

Lipoprotein(a) [Lp(a)][1] has been identified in several studies as an independent risk factor for the development of coronary heart disease (reviewed in ref *1*) and stroke (*2*). Plasma Lp(a) concentrations vary over 1000-fold in the population, ranging from less than 0.1 mg/dL to greater than 100 mg/dL. Roughly 25% of the human population possess

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* To whom correspondence should be addressed. Phone: (613) 545-6586. Fax: (613) 545-2987.

‡ Queen's University.

§ University of Washington.

|| Present address: Academic Medical Center, Meibergdreef 9, Amsterdam AZ, The Netherlands.

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[1] Abbreviations: Lp(a), lipoprotein(a); apo(a), apolipoprotein(a); r-apo(a), recombinant apo(a); LDL, low-density lipoprotein; apoB-100, apolipoprotein B-100; tPA, tissue-type plasminogen activator; LBS, lysine binding sites.

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Lp(a) levels above an apparent coronary risk threshold of 20 mg/dL, which more than doubles their risk of developing coronary heart disease (*3*−*5*). Some recent prospective studies have failed to identify a correlation between Lp(a) levels and risk for coronary heart disease (*6*−*8*). However, the mechanisms by which Lp(a) exerts its pathogenic effects remain unclear at present, which may underlie the conflicting epidemiological data.

Lp(a) closely resembles low-density lipoprotein (LDL) with respect to both lipid composition and the presence of apolipoprotein B-100 (apoB-100) but contains an additional protein moiety designated apolipoprotein(a) [apo(a)] which is linked to apoB-100 by a single disulfide bond (*9, 10*). Human apo(a) consists of multiple tandem repeats of a sequence closely resembling plasminogen kringle IV, followed by sequences exhibiting a high degree of similarity to the kringle V and protease domains of plasminogen (*11*). Owing to the structural homology between apo(a) and plasminogen, it has been postulated that Lp(a) may inhibit the normal fibrinolytic functions of plasmin(ogen), thereby generating a hypercoagulable state *in vivo*. Although the apo(a) protease-like domain contains an intact catalytic triad, there is no apparent protease activity associated with this domain (*12*). It has been demonstrated that there are 10 distinct classes of kringle IV sequences in apo(a) which are present in all individuals; the kringle IV type 2 motif (also referred to as the major repeat kringle) is present in variable numbers, which constitutes the molecular basis of Lp(a) isoform size heterogeneity (*13, 14*). Of the kringle IV sequences in apo(a), the sequence of apo(a) kringle IV type 10 most closely resembles that of plasminogen kringle IV. Like plasminogen kringle IV, apo(a) kringle IV type 10 has lysine binding properties (*15*) and has been postulated to mediate the interaction of Lp(a) with lysine residues present in biological substrates such as fibrin (*16*−*18*). Weak lysine binding sites have also been identified in apo(a) kringle IV types 5−8, which are important in mediating the interaction of apo(a) and apoB-100 to form Lp(a) particles (*19, 20*).

The results of previous studies undertaken to define the effect of Lp(a) on the fibrinolytic system have led to conflicting conclusions. Early studies demonstrated that apo(a) binds to lysine residues present in fibrin, thereby inhibiting the binding of plasminogen (*16*−*18*) and tPA (*17*) to fibrin. Several studies have reported that apo(a) and Lp(a) inhibit tPA-mediated fibrinolysis; a very recent study implicates apo(a) in the inhibition of tPA-mediated fibrinolysis in clots formed in mice expressing apo(a) from a transgene (*21*). However, the nature of this inhibitory effect is unclear at present as both competitive (*22*) and uncompetitive (*17, 23*) mechanisms have been reported. There is also a report that Lp(a) may enhance fibrin clot lysis *in vitro* by promoting the binding of plasminogen to fibrin, thereby resulting in enhanced plasminogen activation in the presence of high concentrations of both D-dimer and tPA (*24*).

In the present study, we have demonstrated that recombinant apo(a) [r-apo(a)] binds with high affinity to plasminogen in solution. This interaction, which is not affected by the number of identically-repeated kringle IV_2 domains, is mediated through lysine binding sites present in plasminogen and involves sequences present in the protease domain of apo(a). Finally, we show that the inhibition of plasminogen binding to fibrin(ogen) by apo(a) can be explained by this interaction; our data suggest that rather than competing with plasminogen for binding to plasmin-modified fibrinogen, apo(a) forms a complex with plasminogen that binds to plasminogen binding sites on plasmin-modified fibrinogen with a very low affinity.

EXPERIMENTAL PROCEDURES

Materials. The cysteine-specific fluorescent probe 5′-(iodoacetamido)fluorescein (5′-IAF) was obtained from Molecular Probes Inc. (Eugene, OR). Dansyl-Glu-Gly-Arg-chloromethyl ketone (dEGRck) and Val-Phe-Lys-chloromethylketone (VFKck) were purchased from CalBiochem, and glutathione was obtained from Sigma. The lysine analogue ϵ-aminocaproic acid (ϵ-ACA) was purchased from Sigma, and lysine−Sepharose CL-4B resin was obtained from Pharmacia. All DNA modifying/restriction enzymes were obtained from New England Biolabs or Promega, and were used according to the manufacturers' specifications.

Construction of Recombinant Apo(a) Expression Plasmids. The r-apo(a) derivatives utilized for this study are shown schematically in Figure 1; the construction of the corresponding expression plasmids is described briefly below. All constructs were based upon the pRK5ha17 17-kringle r-apo(a) expression plasmid, which contains the CMV promoter and the SV40 transcription termination sequences (*25*).

The construction of the following derivatives has been described previously: 17K (*25*), 12K (*12*), KIV_{10} (*15*), 6K, KIV_{6-P}, KIV_{7-P}, KIV_{8-P}, and KIV_{9-P} (*20*). For the generation of KIV_{10-P}, the KIV_{10} expression plasmid pRK5-SK10 (*15*) was digested with *Avr*II; the resultant 580 bp fragment was replaced with a 1678 bp *Avr*II fragment from pRK5ha17 (note that the latter fragment contains sequences encoding the kringle V and protease domains). The final expression construct was designated pRK5haKIV_{10-P}.

In order to disrupt the lysine binding site in kringle IV type 10 in the context of the 17-kringle construct, an Asp→Ala substitution was introduced at amino acid position 67 of this kringle by PCR-mediated mutagenesis. Using pRK5-SK10 (15) as the template, the following primer pairs were utilized for the PCR reaction: primer A (5′ CCA CAG GTG TCC ACT CCC 3′) flanking the multiple cloning site in pRK5, and primer B (5′ CCA AGG GCC TGT **GC**C GGC ATC TGG ATT 3′) which contains mismatches shown in boldface type; primer C (5′ TAA CCA TTA TAA GCT GC 3′) flanking the multiple cloning site in pRK5, and primer D (5′ AAT CCA GAT GCC GGC ACA G**GC** ACA GGC CCT TGG 3′) which contains mismatches shown in boldface type. Using these primer pairs, two overlapping PCR products were generated; the mismatches incorporated into oligos B and D mutate the Asp codon (GAT) to an Ala codon (GGC) and create an *Ngo*MI restriction site. The PCR products were digested with *Eco*RI and *Ngo*MI and with *Ngo*MI and *Sal*I, respectively, and inserted into the pRK5-SK10 expression plasmid that had been digested with *Eco*RI and *Sal*I. A 273 bp *Msc*I/*Avr*II fragment was isolated from this plasmid and inserted into the pRK5ha17 plasmid digested with these enzymes to replace the corresponding wild-type sequence. The mutant expression construct is designated pRK5ha17ΔAsp.

All constructs described above were verified by DNA sequence analysis.

Expression of R-apo(a) Derivatives. 293 cells (human embryonic kidney cells) (*26*) were cultured in 100 mm dishes

in the presence of minimal essential medium (MEM; GIBCO/BRL) supplemented with 5% fetal calf serum. 293 cells were stably transfected with each of the r-apo(a) derivatives shown in Figure 1. For the generation of stably-expressing cell lines, expression plasmids (10 μg) were cotransfected with 1 μg of a plasmid encoding the neomycin resistance gene (*27*) by calcium phosphate coprecipitation (*28*). Transfectants were selected by culturing the cells in the presence of 800 μg/mL G418 (GIBCO/BRL), and expression levels were determined by ELISA as previously described (*20*).

Preparation of Thrombin and Plasmin and Purification of Human Fibrinogen, Plasminogen, Lp(a), and Recombinant Apo(a) Derivatives. Human thrombin was prepared as described previously (*29*). Plasminogen-free, factor XIII-free human fibrinogen (>99% clottable; no detectable α-chain degradation) was prepared by a modified method of Straughn and Wagner (*30*). Human fibrinogen was precipitated from fresh-frozen, barium-adsorbed plasma by the addition of 4 M β-alanine. The precipitate was dissolved in 0.05 M trisodium citrate, pH 6.5, 0.15 M NaCl and was then made 4.5% (w/v) in PEG-8000 by the addition of 40% (w/v) PEG-8000 in H_2O. The precipitate was dissolved in a minimal volume of 0.02 M HEPES, pH 7.4, 0.5 M NaCl and then diluted with 0.02 M HEPES, pH 7.4, to give a final NaCl concentration of 0.025 M. The fibrinogen was passed over lysine−Sepharose, anti-FXIII total IgG (a kind gift of Hugh Hoogendorn; Affinity Biologicals, Hamilton, Canada), and DEAE-cellulose columns linked in series. The DEAE-cellulose was then washed extensively with 0.02 M HEPES, pH 7.4, 0.025 M NaCl. Fibrinogen was eluted with 0.02 M HEPES, pH 7.4, 0.10 M NaCl. The fibrinogen was then concentrated with 30% (w/v) ammonium sulfate, dissolved in 0.02 M HEPES, pH 7.4, 0.15 M NaCl (HBS), and dialyzed extensively against the same buffer. Aliquots were stored at −70 °C.

Glu-Plasminogen was purified according to the method of Castellino and Powell (*31*). Fresh-frozen plasma was passed over lysine−Sepharose CL-4B. Plasminogen was eluted with ϵ-aminocaproic acid (ϵ-ACA) and precipitated with 70% (w/v) ammonium sulfate. The precipitate was dissolved in a minimal volume of 50% (v/v) glycerol and stored at −20 °C. Plasmin was prepared from Glu-plasminogen by treatment with urokinase as previously described (*32*).

In order to isolate Lp(a) from human plasma, blood samples were obtained from a fasting donor with high Lp(a) levels and an apo(a) isoform containing 19 kringle IV repeats, as determined by agarose gel electrophoresis and immunoblotting (*33*, *34*). Lp(a) was purified from the plasma by sequential density gradient ultracentrifugation followed by gel filtration chromatography as previously described (*35*). The purity of isolated Lp(a) was assessed by agarose gel electrophoresis, and the molar protein concentration was determined by a double monoclonal antibody-based ELISA insensitive to apo(a) size heterogeneity (*35*).

All of the r-apo(a) derivatives used in this study were purified by affinity chromatography over lysine−Sepharose columns (*20*). Briefly, conditioned medium (CM) (Opti-MEM; GIBCO/BRL) harvested from 293 cells stably expressing the various r-apo(a) derivatives shown in Figure 1 was passed over 50 mL lysine−Sepharose CL-4B columns. Note that for each r-apo(a) derivative, essentially 100% of the immunoreactive material present in the CM was capable of binding to lysine−Sepharose. The columns were washed with PBS (phosphate-buffered saline) containing 0.5 M NaCl, and protein was eluted with 0.2 M ϵ-ACA in this buffer. Protein-containing fractions were pooled, dialyzed against PBS, and precipitated overnight with ammonium sulfate. The precipitate was pelleted by centrifugation at 12000*g* for 20 min at 4 °C, dissolved in HBS, and dialyzed against this buffer. The protein concentration was determined by measurement of the absorbance at 280 nm.

The molecular weights and extinction coefficients utilized in calculating protein concentrations were as follows: thrombin [37 000, $\epsilon_{1\%}(280) = 18.3$]; Glu-plasminogen [92 000, $\epsilon_{1\%}(280) = 16.1$]; Lys-plasminogen [84 000, $\epsilon_{1\%}(280) = 16.1$]; fibrinogen [340 000, $\epsilon_{1\%}(280) = 16$]. Extinction coefficients for each recombinant apo(a) protein were determined using the tyrosine difference spectral method (*36*). The extinction coefficients and molecular weights used for calculating protein concentrations were as follows: 17K [278 719, $\epsilon_{1\%}(280) = 20.7$]; 17KΔP [249 244, $\epsilon_{1\%}(280) = 22.0$]; 17KΔVP [240 503, $\epsilon_{1\%}(280) = 21.9$]; 12K [207 674, $\epsilon_{1\%}(280) = 22.7$]; 6K [122 542, $\epsilon_{1\%}(280) = 25.6$]; KIV_{6-P} [108 269, $\epsilon_{1\%}(280) = 24.4$]; KIV_{7-P} [94 676, $\epsilon_{1\%}(280) = 24.1$]; KIV_{8-P} [80 022, $\epsilon_{1\%}(280) = 23.4$]; KIV_{9-P} [66 117, $\epsilon_{1\%}(280) = 22.8$]; KIV_{10-P} [52 040, $\epsilon_{1\%}(280) = 21.6$]; KIV_{10} [13 824, $\epsilon_{1\%}(280) = 21.4$].

Purification and Fluorescein Labeling of a Derivative of Recombinant Human Plasminogen [Plg(S741C)]. A derivative of plasminogen [Plg(S741C)] containing a Ser→Cys substitution at the active site serine (S741) was purified from CM harvested from baby hamster kidney (BHK-21) cells stably expressing this variant (*37*). The cultures were seeded in DMEM/F12 supplemented with Ultroser G (GIBCO/BRL) and 0.4% (v/v) methotrexate (Novopharm) and grown to confluence. At confluence, the selection medium was subsequently replaced by serum-free medium (OptiMEM; GIBCO/BRL) containing 50 μM $ZnCl_2$. Medium was harvested every second day, pooled, and treated with glutathione (GSH) (1 mM final) and dEGRck (1 μM final). Pooled medium was then loaded onto a lysine−Sepharose CL-4B column and washed with PBS. The Plg(S741C) was subsequently eluted with PBS containing 10 mM ϵ-ACA and 1 mM EDTA. For fluorescein labeling of Plg(S741C), 5′-IAF (20 mM in *N*,*N*′-dimethylformamide) was added to the pooled Plg(S741C) in a 30-fold molar excess, and the reaction was stored in the dark for 1 h at room temperature. Free label was subsequently removed by adsorption to a 2 mL DEAE-cellulose Fast Flow (Sigma) column which had been preequilibrated in PBS. The flow-through containing labeled Plg(S741C) [i.e., Plg(S741C)-fluorescein] was diluted 1:5 and passed over a 5 mL DEAE-cellulose Fast Flow column which had been preequilibrated with 0.01 M Tris, pH 8.0, containing 0.02% (v/v) Tween 80. The column was then washed with 0.01 M Tris, pH 8.0, containing 0.02% (v/v) Tween 80, followed by 0.02 M HEPES, pH 7.4. The Plg(S741C)-fluorescein was eluted with HBS containing 0.01% (v/v) Tween 80 (HBST). The amount of fluorescein incorporated was determined spectrophotometrically, using an extinction coefficient at 495 nm for fluorescein of 84 000 M^{-1} cm^{-1} (according to manufacturer's specifications). The concentration of the labeled protein was determined from the absorbance at 280 nm after correction for the contribution of fluorescein ($A_{280nm} = 0.19 \times A_{495nm}$). Labeling efficiency was measured by determining the concentration of the 5′-

IAF label and dividing by the plasminogen concentration. Labeling efficiency was typically greater than 80%. Purified Plg(S741C)-fluorescein was aliquoted and stored at −70 °C.

5′-IAF-labeled Lys-plasminogen was derived from incubation of Plg(S741C)-fluorescein (10 μM) with plasmin (0.16 μM) in 23 mL of 0.05 M Tris-HCl, pH 8.0, 0.05 M ϵ-ACA as previously described (*38*). After 2 h, the reaction was stopped by the addition of VFKck (0.16 μM). Analysis by acid−urea gel electrophoresis (*39*) indicated that the preparation contained no Glu-plasminogen. The concentration of the labeled Lys-plasminogen was determined as described above for 5′-IAF-labeled Glu-plasminogen.

Effect of ϵ-ACA on the Binding of the 17K R-apo(a) to Immobilized Glu-Plasminogen. Microtiter plates were coated with 100 μL/well of plasminogen (10 μg/mL in coating buffer) for 12−16 h at 4 °C. The wells were washed 4 times with PBS containing 0.1% (v/v) Tween 20 (PBST) after this and subsequent incubations. After being washed, the wells were blocked with 200 μL/well blocking buffer [PBS containing 0.2% (w/v) BSA] and then incubated with a range of purified 17K r-apo(a) concentrations (in diluent buffer) in the presence and absence of a range of ϵ-ACA concentrations (0−50 mM) for 12−16 h at 4 °C. Following extensive washing, bound r-apo(a) was incubated with 0.5 μg/mL of the apo(a)-specific monoclonal antibody 2G7 (*40*) for 1.5 h at 22 °C. Binding of the monoclonal antibody was detected by incubation with anti-mouse IgG conjugated to horseradish peroxidase (HRP) (Amersham; 100 μL/well) for 1.5 h at 22 °C. Following washing, binding of the secondary antibody was detected by the addition of development buffer containing the substrate *o*-phenylenediamine dihydrochloride (0.42 mg/mL). The color development reaction was stopped with 2 M H_2SO_4, and the absorbance at 492 nm was determined using a Titertek plate reader.

Radioiodination of R-apo(a) and Plasminogen. Purified r-apo(a) and plasma-derived Glu-plasminogen were labeled using IODO-BEADS (Pierce Chemicals). Briefly, two IODO-BEADS were washed with 1 mL of 0.2 M Tris-HCl, pH 7.4, containing 0.1 M NaCl for 1 min at 22 °C. The solution was removed, and 0.5 mL of the same solution containing 1 mCi of $^{125}I_2$ (ICN) was incubated with the IODO-BEADS for 5 min at room temperature. The solution was transferred to a microcentrifuge tube containing 200−300 μg of r-apo(a) or Glu-plasminogen and incubated for 10−15 min at 22 °C before the reaction was stopped with 10 μL of 1 M sodium metabisulfite (BDH). Free ^{125}I was separated from ^{125}I-labeled proteins by passage over a 10DG (BioRad) desalting column preequilibrated with 0.02 M Tris-HCl, pH 7.4, containing 0.1 M NaCl. Fractions containing labeled protein were detected using a γ-radiation counter.

Analysis of the Binding of R-apo(a) Derivatives to Plg-(S741C)-Fluorescein. One hundred and fifty microliters of a solution of Plg(S741C)-fluorescein (10−110 nM) in filtered (0.2 μm) HBST was added to a quartz microcuvette which was thermostated at 22 °C in a Perkin-Elmer LS 50B fluorescence spectrometer. The Plg(S741C)-fluorescein solution in the cuvette was then titrated with filtered HBST containing human plasma-derived Lp(a) or r-apo(a) derivatives. This solution also contained Plg(S741C)-fluorescein at a concentration identical to that present in the cuvette in order to obviate the need to correct for dilution. Changes in fluorescence intensity were monitored continuously during the course of the titration at λ_{ex} = 490 nm and λ_{em} = 535 nm, with a 530 nm cutoff filter in the emission beam.

Binding of Apolipoprotein(a) and Glu-Plasminogen to Immobilized, Plasmin-Modified Fibrinogen. Microtitre wells were coated with 100 μL of 100 μg/mL fibrinogen in HBS for 12−16 h at 4 °C. The wells were washed 4 times with PBST after this and subsequent incubations. The fibrinogen-coated wells were treated with 100 μL/well of 5 nM plasmin (in HBST) for 60 min at room temperature. After incubation, the wells were washed twice with HBST containing 0.2 M ϵ-ACA and 0.5 M NaCl, and 4 times with PBST. In order to inactivate residual plasmin, the wells were incubated with 100 μL/well of 1 μM VFKck for 40 min at room temperature. After four washes with PBST, nonspecific binding sites in the wells were blocked by treatment with 150 μL/well of 0.2% (w/v) BSA in PBS for 2 h.

The immobilized plasmin-modified fibrinogen was incubated with a range of concentrations of radioiodinated Glu-plasminogen (0−4 μM) or 17K r-apo(a) (0−3.1 μM). After incubation, the wells were washed extensively with 200 μL/well PBST. Individual wells were counted directly using a γ-radiation counter to determine the radioactivity associated with each well. Concentrations of bound r-apo(a)/well were determined by dividing the cpm/well by the specific activity of the preparation. Specific binding was obtained by subtracting the nonspecific binding (measured in the presence of ϵ-ACA) from the total binding. In some experiments, a range of concentrations of radioiodinated Glu-plasminogen (0−4 μM), each containing a fixed concentration of unlabeled 17K r-apo(a) (0−4 μM), was incubated with the immobilized plasmin-modified fibrinogen.

RESULTS

Expression and Purification of Recombinant Apo(a) [R-apo(a)] Derivatives. Recombinant apo(a) derivatives shown in Figure 1 were expressed in 293 (human embryonic kidney) cells. Proteins were purified to homogeneity from CM harvested from stably-expressing cell lines by lysine−Sepharose affinity chromatography. Purified proteins (5−10 μg) were analyzed by SDS−PAGE (*41*) and Coomassie blue staining (Figure 2); details of the SDS−PAGE analysis are given in the legend to Figure 2.

Binding of R-apo(a) to Immobilized Glu-Plasminogen and the Effect of ϵ-Aminocaproic Acid on Binding. Glu-plasminogen was immobilized in microtiter wells as described under Experimental Procedures. Solutions containing a range of concentrations of purified 17K r-apo(a) (0−100 nM) and ϵ-ACA (0−50 mM) were added to the wells. Following incubation, bound r-apo(a) was detected by ELISA using an apo(a)-specific monoclonal antibody and a horseradish peroxidase-conjugated secondary antibody; data corresponding to this experiment are shown in Figure 3. The binding of 17K r-apo(a) to plasminogen was completely abolished by the addition of 10 mM ϵ-ACA. This suggests that the r-apo(a)−Glu-plasminogen interaction is lysine-dependent, and therefore involves lysine binding sites present in either plasminogen or apo(a).

Binding of R-apo(a) Derivatives to Fluorescein-Labeled Glu-Plasminogen in Solution. A mutant form of recombinant plasminogen [Plg(S741C); containing a Ser→Cys substitution at the active site] was labeled with the thiol-specific reagent

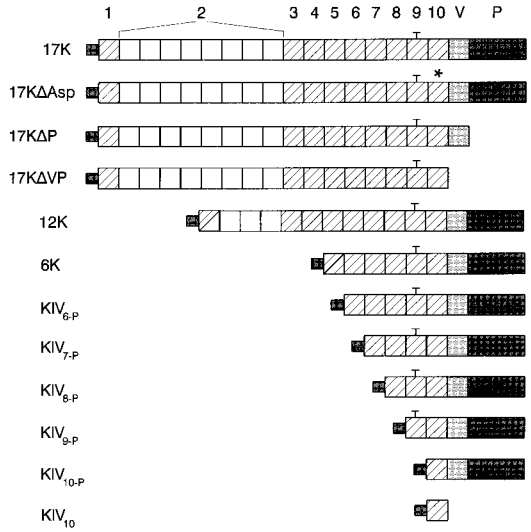

FIGURE 1: Construction of r-apo(a) expression plasmids. The upper line illustrates the organization of the 17K r-apo(a) expression plasmid (pRK5ha17) which was derived from the published cDNA as previously described (Koschinsky *et al.*, 1991). The organization of the r-apo(a) derivatives is shown relative to pRK5ha17. In all cases, open boxes are used to designate the kringle repeats of identical amino acid sequence (i.e., kringle IV type 2) while hatched boxes represent kringle units that contain amino acid substitutions relative to the major kringle repeat; the 10 types of kringle IV sequences are indicated above the 17K derivative. The shaded box represents apo(a) kringle V, while the black bar corresponds to the apo(a) protease-like domain. The position of the free cysteine in apo(a) kringle IV type 9 is shown with a bar. The position of a Asp→Ala substitution in kringle IV type 10 of the 17KΔAsp derivative is indicated by an asterisk. The 12K and 6K constructs contain a hybrid kringle which represents a fusion of kringle IV type 1 with either kringle IV type 2 (for the 12K derivative) or kringle IV type 5 for the 6K. Details of the constructions are provided under Experimental Procedures.

5′-(iodoacetamido)fluorescein to yield Plg(S741C)-fluorescein. This material was used for binding studies with purified r-apo(a) derivatives and plasma-derived Lp(a) in solution; the purified Lp(a) was determined to be free of contaminating plasminogen by Western blot analysis (data not shown). Titration of Plg(S741C)-fluorescein with a solution containing r-apo(a) and Plg(S741C)-fluorescein (the latter at a concentration equal to that present initially in the cuvette) resulted in a saturable decrease in the fluorescence intensity (Figure 4), indicating that binding of apo(a) to labeled plasminogen elicits a quenching of the fluorescein fluorescence signal. The data showing changes in fluorescence intensity as a function of increasing concentrations of plasma-derived Lp(a) and the r-apo(a) derivatives 17K, 17KΔAsp, and 17KΔP are shown in Figure 4 (panels A−D, respectively). Similar analyses was performed using the following purified r-apo(a) derivatives: 17KΔVP, 12K, 6K, KIV_{6-P}, KIV_{7-P}, KIV_{8-P}, KIV_{9-P}, KIV_{10-P}, and single KIV_{10}.

The data were modeled according to a simple bimolecular mechanism

$$\text{apo(a)} + \text{Glu-Pgn} \rightleftharpoons \text{apo(a)}\cdot\text{Glu-Pgn}$$

The fluorescence intensity of a solution of these components at equilibrium is expressed by eq 1:

$$I = I_o - \Delta I 0.5\{K_d + [P]_i + n[A] - [(K_d + [P]_i + n[A])\text{^}2 - 4[P]_i n[A]]\text{^}0.5\} \quad (1)$$

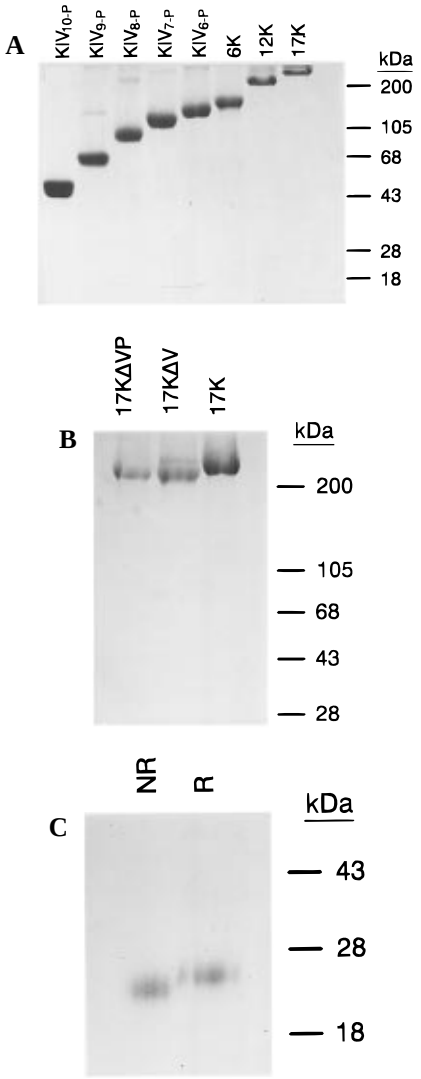

FIGURE 2: SDS−PAGE analysis of purified r-apo(a) derivatives. R-apo(a) was purified from CM harvested from 293 cells stably expressing each derivative by lysine−Sepharose affinity chromatography. Recombinant proteins were analyzed by SDS−PAGE, and proteins were visualized by staining using Coomassie Blue (BioRad). Panel A: 5−10 μg of the indicated purified r-apo(a) derivatives was analyzed by SDS−PAGE under non-reducing conditions using a 4−20% polyacrylamide gradient gel; the positions of protein markers (BioRad) are indicated to the right of the gel. Panel B: 5−10 μg of the indicated purified r-apo(a) derivatives was analyzed by SDS−PAGE under non-reducing conditions using a 3−12% polyacrylamide gradient gel; the positions of protein markers (BioRad) are indicated to the right of the gel. Panel C: Purified KIV_{10} (5 μg) was analyzed by SDS−PAGE (12% polyacrylamide gel) in the absence (NR) or presence (R) of 0.1 M dithiothreitol. The positions of protein markers (BioRad) are indicated to the right of the gel.

where I is the total fluorescence intensity of the solution at any point on the titration curve, $[P]_i$ is the initial concentration of Glu-plasminogen in the cuvette, [A] is the concentration of apo(a) at each point in the titration, I_o is the intensity prior to titration, and ΔI is the total change in intensity at saturation.

The titration data were fit to eq 1 using nonlinear regression analysis and provided a best fit for the following parameters: I_o, ΔI, K_d, the concentration of apo(a) at which half the available binding sites on plasminogen are occupied, and n, the stoichiometry of the interaction [moles of apo(a) per moles of Glu-plasminogen]. The K_d values for the r-apo-

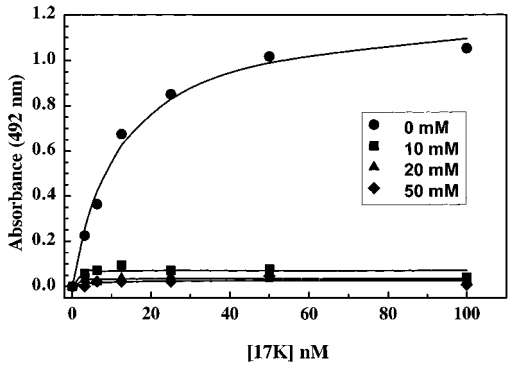

FIGURE 3: Effect of ϵ-aminocaproic acid on the binding of r-apo(a) to immobilized Glu-plasminogen. Glu-plasminogen was immobilized in microtiter wells as described under Experimental Procedures. Solutions containing a range of concentrations of purified 17K r-apo(a) (0−100 nM) and ϵ-ACA (0−50 mM) were added to the wells. Following incubation, bound apo(a) was detected by ELISA using the apo(a)-specific monoclonal antibody 2G7.

(a) species and Lp(a) are presented in Table 1; in order to obtain the estimates of K_d reported in Table 1, n was set to a value of 1. When n was included as a fit parameter, the estimates of K_d were similar, and the estimates of n were close to 1 in all cases [ranging from 0.82 ± 0.11 (for 17KΔP) to 1.16 ± 0.48 (for Lp(a))], suggesting a 1:1 molar ratio for the Lp(a)/apo(a)-plasminogen interaction. Marked increases in K_d values (i.e., from 20−30 nM to >3 μM) were observed using r-apo(a) derivatives which lack the protease-like domain [r-apo(a) species 17KΔP and 17KΔVP; see Figure 4 and Table 1]. The effect of disruption of the lysine binding site in kringle IV type 10 [r-apo(a) species 17KΔAsp] on plasminogen binding of this r-apo(a) derivative appeared to be negligible (compare the K_d of 20.1 ± 3.3 nM for 17K versus the K_d of 25.6 ± 2.6 nM for 17KΔAsp; see Figure 4 and Table 1). Taken together, these data suggest that apo(a)-plasminogen interactions are mediated by lysine binding sites (LBS) present in plasminogen which bind to sequences within the protease domain of apo(a).

Binding of 17K R-apo(a) to Fluorescein-Labeled Lys-Plasminogen in Solution. Fluorescein-labeled Lys-plasminogen was prepared from Plg(S741C)-fluorescein as described under Experimental Procedures. Binding of purified 17K r-apo(a) to labeled Lys-plasminogen was performed as detailed above for Glu-Plg(S741C)-fluorescein. The results obtained for this experiment are shown in Figure 5. The K_d for the binding of r-apo(a) to Lys-plasminogen was determined to be 191.6 ± 1.3 nM, a value approximately an order of magnitude higher than that for the binding of this r-apo(a) species to Glu-plasminogen in solution. When n was used as a fit parameter, the model predicted a value of n = 1.03 ± 0.03, suggesting a 1:1 molar ratio for this interaction.

Influence of the Solution Phase Interaction between Apo(a) and Plasminogen on the Binding of Plasminogen to Plasmin-Modified Fibrinogen. In order to assess the impact of apo(a) and its interaction with plasminogen on the binding of plasminogen to fibrin(ogen), we first measured separately the binding of radioiodinated r-apo(a) (17K species) and plasminogen to plasmin-modified fibrinogen (Figure 6). The binding of both proteins conformed to a rectangular hyperbole, and therefore the data were analyzed according to a single-site model whereby

$$[\text{bound}] = B_{max}[\text{free}]/(K_d + [\text{free}]) \quad (2)$$

in which B_{max} represents the total concentration of binding sites for r-apo(a) or plasminogen on plasmin-modified fibrinogen and [bound] and [free] represent the concentrations of bound and free ligands, respectively. Nonlinear regression analysis yielded B_{max} = 23.4 ± 1.1 nM and K_d = 0.80 ± 0.10 μM for plasminogen and B_{max} = 2.7 ± 0.3 nM and K_d = 1.8 ± 0.42 μM for r-apo(a) (Table 2). The lines on Figure 6 are the regression lines, and the fits were characterized by randomly distributed residuals, which indicates an excellent fit to the single-site model.

We then repeated the measurement of plasminogen binding in the presence of unlabeled 17K r-apo(a) at fixed total concentrations of 0, 250, 500, 1000, 2000, and 4000 nM; the data are shown in Figure 7. The data clearly show that the amount of plasminogen bound is affected by the inclusion of r-apo(a) in a concentration-dependent manner. Qualitatively, apo(a) appears to compete with plasminogen for binding to the plasmin-modified fibrinogen. This can be seen particularly well, for example, by noting the decrease in the binding of plasminogen at a fixed input concentration (e.g., 1.0 μM) at increasing concentrations of input r-apo(a). However, the data cannot be quantitatively rationalized as a consequence of simple competition, in this instance at least, because the concentration of available sites for plasminogen far exceeds the number available for apo(a) [23.4 nM sites for plasminogen, 2.7 nM sites for apo(a); Figure 6]. Thus, we considered whether the solution phase interaction between plasminogen and apo(a) could rationalize the data of Figure 7.

A model was considered in which free plasminogen (P) can bind to a class of sites on plasmin-modified fibrinogen designated F_1 and apo(a) (A) can bind another class of sites designated F_2. Two distinct classes of binding sites were considered in order to account for the fact that the binding capacities of the plasmin-modified fibrinogen are very different for plasminogen and apo(a) (Figure 6) (i.e., if the sites were identical, we would expect identical binding capacities for both proteins). These binding interactions are characterized by the equilibrium expressions $[P][F_1] = K_1[P{\cdot}F_1]$ and $[A][F_2] = K_2[A{\cdot}F_2]$. In addition, a solution phase interaction between plasminogen and apo(a) was considered with the binding characterized by $[A][P] = K_3[A{\cdot}P]$. Finally, equilibrium interactions between A·P, F_1, and F_2 to form $A{\cdot}P{\cdot}F_1$ and $A{\cdot}P{\cdot}F_2$ were allowed, with the equilibrium expression $[A{\cdot}P][F_1] = K_4[A{\cdot}P{\cdot}F_1]$ and $[A{\cdot}P][F_2] = K_5[A{\cdot}P{\cdot}F_2]$. A schematic representation of the above equilibria is presented in Figure 8.

The conservation of total sites on plasmin-modified fibrinogen of the two classes of sites is given by $[F_1]_{total} = [F_1] + [P{\cdot}F_1] + [A{\cdot}P{\cdot}F_1]$ and $[F_2]_{total} = [F_2] + [A{\cdot}F_2] + [A{\cdot}P{\cdot}F_2]$. These relationships can be used to solve for fibrinogen-bound plasminogen ($[P]_{bound} = [P{\cdot}F_1] + [A{\cdot}P{\cdot}F_1] + [A{\cdot}P{\cdot}F_2]$), and the result is given in eq 3:

$$[P]_{bound} = [F_1]_{total}([P]/K_1 + [A{\cdot}P]/K_4)/(1 + [P]/K_1 + [A{\cdot}P]/K_4) + [F_2]_{total}([A]/K_2 + [A{\cdot}P]/K_5)/(1 + [A]/K_2 + [A{\cdot}P]/K_5) \quad (3)$$

Free plasminogen (P), free apo(a) (A), and their complex (A·P) are related by the equilibrium expression $[A][P] = K_3[A{\cdot}P]$ or $([P]_0 - [A{\cdot}P])([A]_0 - [A{\cdot}P]) = K_3[A{\cdot}P]$, where $[P]_0$ and $[A]_0$ are the *total* concentrations of plasminogen

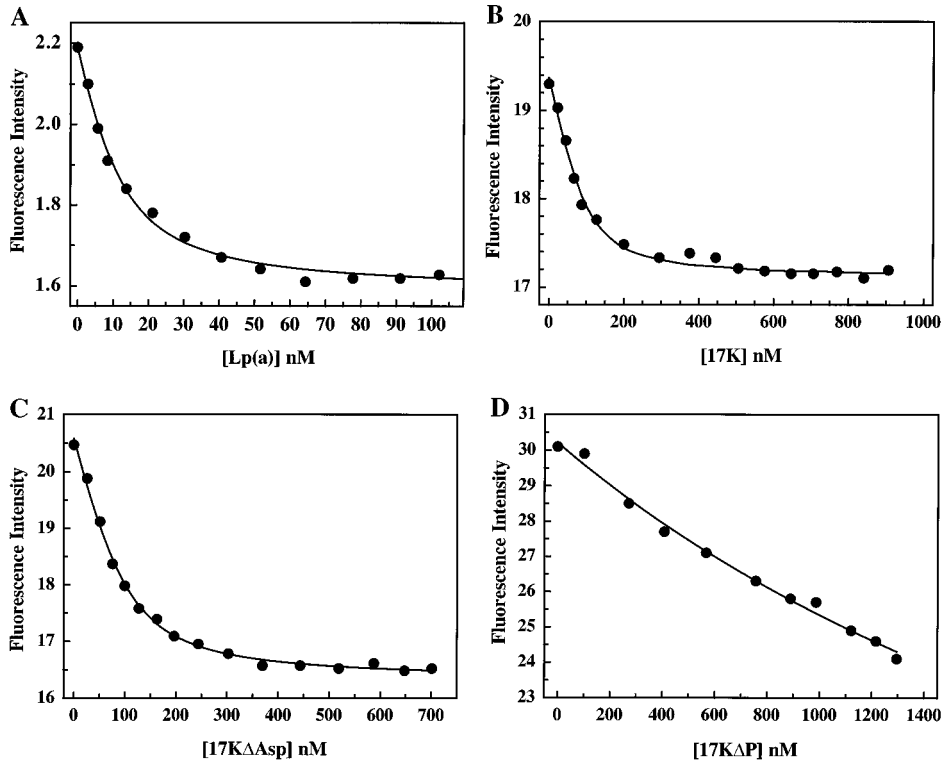

FIGURE 4: Binding of r-apo(a) derivatives to fluorescein-labeled Glu-plasminogen in solution. A variant of recombinant plasminogen [Plg-(S741C); containing a Ser→Cys substitution at the active site] was labeled with the thiol-specific reagent 5′-(iodoacetamido)fluorescein. Changes in fluorescence intensity upon titration of the labeled Glu-plasminogen [Plg(S741C)-fluorescein] with solutions containing Lp(a) (panel A) or r-apo(a) species (17K, 17KΔAsp, and 17KΔP; panels B−D, respectively) and labeled Glu-plasminogen (identical concentration to that initially present in the cuvette) are shown by the closed circles. The data were analyzed by nonlinear regression to eq 1; the solid lines on the graphs are the regression lines.

Table 1: Binding of Lp(a) and R-apo(a) Derivatives to Plg(S741C)-Fluorescein in Solution[a]

ligand	K_d (nM)[b]	ligand	K_d (nM)[b]
Lp(a)	5.58 ± 0.80	KIV_{8-P}	41.8 ± 7.9
17K	20.1 ± 3.3	KIV_{9-P}	40.4 ± 4.5
17KΔAsp	25.6 ± 2.6	KIV_{10-P}	27.9 ± 2.7
12K	29.0 ± 4.0	KIV_{10}	3150 ± 290
6K	10.7 ± 2.5	17KΔVP	3700 ± 300
KIV_{6-P}	21.7 ± 3.4	17KΔP	3160 ± 140
KIV_{7-P}	17.0 ± 7.0		

[a] A solution of fluorescein-labeled Glu-plasminogen [Plg(S741C)-fluorescein] was titrated with a solution containing the indicated ligands and an equal concentration of Plg(S741C)-fluorescein in a Perkin-Elmer LS 50B fluorescence spectrometer. Data were obtained which relate the change in fluorescence to the concentration of ligand added; these data were fit to eq 1 by nonlinear regression with K_d as the fit parameter. [b] For determination of K_d values, n was set to a value of 1 for the nonlinear regression.

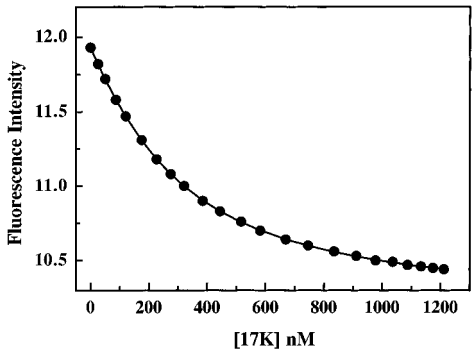

FIGURE 5: Binding of 17K r-apo(a) to fluorescein-labeled Lys-plasminogen in solution. Fluorescein-labeled Lys-plasminogen was derived from Plg(S741C)-fluorescein as described under Experimental Procedures. Changes in fluorescence intensity upon titration of the labeled Lys-plasminogen with a solution containing 17K r-apo(a) and fluorescein-labeled Lys-plasminogen (identical concentration to that in the cuvette) are shown by the closed circles. The data were analyzed by nonlinear regression to eq 1; the solid lines on the graphs are the regression lines.

and apo(a). The values of [P], [A], and [A·P] were calculated from the *total* concentrations and the quadratic equation:

$$[A\cdot P] = 0.5\{[P]_0 + [A]_0 + K_3 - [([P]_0 + [A]_0 + K_3)\text{^}2 - 4[A]_0[P]_0]\text{^}0.5\}$$

The value of K_3 was set at 20.1 nM, which is the value of the dissociation constant for the solution phase interaction between 17*K* r-apo(a) and Glu-plasminogen as determined in this study (Table 1). The data of Figure 7 were then fit globally by nonlinear regression analysis to eq 3 with $[P]_{bound}$, $[P]_0$, and $[A]_0$ as input parameters and $[F_1]_{total}$, $[F_2]_{total}$, K_1, K_2, K_4, and K_5 as parameters to be optimized. The results are shown in Table 2; the fit of the model to the data was excellent, with essentially randomly distributed residuals. This is shown by Figure 7, where the indicated lines are the regression lines that resulted from fitting the data globally to eq 3.

The data in Table 2 indicate that although free plasminogen binds to plasminogen binding sites (i.e., F_1) in a plasmin-modified fibrinogen well (K_d = 0.78 μM), the complex of plasminogen with r-apo(a) does not (K_d = 9.4 μM). The A·P complex, however, does bind the second class of low-capacity sites to which r-apo(a) binds (i.e., F_2; K_d = 0.56 μM). Because this second class of sites is of relatively low

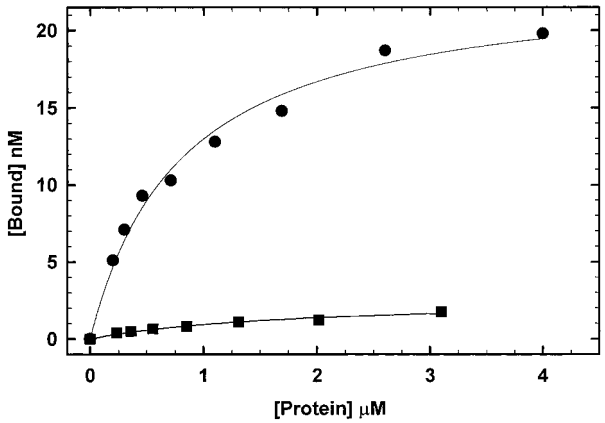

FIGURE 6: Binding of Glu-plasminogen and 17K r-apo(a) to plasmin-modified fibrinogen. Fibrinogen was immobilized in the wells of microtiter plates and subsequently partially degraded by the addition of plasmin. A range of concentrations of radioiodinated Glu-plasminogen (0−4 μM) or 17K r-apo(a) (0−3.1 μM) was incubated with the immobilized fibrinogen; radioiodinated proteins were >97% trichloroacetic acid (TCA)-precipitable, and the specific activity of the labeled proteins was ~2000 cpm/ng in all cases. Following washing, the bound species were quantified by γ-radiation counting. Nonspecific binding was considered to be that observed in the presence of 0.2 M ϵ-ACA. The binding data were fit to eq 2 by nonlinear regression; the solid lines in the figure are the regression lines. Solid circles, Glu-plasminogen; solid squares, 17K r-apo(a).

Table 2: Binding of R-apo(a) and Glu-Plasminogen to Plasmin-Modified Fibrinogen[a]

	Glu-plasminogen[b]	17K r-apo(a)[b]	Glu-plasminogen + 17K r-apo(a)[c]
$[F_1]_{total}$ (nM)	23.4 ± 1.1		22.0 ± 1.0
$[F_2]_{total}$ (nM)		2.7 ± 0.3	11.5 ± 1.2
K_1 (μM)	0.8 ± 0.1		0.78 ± 0.08
K_2 (μM)		1.8 ± 0.42	1.3 ± 0.5
K_4 (μM)			9.4 ± 3.0
K_5 (μM)			0.56 ± 0.11

[a] Various concentrations of radioiodinated purified 17K-r-apo(a) or Glu-plasminogen, or of radioiodinated Glu-plasminogen containing a fixed concentration of unlabeled 17K r-apo(a), were allowed to bind to plasmin-modified fibrinogen immobilized in the wells of microtiter plates. Following washing of the wells, the radioactivity associated with the plasmin-modified fibrinogen was quantified by γ-radiation counting. Nonspecific binding was considered to be that observed in the presence of 0.2 M ϵ-ACA. [b] Binding data were fit to eq 2 by nonlinear regression. [c] Binding data were fit to eq 3 by nonlinear regression.

capacity, however, the net result of the solution phase interaction between apo(a) and plasminogen is a reduction in the amount of plasminogen bound. This reduction is the result of the formation in solution of A·P which does not bind well to the same class of sites to which plasminogen binds, rather than the consequence of direct competition between apo(a) and plasminogen for the same class of sites on plasmin-modified fibrinogen.

DISCUSSION

The results of the present study demonstrate that both apo(a) and Lp(a) bind tightly to plasminogen, and that the binding of apo(a) to plasminogen is a lysine-dependent interaction which can be abolished by addition of the lysine analogue ϵ-aminocaproic acid. We chose to analyze the interaction of apo(a)/Lp(a) with plasminogen in solution in order to rigorously quantify the binding of the respective proteins to each other in their native state. We utilized a

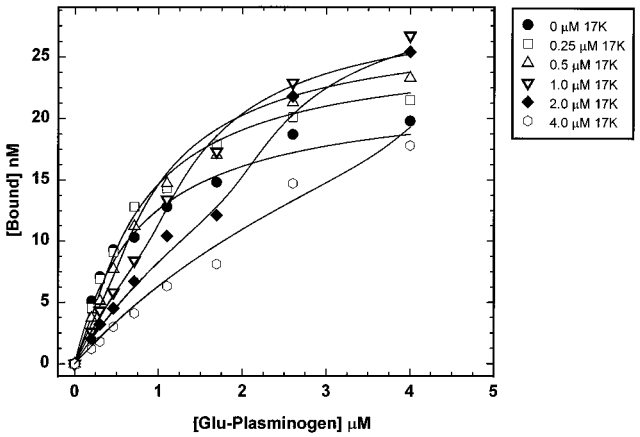

FIGURE 7: Effect of 17K r-apo(a) on the binding of Glu-plasminogen to plasmin-modified fibrinogen. A range of concentrations of radioiodinated Glu-plasminogen (0−4 μM), each containing a single fixed concentration of unlabeled 17K r-apo(a) (0−4 μM), was incubated with plasmin-modified fibrinogen immobilized in the wells of microtiter plates. After washing, the bound Glu-plasminogen was quantified by γ-radiation counting; nonspecific binding was considered to be that observed in the presence of 0.2 M ϵ-ACA. The binding data were fit to eq 3 by nonlinear regression; the solid lines in the figure are the regression lines.

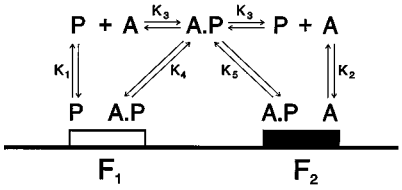

FIGURE 8: Model for the effect of apo(a) on the binding of plasminogen to fibrin(ogen). Plasminogen (P) and apo(a) (A) bind to distinct sites on fibrin(ogen) (F_1 and F_2, respectively). Plasminogen and apo(a) can also interact in solution; the resultant complex (A·P) binds with similar affinity to apo(a) binding sites (F_2) as apo(a) alone (i.e., $K_2 \approx K_5$) but exhibits a greatly reduced affinity for plasminogen binding sites relative to plasminogen alone (i.e., $K_4 \gg K_1$). Since the number of plasminogen sites is greater than the number of apo(a) sites (i.e., $[F_1] > [F_2]$), the formation of the apo(a)/plasminogen complex results in a decrease in the binding of plasminogen to fibrin(ogen).

fluorescein-labeled plasminogen derivative for these studies; changes in fluorescence upon apo(a) binding were analyzed using nonlinear regression to determine K_d and n values. Using this system, we determined that the 17K r-apo(a) binds to plasminogen in a 1:1 stoichiometry with a K_d of 20.1 ± 3.3 nM. Analysis of the binding of 12K r-apo(a) to fluorescein-labeled plasminogen yielded a comparable K_d value of 29.0 ± 4.0 nM, suggesting that the number of major repeat kringles in apo(a) (i.e., kringle IV type 2) does not affect the affinity of this interaction. Additionally, analysis of the binding of the r-apo(a) derivatives 6K, KIV_{6-P}, KIV_{7-P}, KIV_{8-P}, KIV_{9-P}, and KIV_{10-P} to fluorescein-labeled Glu-plasminogen yielded K_d values similar to those obtained using the 17K and 12K species (see Table 1). These data suggest that apo(a) kringle IV types 2−9 (one or more of which are lacking in these constructs) also are not involved in the interaction of apo(a) with plasminogen. Plasminogen binding results obtained with these derivatives also indicate that weak LBS [present in apo(a) kringle IV types 5−8] (*19*−*20*), which are involved in the initial noncovalent interactions between apo(a) and LDL to form covalent Lp(a) particles, are not required for the binding of apo(a) to plasminogen. Removal of the LBS in kringle IV type 10 by site-directed

mutagenesis also did not alter the K_d of the resulting apo(a) derivative (17KΔAsp; see Table 1) compared to the K_d obtained using the 17K r-apo(a), suggesting that the strong LBS in this kringle is not required for the apo(a)−plasminogen interaction. Data obtained using apo(a) derivatives which lack the protease domain exhibit severely reduced affinity of binding to fluorescently-labeled plasminogen (17KΔVP; 17KΔP; see Table 1). This suggests that sequences within the protease-like domain of apo(a) mediate its binding to plasminogen. This is the first functional role that has been ascribed to this apo(a) domain, since it has been reported to be proteolytically inactive (*12*). Since we have demonstrated that the apo(a)−plasminogen interaction is lysine-dependent, we would conclude that lysine residues in the protease domain of apo(a) interact with LBS in plasminogen. In this context, it is interesting to note that there are a total of 23 lysine residues in the apo(a) molecule, with 14 in the protease-like sequence, 6 in the kringle V domain, and a total of 3 in the nonidentically repeated kringle sequences (*11*).

The striking homology which has been observed between apo(a) and plasminogen has led to the hypothesis that the pathological effects of Lp(a) could be attributable to the ability of apo(a) to interfere with activation of plasminogen by plasminogen activators. Consistent with this hypothesis, Lp(a) has been found to compete with plasminogen for binding to fibrin (*16*−*18*, *42*, *43*), endothelial and mononuclear cells (*44*, *45*), and platelets (*46*). Furthermore, Lp(a) has been shown to inhibit plasminogen activation by tPA on platelets (*46*) and in the presence of CNBr fibrinogen fragments (*23*) or D-dimer (*24*). Clots introduced into the lungs of mice overexpressing apo(a) from a transgene exhibit retarded clot lysis initiated by the infusion of pharmacological doses of tPA (*21*). Similarly, we have demonstrated using an *in vitro* fibrinolysis model consisting of purified components that the addition of recombinant apo(a) resulted in inhibition of fibrin clot lysis through decreased tPA-dependent plasminogen activation (*47*). Finally, inhibition of plasmin generation by apo(a) has been shown to enhance vascular smooth muscle cell proliferation and migration in culture (*48*) and in mice overexpressing apo(a) from a transgene (*49*), an effect attributable to decreased TGF-β activation.

Owing to the structural similarity between apo(a) and plasminogen, it has generally been hypothesized that the ability of apo(a)/Lp(a) to inhibit plasminogen binding to fibrin is a consequence of direct competition between the two proteins for similar or identical binding sites on fibrin. However, our demonstration of a solution phase interaction between apo(a) and plasminogen and consideration of the effect of this interaction on plasminogen binding to plasmin-modified fibrinogen in the presence of apo(a) strongly suggest that apo(a) binds to plasminogen in solution, thereby decreasing the affinity of the resultant complex for the cognate binding sites for plasminogen on this substrate (Table 2; Figure 8). Although the apo(a)/plasminogen complex binds to apo(a) sites on plasmin-modified fibrinogen with an affinity similar to apo(a) alone (Table 2), this second class of sites is of lower abundance than the plasminogen binding sites (Figure 6), thus resulting in a net decrease in the amount of plasminogen bound to plasmin-modified fibrinogen (Figure 7).

The binding of plasminogen to Lp(a) has been suggested by the work of Liu and co-workers (*24*, *50*) in which they reported that Lp(a) enhances the binding of plasminogen to D-dimer. However, they did not observe direct binding of plasminogen to immobilized Lp(a) which was interpreted to suggest that plasminogen binding sites on Lp(a) were masked prior to a fibrin-induced conformational change in Lp(a) (*50*). However, this finding does not exclude the possibility of a solution phase interaction between Lp(a) and plasminogen, as we have demonstrated in the current study. Furthermore, their finding that Lp(a) at a concentration of 0.2 μM enhanced plasminogen binding to D-dimer is in agreement with our data at a similar concentration of r-apo(a) (see Figure 7; 0.25 μM 17K).

A recent study reported by Hervio and co-workers (*43*) examined the ability of Lp(a) to inhibit the binding of plasminogen to plasmin-degraded fibrin surfaces. They found that Lp(a) and plasminogen bind to this surface through different sites with the degraded fibrin surface possessing an approximately 1000-fold greater maximal binding capacity for plasminogen than Lp(a); Lp(a) binding was characterized by a 10-fold lower dissociation constant than plasminogen. Despite this large difference in binding capacities, Hervio *et al.* concluded that binding of Lp(a) to degraded fibrin directly inhibited the binding of plasminogen to its cognate sites (*43*). However, these data are also compatible with the existence of a solution phase Lp(a)/plasminogen complex that binds to plasminogen binding sites on fibrin with a greatly reduced affinity, thus accounting for the ability of Lp(a) to inhibit plasminogen binding despite being itself bound to the fibrin surface at a concentration approximately 1000-fold lower than that of bound plasminogen. Interestingly, Hervio *et al.* reported an inhibition constant for inhibition of plasminogen binding to fibrin by Lp(a) of 32 nM (*43*); this value corresponds closely to the K_d for the plasminogen/Lp(a) complex which we report in the current study (Table 1).

A number of studies (*16*, *18*, *51*) have reported that in experiments in which apo(a) or Lp(a) is used to compete with plasminogen for binding to fibrin(ogen), the inhibition has been significantly less than 100% at a saturating concentration of apo(a)/Lp(a). These findings are in concordance with our prediction that apo(a)/plasminogen complexes are capable of binding to plasmin-modified fibrin(ogen); a significant proportion of the bound plasminogen detected in these experiments may in fact correspond to bound apo(a)/plasminogen or bound Lp(a)/plasminogen complexes.

The mechanism by which apo(a) inhibits tPA-mediated activation of plasminogen remains controversial. Several reports suggest that apo(a)/Lp(a) binds to fibrin with high affinity and can compete with plasminogen for binding sites on fibrin (*16*−*18*, *42*). This would subsequently lead to inhibition of fibrinolysis through decreased formation of the ternary complex of plasminogen, tPA, and fibrin which is required for efficient plasminogen activation (*52*). Our data are consistent with this scenario, but also suggest that a significant component of the fibrin(ogen)-bound plasminogen would be in complex with apo(a)/Lp(a). It is conceivable that plasminogen, when bound to apo(a)/Lp(a) on fibrin(ogen), is resistant to activation by plasminogen activators, thereby further contributing to the antifibrinolytic potential of Lp(a).

A number of studies have been undertaken to define at the kinetic level the inhibitory effect of Lp(a) on plasminogen activation. Liu and co-workers (*24*) reported that Lp(a) inhibition of tPA activation was of the uncompetitive type, therefore suggesting that it could not be attributed to inhibition of plasminogen binding to fibrin by Lp(a). Other investigators have also reported that apo(a) inhibition of tPA-mediated plasminogen activation is uncompetitive (*23*), while the findings of Edelberg and co-workers (*22*) point to inhibition of a competitive type. However, in the studies reported to date, the inhibition by Lp(a) was generally sensitive to the concentration of plasminogen, tPA, and fibrin cofactor, reflecting the complexity of the mechanism of fibrin-dependent plasminogen activation by tPA. Our data which show that apo(a) interacts directly with plasminogen suggest that this binding may result in decreased binding of plasminogen to fibrin, inhibition of formation of the ternary complex with tPA and fibrin, or interference with the cleavage of plasminogen by tPA. These effects are compatible with either competitive, uncompetitive, or mixed inhibition of tPA-mediated plasminogen activation; additional detailed kinetic studies will be required to address this question.

Surprisingly, we found that r-apo(a) bound to Lys-plasminogen in solution with an affinity that was approximately an order of magnitude less than that observed for Glu-plasminogen; the affinity of r-apo(a) for Lys-plasminogen in solution was similar in magnitude to that observed for binding of apo(a) to immobilized Glu-plasminogen. Lys-plasminogen, formed by cleavage of native Glu-plasminogen between Lys77 and Lys78 by plasmin, displays enhanced binding affinity for fibrin (*32*) and is a better substrate for tPA (*52*), which has been interpreted to suggest a role for Lys-plasminogen in positive feedback during fibrinolysis. Lys-plasminogen is characterized by an "open" conformation relative to the "closed" conformation adopted by Glu-plasminogen, which reflects removal of lysine-dependent intramolecular interactions in the "open" form (*53*). In addition, the high-affinity LBS in plasminogen becomes slightly weaker whereas the low-affinity LBS becomes 20-fold stronger upon conversion from the Glu to the Lys form (*54*−*56*). Interestingly, the transition from the Glu to the Lys form appeared to decrease the affinity of apo(a) for plasminogen, the basis for which remains to be explored. However, these data are consistent with our earlier *in vitro* fibrinolysis data, which showed that apo(a) had only a minimal effect on Lys-plasminogen-mediated fibrin clot lysis (*47*).

The binding affinity of Lp(a)/apo(a) for plasminogen that we have determined in this study is clearly compatible with the existence of Lp(a)/plasminogen complexes in plasma *in vivo*, though demonstration of such complexes awaits further study. In addition, Lp(a) has been found in an ϵ-ACA-releasable fraction from the aortic intima (*57, 58*); although this was interpreted to reflect the presence of Lp(a)/fibrin complexes in the intima, these data are also consistent with the existence of Lp(a)/plasminogen complexes in the intima (possibly bound to fibrin; see above). Interestingly, although plasminogen was only found in ∼20% of intimal ϵ-ACA extracts, a statistically significant positive correlation was observed between the amounts of Lp(a) and plasminogen released by ϵ-ACA (*57*), rather than the inverse correlation that would be expected if Lp(a) and plasminogen competed for the same binding sites on fibrin.

In conclusion, we have shown that apo(a) and Lp(a) bind with a high affinity to Glu-plasminogen in solution. This interaction is mediated by lysine binding sites within plasminogen and by lysine residues present in the protease-like domain of apo(a). We found that apo(a) and plasminogen bound to distinct sites on plasmin-modified fibrinogen and that the formation of the apo(a)/plasminogen complex results in a decrease in plasminogen binding to its cognate site on this substrate. The ability of apo(a) to bind to plasminogen and influence its binding to fibrin may provide the basis for the ability of Lp(a) to interfere with tPA-mediated plasminogen activation, thereby attenuating fibrinolysis and promoting vascular smooth muscle cell migration and proliferation. Collectively, these effects may account for the risk for atherothrombotic disorders associated with elevated plasma levels of Lp(a).

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