Interaction of Fibrin(ogen) with Apolipoprotein(a): Further Characterization and Identification of a Novel Lysine-Dependent Apolipoprotein(a)-Binding Site within the γ Chain 287−411 Region†

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Received March 15, 2006; Revised Manuscript Received June 19, 2006

ABSTRACT: Interaction of lipoprotein(a) with fibrin associated with atherosclerotic lesions promotes its accumulation in the lesions, thereby contributing to the development of atherothrombosis. Numerous studies revealed that this interaction occurs through the apolipoprotein(a) [apo(a)] component of lipoprotein(a) and COOH-terminal Lys residues generated by partial degradation of fibrin with plasmin (a COOH-Lys-dependent mechanism). At the same time, the mechanism of the interaction of apo(a) with intact fibrin(ogen) remained unclear. Our recent study identified the Lys-independent apo(a)-binding sites within the fibrin(ogen) αC domains which contribute to an alternative Lys-independent mechanism. In this study, we performed direct measurements of the interaction between apo(a) and various fibrin(ogen) fragments representing the whole fibrin(ogen) molecule except the αC regions. The experiments revealed that the apo(a)-binding site, identified previously within fibrinogen γ chain residues 207−235 [Klose, R., et al. (2000) *J. Biol. Chem. 275,* 38206−38212], is a high-affinity site and mainly Lys-independent, suggesting that it should also contribute to the Lys-independent mechanism. The experiments also identified a novel Lys-dependent high-affinity apo(a)-binding site within the sequence of γ chain residues 287−411. This site may provide interaction of apo(a) with intact fibrin(ogen) through another alternative mechanism, which depends on internal Lys residues. Thus, apo(a) may interact with intact fibrin through the Lys-independent and Lys-dependent mechanisms, while the COOH-Lys-dependent mechanism may prevail in the presence of fibrinolytic activity.

It is well established that elevated plasma levels of lipoprotein(a) [Lp(a)][1] and fibrinogen are independent risk factors for atherosclerotic cardiovascular diseases (*1, 2*). Lp(a) and fibrin deposits have been detected in atherosclerotic lesions, and the accumulation of these components has been connected with their development (*2, 3*). Numerous experiments with transgenic animals have provided direct evidence of the involvement of both fibrin and apo(a), a protein component of Lp(a), in the development and progression of atherosclerosis (*4*−*8*). Specifically, it was shown that fibrinogen deficiency in apo(a) transgenic mice substantially decreased the level of accumulation of apo(a) in the vessel walls as well as lesion development, and it was suggested that fibrin(ogen) provides one of the major sites to which Lp(a) binds to the vessel wall and participates in generation of atherosclerosis (*8*).

Lp(a) interacts with fibrin(ogen) via its apo(a) component, and this interaction occurs in Lys-dependent and Lys-independent manners (*9*−*11*). Numerous studies have characterized the Lys-dependent interaction of apo(a) with COOH-terminal Lys residues generated by limited plasminolysis of fibrin (reviewed in ref *12*). It was also shown using the yeast two-hybrid system that apo(a)-binding sites are located in the fibrinogen D region within the sequence of residues 207−235 of its γ chain and the corresponding homologous portion of its β chain (*10*) (Figure 1A−C). Further binding experiments with apo(a) and/or Lp(a) and synthetic peptides, including γ chain residues 207−235, revealed that the interaction between them is only partially Lys-dependent (*10*). More recent experiments with recombinant fibrinogen αC fragments identified a Lys-independent apo(a)-binding site in the fibrin(ogen) αC region and further localized it within the sequence of residues Aα392−610 forming the αC domain (*11*) (Figure 1A). The apo(a)-binding

† This work was supported by National Institutes of Health Grant HL-56051 to L.M. and by a grant from Institut National de la Santé et de la Recherche Médicale, Paris, France, to E.A.-C.

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[1] Abbreviations: Lp(a), lipoprotein(a); apo(a), apolipoprotein(a), a protein component of Lp(a); TBS-Ca buffer, 20 mM Tris buffer (pH 7.4) containing 150 mM NaCl and 1 mM $CaCl_2$; SPR, surface plasmon resonance; ϵ-ACA, ϵ-aminocaproic acid.

10.1021/bi060526h CCC: $33.50
Published on Web 08/10/2006

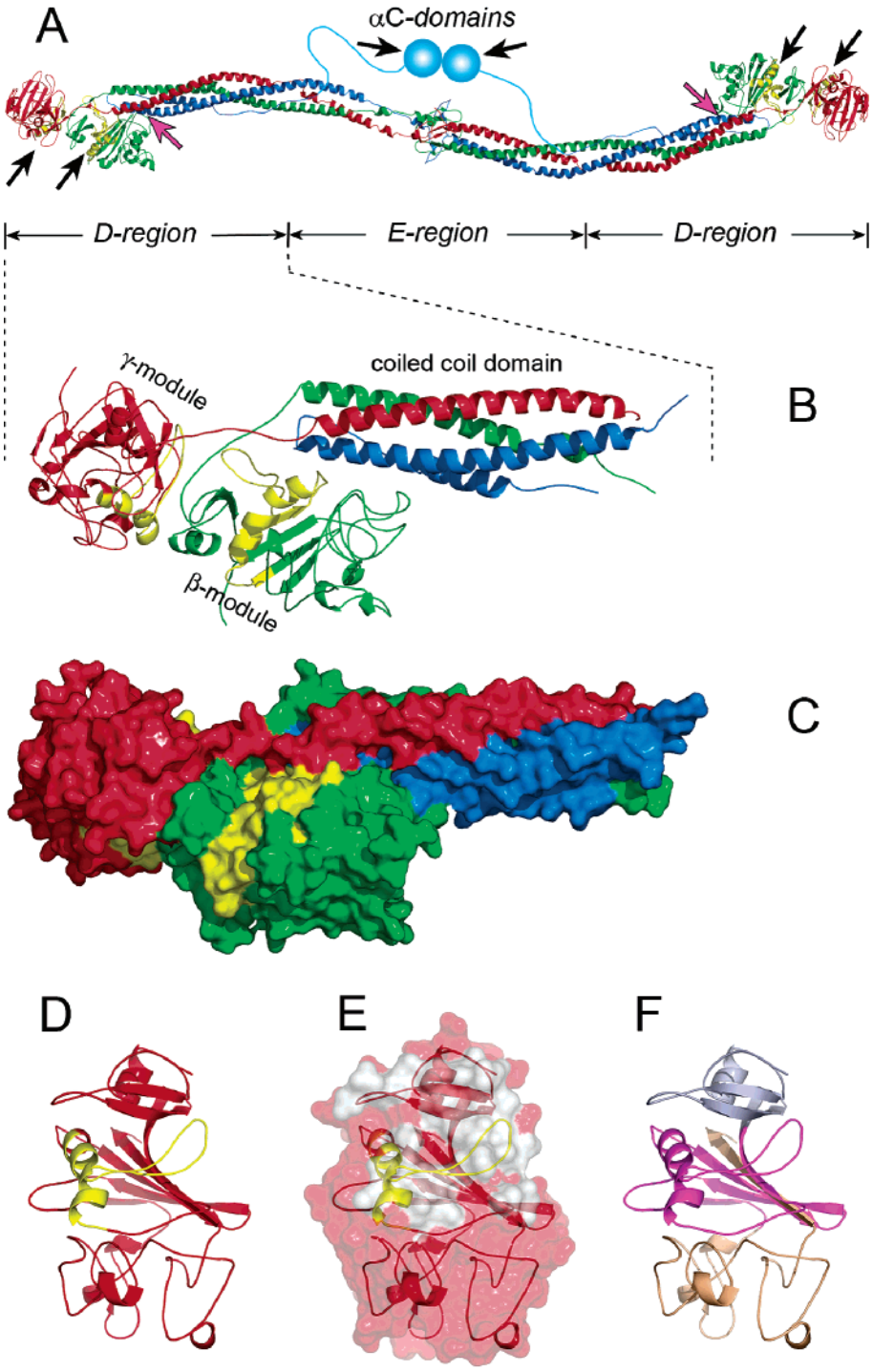

FIGURE 1: Location of the apo(a)-binding sites in fibrinogen and its fragments. (A) Location of the previously identified apo(a)-binding sites in the fibrin(ogen) D regions (*10*) and αC domains (*11*). Fibrinogen is represented by a ribbon diagram based upon its crystal structure (*31*); its interacting αC domains (*41*) are shown as two spheres. The individual fibrinogen chains, Aα, Bβ, and γ, are colored blue, green, and red, respectively; black arrows mark the apo(a)-binding sites, while magenta arrows mark the Aα148−160-containing plasminogen-binding sites located in the D regions (*13*). (B and C) Ribbon diagram and solvent-accessible surface, respectively, of the D fragment based upon its crystal structure (*27*). The D fragment consists of the coiled coil domain (triple blue, green, and red helix) and the β and γ modules formed by the COOH-terminal portions of the Bβ and γ chains, respectively. In both panels, the apo(a)-binding sequences, β247−310 and γ207−235 (*10*), are colored yellow. (D−F) Ribbon diagrams of the isolated γ module (*28*). The color scheme in panels D and E is the same as in the previous panels. The diagram in panel E is covered with semitransparent solvent accessible surface; the surface which is buried in the D fragment and becomes exposed in the isolated γ module is highlighted in white. The diagram in panel F shows the domain structure of the γ module according to ref *28*. The NH_2-terminal (residues γ148−191), central (residues γ192−286), and COOH-terminal (residues γ287−380) domains are colored light blue, magenta, and beige, respectively; the γ381−390 β strand inserted into an antiparallel β sheet of the central domain is also colored beige. This figure was prepared with PyMol (*42*).

sites identified in the D and αC regions are not related to COOH-terminal Lys residues and therefore may play a role in the interaction of apo(a) with intact fibrin(ogen). Whether these sites account for all the apo(a) binding activity of fibrin(ogen) or if there are some additional putative apo(a)-binding sites remains to be established.

Apo(a) consists of the COOH-terminal protease- and kringle 5-like domains and of multiple copies of kringle 4-like domains of plasminogen. The kringle domains of both apo(a) and plasminogen contain Lys-binding sites which provide their interactions with various proteins and thereby participation in different physiological and pathological processes. Specifically, they play an important role in fibrin-dependent tPA-mediated activation of plasminogen into plasmin which initiates fibrinolysis (*13*). Because of their structural homology and because both proteins interact with fibrin via their Lys-binding sites, it was proposed that apo(a) may compete with plasminogen for the Lys-dependent binding sites of fibrino(gen); such competition may inhibit generation of plasmin on fibrin associated with atherosclerotic lesions, resulting in inhibition of fibrinolysis and thereby promoting atherogenesis (*12*).

Interaction of plasminogen with fibrin(ogen) occurs in a Lys-dependent manner. There are two identified plasminogen-binding sites in each half of the intact fibrinogen molecule; one, which includes residues Aα148−160, is in the coiled coil domain of the D region, and the other is located in the αC domain (reviewed in ref *13*) (Figure 1A). In addition, the αC domain contains the Lys-dependent tPA-binding site (*14*). All these sites are important for the initiation of fibrinolysis, while COOH-terminal Lys residues generated upon fibrinolysis are involved in its propagation (*13*). Although all three proteins, apo(a), plasminogen, and tPA, interact with the αC domain, there is practically no competition between them for the αC domain specific binding sites (*11*). However, apo(a) does compete with plasminogen for COOH-terminal Lys residues on partially digested fibrin (*12*). Whether it also competes for the plasminogen-binding sites localized in the D regions remains to be established.

The major goals of this study were to further characterize the interaction between fibrin(ogen) and apo(a), to clarify whether the above-mentioned binding sites account for all the apo(a) binding capacity of fibrin(ogen), and to test which binding sites in fibrin(ogen) are shared by apo(a) and plasminogen.

EXPERIMENTAL PROCEDURES

Proteins. Plasminogen-depleted human fibrinogen and bovine serum albumin were purchased from Calbiochem. Recombinant tPA was a product of Genentech. Human Glu-plasminogen was prepared from citrated human plasma by affinity chromatography on Lys-Sepharose 4B (*15*) and further purified by size exclusion chromatography on Superdex 200 (Pharmacia). Bovine α-thrombin, aprotinin, and carboxypeptidase B were from Sigma.

Antibodies. The rabbit anti-plasminogen polyclonal antibodies were purchased from Chemicon. The peroxidase-conjugated anti-sheep, anti-mouse, and anti-rabbit polyclonal antibodies were from Sigma. The sheep polyclonal anti-apo(a) antibodies and monoclonal antibody mAb A10.2 directed against the lysine-binding site of kringle IV type 10 of apo(a) were obtained as described in refs *16* and *17*. Monoclonal antibodies Fd4-7B3 and 2N3H10 directed against fibrinogen D and E fragments, respectively, were a gift from B. Kudryk (New York Blood Center, New York, NY).

Preparation of Recombinant and Proteolytic Fibrin(ogen) Fragments. The recombinant γ module, including human fibrinogen γ chain residues 148−411 and its subfragments, γ148−191, γ192−286, and γ287−411, were prepared as described in refs *18* and *19*. Recombinant apo(a) A10 was expressed and purified as described previously (*20*). Fibrinogen-derived fragments D_1 and E_3 were prepared by the procedures described in ref *21*. The fibrin-derived E_1 fragment was prepared as described previously (*22*). The NH_2-terminal sequences of D_1, E_1, and E_3 were determined by direct sequencing for 10 cycles using a Hewlett-Packard model G 1000S sequenator. Their NH_2-terminal residues were essentially the same as those reported previously (*23*, *24*), namely, AαAsp^{105}, AαVal^{111}, BβAsp^{134}, γSer^{86}, and γMet^{89} for the D_1 fragment; AαGly^{17}, BβGly^{15}, and γTyr^{1} for the E_1 fragment; and AαVal^{20}, AαHis^{24}, BβLys^{54}, and γTyr^{1} for the E_3 fragment. Note that in E_1 the NH_2-terminal residues were the same as those in intact fibrin while in E_3 the NH_2-terminal portions of the Aα and Bβ chains were missing. Since all three fragments were prepared with plasmin, their COOH-terminal residues were supposedly the same as those reported in refs *23* and *24*.

Generation of Fibrin Surfaces. To prepare a surface with intact fibrin, commercially available fibrinogen (Calbiochem) was additionally purified on Superdex 200, immobilized onto plastic at 10 μg/mL by incubation overnight at 4 °C, and converted into fibrin by treatment with 100 μL of a mixture of thrombin (1 NIH unit/mL) and aprotinin (400 units/mL) per well. Plasmin-degraded fibrin surfaces were prepared via a procedure similar to that described in ref *25* with some modifications. Briefly, immobilized intact fibrin was incubated with 0.1 nM plasmin in 20 mM Tris buffer (pH 7.4) containing 150 mM NaCl and 1 mM $CaCl_2$ (TBS-Ca buffer) for 30 min at 37 °C, washed with 20 mM Tris buffer (pH 7.4) containing 200 mM ϵ-ACA and 500 mM NaCl, and then with TBS-Ca buffer, and incubated with 400 units/mL aprotinin in TBS-Ca buffer for 1 h at 37 °C. Such treatment generated a fibrin surface with maximal apo(a) binding capacity as revealed by the control experiments (not shown). In some experiments, plasmin-degraded fibrin surfaces were treated with 5 μg/mL carboxypeptidase B in TBS-Ca buffer for 1 h at 37 °C to remove COOH-terminal Lys residues.

Solid-Phase Binding Assay. Solid-phase binding was performed in plastic microtiter plates using an enzyme-linked immunosorbent assay (ELISA) as described in ref *11* with some modifications. Microtiter plate wells (Fisher) were coated overnight with fibrinogen or fibrin(ogen) fragments, recombinant γ module or its truncated variants, apo(a) A10, tPA, or plasminogen, all at 10 μg/mL in TBS-Ca buffer following washing with the same buffer. The wells were then blocked with 2% bovine serum albumin in TBS-Ca buffer containing 0.01% Tween 20. Treatment of immobilized proteins with carboxypeptidase B in some experiments was performed as described above. Following washing with TBS-Ca buffer containing 0.01% Tween 20, the indicated concentrations of the proteins used as ligands were added to the wells and incubated for 1 h. Bound proteins were detected by reactions with specific polyclonal or monoclonal antibodies and the peroxidase-conjugated anti-sheep, anti-mouse, or anti-rabbit polyclonal antibodies. A TMB Microwell Peroxide Substrate (Kirkegaard & Perry Laboratories Inc.) was added to the wells, and the amount of bound ligand was measured spectrophotometrically at 450 nm. Data were analyzed by nonlinear regression analysis using eq 1:

$$A = A_{max}/(1 + K_d/[L]) \quad (1)$$

where A represents the absorbance of the oxidized substrate, which is assumed to be proportional to the amount of ligand bound, A_{max} is the absorption at saturation, [L] is the molar concentration of the ligand, and K_d is the dissociation constant.

Biosensor Assay. The interaction of fibrinogen or fibrin(ogen)-derived fragments with apo(a) A10 was studied by surface plasmon resonance (SPR) using the BIAcore 3000 biosensor (Biacore AB, Uppsala, Sweden) which assesses association and dissociation of proteins in real time. Apo(a) A10 at 50 μg/mL was immobilized to the CM5 sensor chip using the amine coupling kit (BIAcore AB) as specified by the manufacturer. Binding experiments were performed in TBS-Ca buffer containing 0.01% Tween 20 at a flow rate of 10 μL/min. The association between the immobilized fragments and the added proteins was monitored as the change in the SPR response; the dissociation was assessed upon replacement of the ligand solution for the buffer without ligand. To regenerate the chip surface, complete dissociation of the complex was achieved by adding 10 mM HCl for 30 s following re-equilibration with binding buffer.

Protein Structure Analysis. The solvent accessible surface areas of residues 207−235 and 287−411 of the γ chains in the D_1 fragment and the isolated γ module were calculated on the basis of atomic coordinates available from the Protein Data Bank (*26*) as entries 1FZA and 1FIB, respectively (*27*, *28*). All calculations were performed using NACCESS (*29*).

RESULTS

The fibrinogen D regions and αC domains have been implicated in the interaction with apo(a) (*10*, *11*) (Figure 1A). To check if other fibrinogen regions and/or domains are involved in apo(a) binding and to further characterize apo(a)-binding sites of the D regions, we tested the interaction of recombinant apo(a) A10 with various fibrin(ogen)-derived proteolytic fragments and their recombinant subfragments.

Interaction of Apo(a) with Immobilized Fibrin(ogen)-Derived Proteolytic Fragments. In an ELISA, when the immobilized D_1 and E_3 fragments, derived from the fibrinogen D and E regions, respectively (Figure 1A,B), were incubated with increasing concentrations of apo(a) A10, only D_1 exhibited dose-dependent binding (Figure 2). Like E_3, the more intact fibrin-derived E_1 fragment (see Experimental Procedures) also failed to interact with apo(a). The interaction between apo(a) A10 and D_1 occurred with a high affinity (K_d = 7.7 nM) (Table 1). This interaction was influenced by the Lys analogue, ϵ-aminocaproic acid (ϵ-ACA), the addition of which reduced the amount of bound apo(a) A10 and increased the apparent K_d value to 28.4 nM (Figure 2 and Table 1). At the same time, no changes in binding were observerd after the D_1 fragment was treated with carboxypeptidase B to remove COOH-terminal Lys residues (not shown). All these experiments indicate that the fibrin(ogen) E region does not contain apo(a)-binding sites and that those localized in the D regions are not related to COOH-terminal Lys residues.

To further characterize the interaction between apo(a) and the D regions, we tested if plasminogen, which also binds to these regions through the Aα148−160-containing sites (*13*) (Figure 1A), would interfere with this interaction. In an ELISA, when apo(a) at 0.1 μM (saturating concentration) was added to the immobilized D_1 fragment in the presence of increasing concentrations of plasminogen (up to 2.5 μM), virtually no differences in apo(a) binding were observed (Figure 2, inset). At the same time, when plasminogen at 0.1 μM was added to D_1 in the presence of increasing concentrations of apo(a) A10, the latter inhibited binding of the former by ~40%. This inhibition reflects most likely the competition of apo(a) A10 for the Aα148−160-containing plasminogen-binding site located in the coiled coil domain of D_1 (Figure 1A,B).

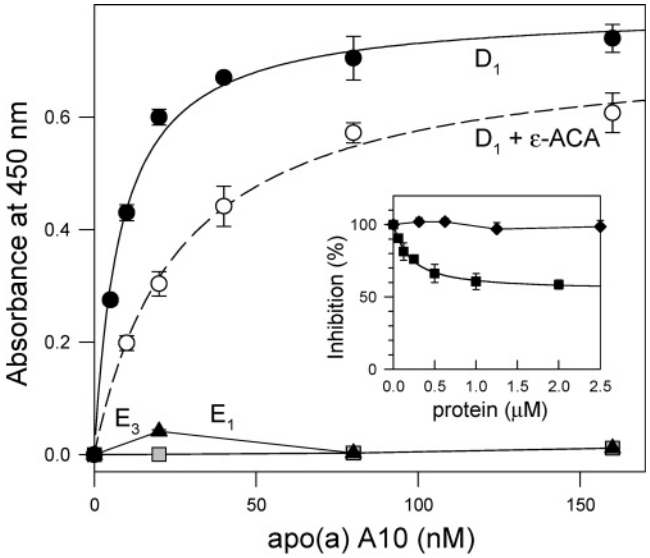

FIGURE 2: Analysis of binding of apo(a) A10 to the immobilized fibrin(ogen) fragments. Increasing concentrations of apo(a) A10 were added to the immobilized D_1 fragment (●), E_3 fragment (▲), and E_1 fragment (■). The data for the binding of apo(a) A10 to the immobilized D_1 fragment in the presence of 0.1 μM ϵ-ACA are shown with empty circles. Bound apo(a) A10 was detected with specific anti-apo(a) antibodies. The curves represent the best fit of the data to eq 1. The inset shows results of the experiment in which apo(a) A10 at 0.1 μM was incubated with microtiter wells coated with the D_1 fragment in the presence of increasing concentrations of plasminogen (◆); similarly, plasminogen at 0.1 μM was incubated with the immobilized D_1 fragment in the presence of increasing concentrations of apo(a) A10 (■). Bound proteins were detected with specific anti-apo(a) or anti-plasminogen polyclonal antibodies, as described in Experimental Procedures. All results are means ± the standard deviation of three independent determinations.

Table 1: Dissociation Constants (K_d) for the Interaction of Apo(a) A10 with the Fibrin(ogen) Fragments Detected by an ELISA[a]

fragment	K_d (nM) without ϵ-ACA	K_d (nM) with 100 mM ϵ-ACA
D_1 fragment	7.7 ± 0.5	28.4 ± 2.2
E_1 fragment	nb[b]	nb[b]
E_3 fragment	nb[b]	nb[b]
γ module	1.8 ± 0.5	21.1 ± 1.4
γ148−191	nb[b]	nb[b]
γ192−286	1.1 ± 0.2	5.5 ± 2.1
γ287−411	60.6 ± 3.5	nb[b]

[a] Values are means ± the standard deviation of at least three independent experiments. [b] No binding observed.

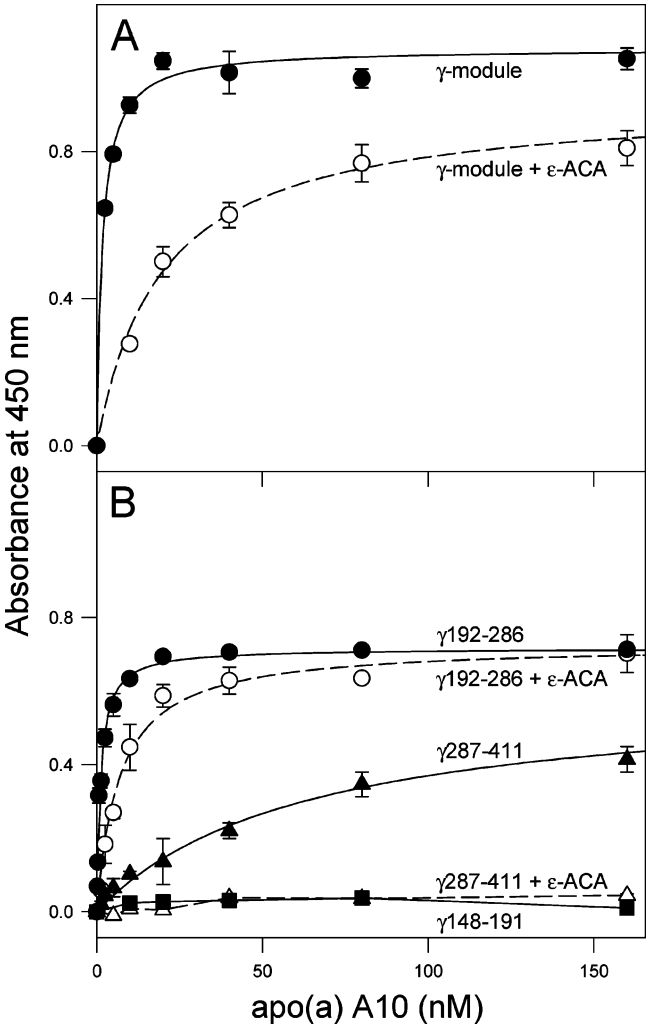

FIGURE 3: Analysis of binding of apo(a) to the recombinant γ module and its subfragments. (A) Increasing concentrations of apo(a) A10 were incubated with microtiter wells coated with the γ module in the absence (●) or presence (○) of 100 mM ϵ-ACA. (B) Increasing concentrations of apo(a) A10 were incubated with microtiter wells coated with the γ148−191 (squares), γ192−286 (circles), and γ287−411 (triangles) fragments; the experiments with γ192−286 and γ287−411 were performed in the absence (filled symbols) or presence (empty symbols) of 100 mM ϵ-ACA. Bound apo(a) A 10 was detected with specific anti-apo(a) antibodies as described in Experimental Procedures. The curves represent the best fit of the data to eq 1. Results are means ± the standard deviation of three independent determinations.

Interaction of Apo(a) A10 with the Recombinant γ Module and Its Subfragments. Because the γ module is a part of the D_1 fragment (Figure 1B) and because the previously identified apo(a)-binding site was localized within its γ207−235 region (*10*), we next focused on the interaction of this module with apo(a) A10. In an ELISA, the immobilized recombinant γ module exhibited a dose-dependent binding to apo(a) A10 with an apparent K_d of 1.8 nM (Figure 3A and Table 1). This binding was influenced by ϵ-ACA, the addition of which reduced the amount of bound apo(a) A10 and increased the K_d value to 21.1 nM. Note that the affinities of apo(a) A10 for the γ module and D_1, both in the absence and in the presence of ϵ-ACA, were comparable (Table 1). These data indicate that binding of apo(a) A10 to the immobilized γ module, like that to the immobilized D_1 fragment, occurs with high affinity and that such binding is only partially Lys-dependent.

It was shown that the γ module consists of three independently folded domains (*18*, *28*) (Figure 1F). We have recently expressed the recombinant fragments, γ148−191, γ192−286, and γ287−411, corresponding to the NH_2-terminal domain, the central domain, and the COOH-terminal domain with the γ381−411 extension, respectively (*19*). These fragments were used to test the involvement of individual γ module domains in the interaction with apo(a). In an ELISA, the immobilized γ148−191 fragment did not bind apo(a) A10, indicating that the NH_2-terminal domain is not involved in this interaction (Figure 3B). As expected, the immobilized γ192−286 fragment containing the apo(a)-binding γ207−235 sequence (*10*) bound apo(a) A10. The binding occurred with high affinity; the apparent K_d was found to be 1.1 nM (Table 1). This binding was only slightly inhibited by ϵ-ACA which, when added, increased the K_d value to 5.5 nM but had practically no impact on the amount of bound protein (Figure 3B and Table 1). This is in agreement with a previous finding that the interaction of the γ207−235 peptide with Lp(a) or apo(a) was only partially Lys-dependent (*10*). The immobilized COOH-terminal γ287−411 fragment also bound apo(a) with a comparatively high affinity (K_d = 60.6 nM); however, this binding was completely abolished by ϵ-ACA. Since this fragment does not contain COOH-terminal Lys, the binding most probably occurred through internal Lys residue(s). In agreement, α-*N*-acetyl-L-lysine methyl ester, which may better mimic internal Lys, also completely inhibited the interaction with apo(a) A10 (not shown). These experiments confirm the presence of the apo(a)-binding site in the central domain of the γ module and reveal that it is of high affinity and practically Lys-independent. They also identify a novel Lys-dependent apo(a)-binding site within the γ287−411 portion of this module.

Interaction of Fibrin(ogen) Fragments with Immobilized Apo(a) A10. In reverse ELISA experiments, when the D_1, E_1, or E_3 fragments, all at 5 μM, were added to immobilized apo(a) A10, no binding was observed (Figure 4). In the control experiment, no binding was observed with fibrinogen, in agreement with our previous observation (*11*). The lack of interaction between immobilized apo(a) A10 and these fragments may be connected with the cryptic character of the fibrin(ogen) apo(a)-binding sites. At the same time, one cannot exclude the possibility that immobilization of apo(a) A10 by adsorption to a surface may alter its conformation and inactivate its binding sites. To check for such a possibility, we tested the interaction between apo(a) A10 and the same fragments by another method, surface plasmon resonance (SPR), in which chemical coupling of a protein to a surface provides less structural perturbation. When apo(a) A10 was coupled to the surface of a sensor chip and D_1, E_1, and E_3 were added, none of them exhibited binding while anti-apo(a) mAb A10.2, used as a control, bound to apo(a) A10 well (Figure 5). These results suggest that apo(a)-binding sites are cryptic in D_1. In agreement, visual inspection of the γ207−235 and β247−310 residues in the three-dimensional structure of the D fragment revealed that most of them are buried (Figure 1B,C).

Interaction of the Recombinant γ Module with Immobilized Apo(a) A10. In contrast to the D_1 fragment, the recombinant γ module, representing a portion of this fragment (Figure

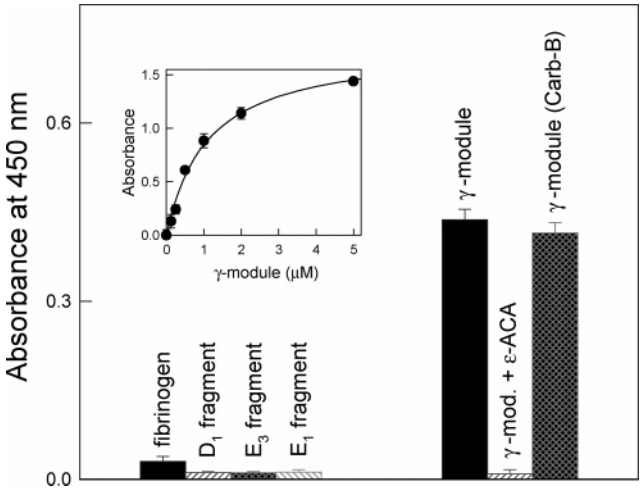

FIGURE 4: Analysis of binding of the fibrin(ogen)-derived fragments and the recombinant γ module to immobilized apo(a) A10. Fibrinogen, fibrin(ogen)-derived D_1, E_1, and E_3 fragments, or the recombinant γ module, each at 5 μM, was incubated with microtiter wells coated with apo(a) A10. Binding of the γ module was tested in the absence and presence of 100 mM ϵ-ACA as well as after its treatment with carboxypeptidase B (Carb-B). Bound species were detected with monoclonal and/or polyclonal antibodies against fibrinogen fragments as described in Experimental Procedures. The inset shows results of the experiment in which increasing concentrations of the γ module were added to immobilized apo(a) A10, and the bound protein was detected with specific polyclonal antibodies against γ module. The curve represents the best fit of the data to eq 1. Results are means $\pm$ the standard deviation of three independent determinations.

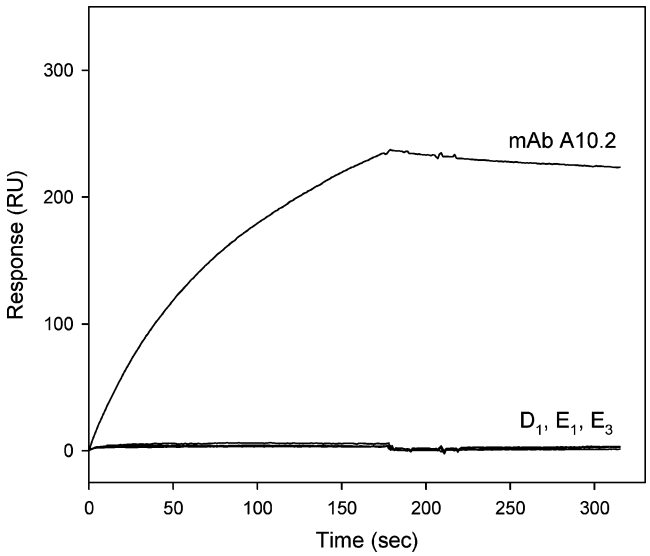

FIGURE 5: Analysis of binding of the fibrin(ogen)-derived fragments by surface plasmon resonance. The D_1, E_1, and E_3 fragments, all at 10 μM, as well as anti-apo(a) A10 monoclonal antibody A10.2 were added to immobilized apo(a) A10, and their association and dissociation were monitored in real time while registering the resonance signal (response).

1), exhibited binding when added to immobilized apo(a) A10 (Figure 4). The binding was Lys-dependent since it was abolished by the addition of 100 mM ϵ-ACA. The fact that treatment of this module with carboxypeptidase B had no effect on the binding excluded the possibility of the involvement of COOH-terminal Lys residues. The binding was dose-dependent (Figure 4, inset); the apparent K_d was found to be 1.2 μM. This value is much higher than that for the interaction between apo(a) A10 and the immobilized γ module (Table 1), suggesting that the apo(a)-binding sites in the soluble nonimmobilized γ module are only partially exposed.

Although the γ module in the D fragment[2] and fibrinogen has the same overall fold as the isolated γ module (*27*, *28*, *30*, *31*), the presence of the neighboring β module in the parent molecules (Figure 1A,B) may influence the exposure of its apo(a)-binding sites. To check if this is the case, we compared the accessibilities of the apo(a)-binding portions, γ207−235 and γ287−411, in the D fragment with those in the isolated γ module. Calculation of the solvent accessible surface areas revealed that the degree of exposure of γ287−411 in both the D fragment and the γ module is similar while γ207−235 is more exposed in the latter (602 Å^2 in D_1 vs 1190 Å^2 in the γ module). Further, the accessibilities of Gln^{210}, Glu^{213}, His^{217}, Leu^{218}, Thr^{224}, and Lys^{232} side chains in the γ module were found to be more than 2-fold higher than those in the D fragment (not shown). The exposure of these residues could account for the observed apo(a) binding activity of the isolated γ module.

Competition between Apo(a) and Plasminogen for the Interaction with Fibrin. The results presented above, as well as those reported previously (*14*), suggest that plasminogen does not compete with apo(a) for binding to the isolated D_1 fragment and αC domain. At the same time, apo(a) exhibited some competition for binding to D_1 (Figure 2, inset) and also effectively competed for COOH-terminal Lys residues generated by treatment of fibrin with plasmin (*32*). To further test competition between plasminogen and apo(a) for binding to fibrin, the following experiments have been performed.

First, we generated intact and plasmin-treated fibrin surfaces (see Experimental Procedures) and characterized their apo(a) and plasminogen binding properties. In an ELISA, apo(a) A10 bound to immobilized intact fibrin with a high affinity (Figure 6A and Table 2); the apparent K_d value was found to be 13.2 nM, close to that reported previously (*11*, *32*). Plasmin-treated fibrin exhibited an increased level of apo(a) A10 binding; the affinity of the binding also increased [K_d = 2.3 nM (Table 2)]. This extra binding occurred due to the plasmin-generated COOH-terminal Lys residues because after incubation of this fibrin with carboxypeptidase B its apo(a) binding became similar to that of intact fibrin (dashed line in Figure 6A). In contrast, binding of plasminogen to intact fibrin occurred with a much lower affinity (K_d = 860 nM), and the treatment of fibrin with plasmin increased the level of this binding more significantly, while not affecting the affinity (Figure 7A and Table 2).

Next, we tested the ability of apo(a) and plasminogen to compete for binding to intact and plasmin-treated fibrin. In an ELISA, when increasing concentrations of plasminogen (up to 2 μM) were added to 0.1 μM apo(a) A10 (saturation concentration), there was virtually no inhibition of interactions between the latter and the immobilized fibrins, both intact and plasmin-treated (Figure 6B). This result indicates that plasminogen at near-physiological concentrations does not compete for apo(a)-binding sites of fibrin. This is not surprising since its affinity for both fibrins is much lower than that of apo(a) (Table 2). In contrast, apo(a) A10, added at concentrations up to 2 μM, decreased the level of binding

[2] This D fragment is equivalent to the D_1 fragment used in this study.

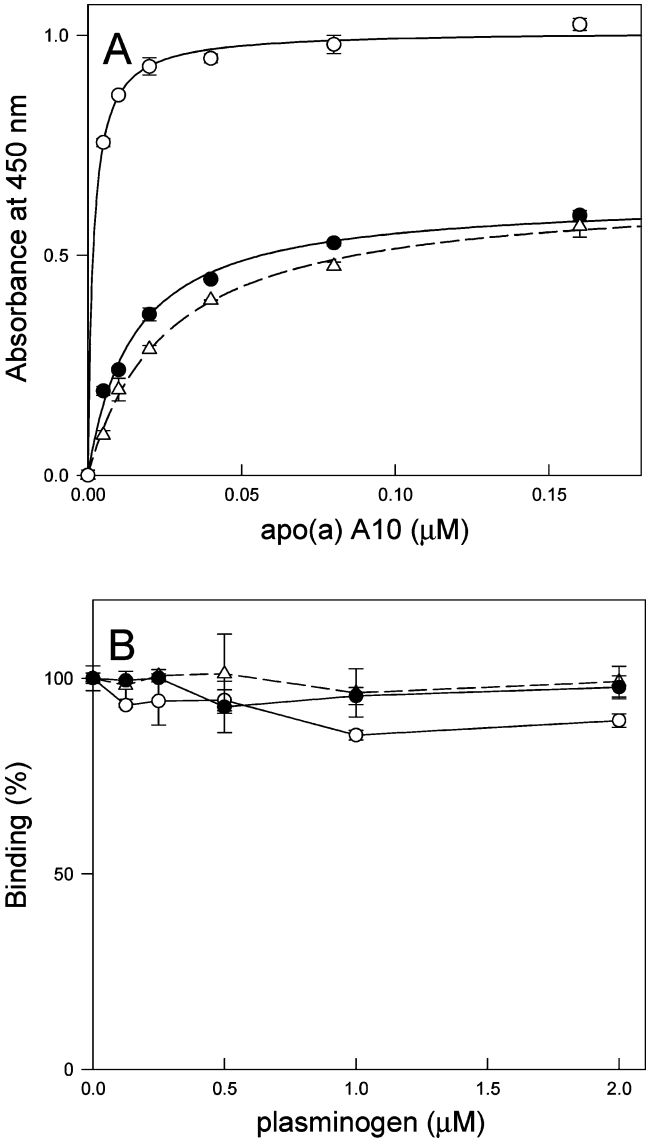

FIGURE 6: Analysis of binding of apo(a) A10 to fibrin species in the absence or presence of plasminogen. (A) Increasing concentrations of apo(a) A10 were added to the following immobilized species: intact fibrin (●), fibrin treated with plasmin for 30 min (○), or fibrin treated with plasmin for 30 min followed by treatment with carboxypeptidase B (△). Bound apo(a) A10 was detected with specific anti-apo(a) polyclonal antibodies. The curves represent the best fit of the data to eq 1. (B) Apo(a) at 0.1 μM in the presence of increasing concentrations of plasminogen was incubated with microtiter wells coated with fibrin (●), fibrin treated with plasmin for 30 min (○), and fibrin treated with plasmin for 30 min followed by incubation with carboxypeptidase B (△). Bound apo(a) A10 was detected with specific anti-apo(a) polyclonal antibodies. Results of all experiments are means ± the standard deviation of three independent determinations.

of 0.1 μM plasminogen to intact and plasmin-treated fibrins by 16 and 70%, respectively (Figure 7B). The 16% decrease was most probably due to the competition of apo(a) A10 for plasminogen-binding sites of the D regions (Figure 1A), while the more dramatic 70% decrease also reflects its competition for the newly generated COOH-terminal Lys residues. It should be noted that since the concentration of plasminogen used in these experiments was below the physiological concentration, we also tested the effect of apo(a) on the binding of 1 μM plasminogen (near-physiological concentration) to plasmin-treated fibrin. The concentration of apo(a) which inhibited this binding by 50% ($IC_{50\%}$) was found to be 137 nM (not shown), very similar to the $IC_{50\%}$ of 123 nM determined from data presented in Figure 7B. This indicates that apo(a) efficiently competes for the plasminogen-binding sites and COOH-terminal Lys residues at physiological concentrations of plasminogen.

Table 2: ELISA-Detected Dissociation Constants (K_d) for the Interaction of Apo(a) A10 and Plasminogen with Fibrin, Plasmin-Treated Fibrin, and Plasmin-Treated Fibrin after Incubation with Carboxypeptidase B[a]

	K_d (nM)	
	apo(a) A10	plasminogen
intact fibrin	13.2 ± 2.5	860 ± 77
plasmin-treated fibrin	2.3 ± 0.6	920 ± 64
plasmin-treated fibrin incubated with carboxypeptidase B	21.3 ± 6.2	1100 ± 278

[a] Values are means ± the standard deviation of at least three independent experiments.

DISCUSSION

In a previous study (*10*), use of the yeast two-hybrid system allowed localization of the apo(a)-binding sites within the Bβ247−310 and γ189−246 portions of the fibrinogen β and γ modules, respectively (Figure 1). Further localization of one of the sites within γ207−235 has been performed using synthetic peptides (*10*). In this study, we performed direct measurements of the interaction of recombinant apo(a) A10 with fibrin(ogen) and its various fragments. The experiments with the recombinant fibrinogen γ module and its subfragments identified a novel high-affinity Lys-dependent apo(a)-binding site within its COOH-terminal γ287−411 portion and revealed that the previously localized γ207−235 apo(a)-binding site is of high affinity and mainly Lys-independent. Although we did not test the apo(a) binding properties of the β module, whose sequence and overall fold are homologous to those of the γ module (*27*), on the basis of previous data (*10*) one can expect its central domain also to contain an apo(a)-binding site. Whether its COOH-terminal portion contains another binding site similar to that in the γ module remains to be clarified.

Interaction of Lp(a) with fibrin(ogen) through its apo(a) component contributes to the atherogenic processes (*8*). Competition between apo(a) and plasminogen for Lys-dependent binding sites of fibrin associated with atherosclerotic lesions results in inhibition of fibrinolysis and is regarded as the major established mechanism by which apo(a) and fibrin(ogen) promote atherogenesis (*12*). At the same time, as demonstrated in this and some previous studies (*12*), such competition occurs mostly for plasmin-generated COOH-terminal Lys residues, suggesting that this COOH-Lys-dependent mechanism may play a substantial role only when fibrin is partially degraded with plasmin and that in the absence of fibrinolytic activity apo(a) should interact with intact fibrin through alternative mechanisms. The existence of an alternative Lys-independent mechanism was proposed after the discovery of a high-affinity Lys-independent apo(a)-binding site in the fibrinogen αC domain (*11*). In agreement, the recent in vivo study with apo(a) transgenic mice in a plasminogen deficient background provided strong evidence for a prothrombotic role of apo(a) that is indepen-

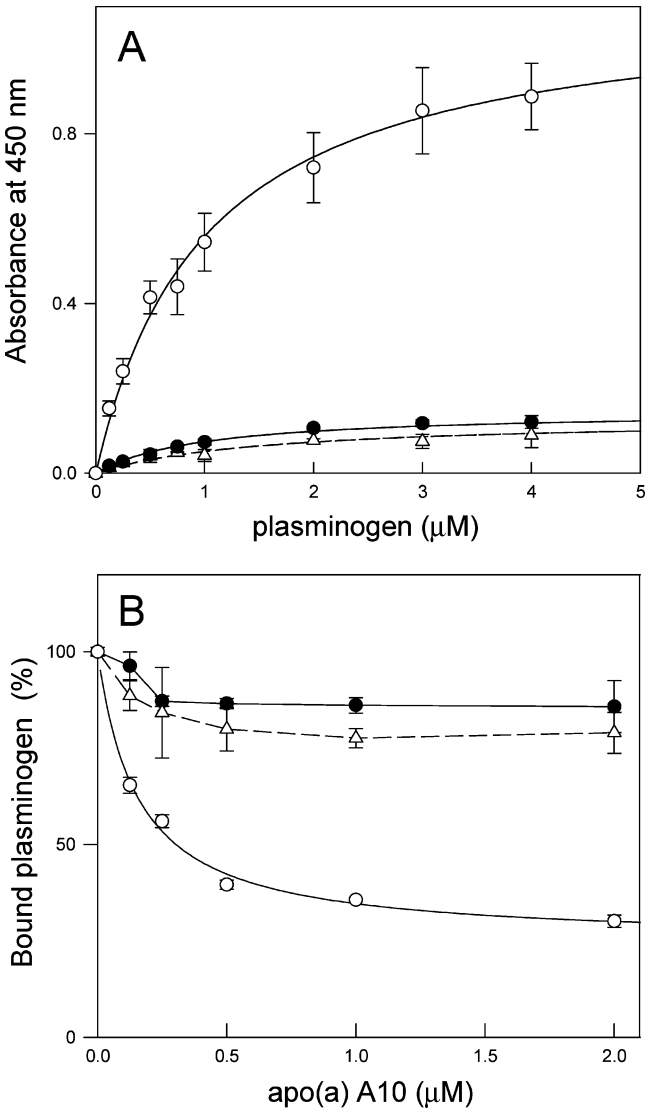

FIGURE 7: Analysis of binding of plasminogen to fibrin species in the absence or presence of apo(a) A10. (A) Increasing concentrations of plasminogen were added to the following immobilized species: intact fibrin (●), fibrin treated with plasmin for 30 min (○), and fibrin treated with plasmin for 30 min followed by incubation with carboxypeptidase B (△). Bound plasminogen was detected with specific anti-plasminogen polyclonal antibodies. The curves represent the best fit of the data to eq 1. (B) Plasminogen at 0.1 μM in the presence of increasing concentrations of apo(a) A10 was incubated with microtiter wells coated with fibrin (●), fibrin treated with plasmin for 30 min (○), or fibrin treated with plasmin for 30 min followed by incubation with carboxypeptidase B (△). Bound plasminogen was detected with specific anti-plasminogen polyclonal antibodies. Results of all experiments are means ± the standard deviation of three independent determinations.

dent of plasmin (*33*). In this study, we found that the apo(a)-binding site located within the γ207−235 portion of the central domain of the γ module is mainly Lys-independent and therefore should also contribute to the Lys-independent apo(a) binding mechanism. Furthermore, since the affinity of this site for apo(a) is very high, its contribution to the Lys-independent mechanism could be comparable with that of the high-affinity site of the αC domain.

Our previous study (*11*) revealed that Lys-independent interaction accounts for almost half of the overall binding of apo(a) to intact fibrin, while the other half is Lys-dependent. Since intact fibrin has no COOH-terminal Lys residues, the Lys-dependent interaction occurs most probably through the novel γ287−411 Lys-dependent apo(a)-binding site. This site is of high affinity and therefore may contribute substantially to the Lys-dependent interaction between apo(a) and intact fibrin. Such interaction may represent another alternative mechanism, a Lys-dependent one, which involves internal Lys residues (internal Lys-dependent mechanism). In addition, since competition experiments with the D_1 fragment revealed that apo(a) competes for the Aα148−160 plasminogen-binding site (Figure 2, inset), binding of apo(a) through this site may also contribute to the internal Lys-dependent mechanism. This mechanism, in contrast to the COOH-Lys-dependent one, does not require COOH-terminal Lys residues, i.e., it is plasmin-independent, and therefore may mediate binding of apo(a) to intact fibrin in the absence of fibrinolytic activity.

It should be noted that in our experiments the fibrinogen-derived D_1 fragment exhibited its apo(a) binding activity only when immobilized onto plastic; in solution, it was inactive. The absence of this activity in solution is in agreement with our findings that in the crystal structure of the D fragment most of the γ207−235 residues are totally or partially buried (this study) and that apo(a)- and plasminogen-binding sites in intact fibrinogen are cryptic (*11*, *13*, *14*). At the same time, the appearance of the apo(a) binding activity upon immobilization of D_1 is in agreement with the previous studies indicating that surface-adsorbed fibrinogen or its fragments have altered conformation and that their cryptic binding sites and fibrin specific epitopes are exposed (*19*, *34*−*38*). Further, the previous reports that the fibrinogen D regions undergo conformational changes upon conversion of fibrinogen into fibrin and that such changes result in exposure of some cryptic sites (*22*, *39*, *40*) strongly suggest that the apo(a)-binding sites are also exposed in fibrin deposited on the vessel walls or in atherosclerotic lesions. In such a case, the interaction of apo(a) with fibrin through these sites may play an important role in attraction of Lp(a) to the lesions in the absence of fibrinolytic activity required for generation of COOH-terminal Lys residues.

In summary, the results of this and the previous studies (*10*−*12*) suggest that apo(a) may interact with fibrin(ogen) through at least three alternative mechanisms which utilize different types of apo(a)-binding sites. They include (i) plasmin-generated COOH-terminal Lys residues (COOH-Lys-dependent sites), (ii) Lys-dependent apo(a)- and plasminogen-binding sites localized in the γ287−411 and Aα148−160 portions of the D region, respectively (internal Lys-dependent sites), and (iii) Lys-independent apo(a)-binding sites localized in the αC domain and in the γ207−235 portion of the central domain of the γ module (and most probably in the homologous portion of the β module). The Lys-independent and internal Lys-dependent mechanisms may provide an interaction of apo(a) with intact fibrin and therefore may be important at the early stage of accumulation of Lp(a) in atherosclerotic lesions, whereas the COOH-Lys-dependent one may prevail in the presence of fibrinolytic activity which generates COOH-terminal Lys residues in lesion-associated fibrin.

ACKNOWLEDGMENT

We thank Dr. Benoît Ho-Tin-Noe for assistance in the preparation and purification of monoclonal antibody A10.2.

REFERENCES

1. Danesh, J., Collins, R., and Peto, R. (2000) Lipoprotein(a) and coronary heart disease. Meta-analysis of prospective studies, *Circulation 102*, 1082−1085.
2. Bini, A., and Kudryk, B. J. (1995) Fibrinogen in human atherosclerosis, *Ann. N.Y. Acad. Sci. 748*, 461−471.
3. Smith, E. B., and Crosbie, L. (1991) Does lipoprotein(a) (Lp[a]) complete with plasminogen in human atherosclerotic lesions and thrombi? *Atherosclerosis 89*, 127−136.
4. Lawn, R. M., Wade, D. P., Hammer, R. E., Chiesa, G., Verstuyft, J. G., and Rubin, E. M. (1992) Atherogenesis in transgenic mice expressing human apolipoprotein(a), *Nature 360*, 670−672.
5. Boonmark, N. W., Lou, X. J., Yang, Z. J., Schwartz, K., Zhang, J. L., Rubin, E. M., and Lawn, R. M. (1997) Modification of apolipoprotein(a) lysine binding site reduces atherosclerosis in transgenic mice, *J. Clin. Invest. 100*, 558−564.
6. Fan, J., Shimoyamada, H., Sun, H., Marcovina, S., Honda, K., and Watanabe, T. (2001) Transgenic rabbits expressing human apolipoprotein(a) develop more extensive atherosclerotic lesions in response to a cholesterol-rich diet, *Arterioscler. Thromb. Vasc. Biol. 21*, 88−94.
7. Sun, H., Unoki, H., Wang, X., Liang, J., Ichikawa, T., Arai, Y., Shiomi, M., Marcovina, S. M., Watanabe, T., and Fan, J. (2002) Lipoprotein(a) enhances advanced atherosclerosis in WHHL transgenic rabbits expressing human apolipoprotein(a), *J. Biol. Chem. 277*, 47486−47492.
8. Lou, X. J., Boonmark, N. W., Horrigan, F. T., Degen, J. L., and Lawn, R. M. (1998) Fibrinogen deficiency reduces vascular accumulation of apolipoprotein(a) and development of atherosclerosis in apolipoprotein(a) transgenic mice, *Proc. Natl. Acad. Sci. U.S.A. 95*, 12591−12595.
9. Harpel, P. C., Gordon, B. R., and Parker, T. S. (1989) Plasmin catalyzes binding of lipoprotein(a) to immobilized fibrinogen and fibrin, *Proc. Natl. Acad. Sci. U.S.A. 86*, 3847−3851.
10. Klose, R., Fresser, F., Kochl, S., Parson, W., Kapetanopoulos, A., Fruchart-Najib, J., Baier, G., and Utermann, G. (2000) Mapping of a minimal apolipoprotein(a) interaction motif conserved in fibrin(ogen) β- and γ-chains, *J. Biol. Chem. 275*, 38206−38212.
11. Tsurupa, G., Ho-Tin-Noe, B., Angles-Cano, E., and Medved, L. (2003) Identification and characterization of novel lysine-independent apolipoprotein(a)-binding sites in fibrin(ogen) αC-domains, *J. Biol. Chem. 278*, 37154−37159.
12. Angles-Cano, E., de la Pena Diaz, A., and Loyau, S. (2001) Inhibition of fibrinolysis by lipoprotein(a), *Ann. N.Y. Acad. Sci. 936*, 261−275.
13. Medved, L., and Nieuwenhuizen, W. (2003) Molecular mechanisms of initiation of fibrinolysis by fibrin, *Thromb. Haemostasis 89*, 409−419.
14. Tsurupa, G., and Medved, L. (2001) Identification and characterization of novel tPA- and plasminogen-binding sites within fibrin(ogen) αC-domains, *Biochemistry 40*, 801−808.
15. Deutsch, D. G., and Mertz, E. T. (1970) Plasminogen: Purification from human plasma by affinity chromatography, *Science 170*, 1095−1096.
16. Kang, C., Dominguez, M., Loyau, S., Miyata, T., Durlach, V., and Angles-Cano, E. (2002) Lp(a) particles mold fibrin-binding properties of apo(a) in size-dependent manner: A study with different-length recombinant apo(a), native Lp(a), and monoclonal antibody, *Arterioscler. Thromb. Vasc. Biol. 22*, 1232−1238.
17. Dominguez, M., Rojas, G., Loyau, S., Bazurco, M., Sorell, L., and Angles-Cano, E. (2001) Kringles of the plasminogen-prothrombin gene family share conformational epitopes with recombinant apolipoprotein(a): Specificity of the fibrin-binding site, *Biochim. Biophys. Acta 1548*, 72−80.
18. Medved, L., Litvinovich, S., Ugarova, T., Matsuka, Y., and Ingham, K. (1997) Domain structure and functional activity of the recombinant human fibrinogen γ-module (γ148−411), *Biochemistry 36*, 4685−4693.
19. Yakovlev, S., Zhang, L., Ugarova, T., and Medved, L. (2005) Interaction of fibrin(ogen) with leukocyte receptor $\alpha_M \beta_2$ (Mac-1): Further characterization and identification of a novel binding region within the central domain of the fibrinogen γ-module, *Biochemistry 44*, 617−626.
20. Angles-Cano, E., Loyau, S., Cardoso-Saldana, G., Couderc, R., and Gillery, P. (1999) A novel kringle-4 number-based recombinant apo[a] standard for human apo[a] phenotyping, *J. Lipid Res. 40*, 354−359.
21. Yakovlev, S., Gorlatov, S., Ingham, K., and Medved, L. (2003) Interaction of fibrin(ogen) with heparin: Further characterization and localization of the heparin-binding site, *Biochemistry 42*, 7709−7716.
22. Yakovlev, S., Makogonenko, E., Kurochkina, N., Nieuwenhuizen, W., Ingham, K., and Medved, L. (2000) Conversion of fibrinogen to fibrin: Mechanism of exposure of tPA- and plasminogen-binding sites, *Biochemistry 39*, 15730−15741.
23. Olexa, S. A., Budzynski, A. Z., Doolittle, R. F., Cottrell, B. A., and Greene, T. C. (1981) Structure of fragment E species from human cross-linked fibrin, *Biochemistry 20*, 6139−6145.
24. Henschen, A., and McDonagh, J. (1986) Fibrinogen, fibrin and factor XIII, in *Blood Coagulation* (Zwaal, R. F. A., and Hemker, H. C., Eds.) pp 171−241, Elsevier Science Publishers, Amsterdam.
25. Fleury, V., and Angles-Cano, E. (1991) Characterization of the binding of plasminogen to fibrin surfaces: The role of carboxy-terminal lysines, *Biochemistry 30*, 7630−7638.
26. Berman, H. M., Westbrook, J., Feng, Z., Gilliland, G., Bhat, T. N., Weissig, H., Shindyalov, I. N., and Bourne, P. E. (2000) The Protein Data Bank, *Nucleic Acids Res. 28*, 235−242.
27. Spraggon, G., Everse, S. J., and Doolittle, R. F. (1997) Crystal structures of fragment D from human fibrinogen and its crosslinked counterpart from fibrin, *Nature 389*, 455−462.
28. Yee, V. C., Pratt, K. P., Cote, H. C., Trong, I. L., Chung, D. W., Davie, E. W., Stenkamp, R. E., and Teller, D. C. (1997) Crystal structure of a 30 kDa C-terminal fragment from the γ chain of human fibrinogen, *Structure 5*, 125−138.
29. Hubbard, S. J., and Thornton, J. M. (1993) *NACCESS*, Department of Biochemistry and Molecular Biology, University College, London.
30. Brown, J. H., Volkmann, N., Jun, G., Henschen-Edman, A. H., and Cohen, C. (2000) The crystal structure of modified bovine fibrinogen, *Proc. Natl. Acad. Sci. U.S.A. 97*, 85−90.
31. Yang, Z., Kollman, J. M., Pandi, L., and Doolittle, R. F. (2001) Crystal structure of native chicken fibrinogen at 2.7 Å resolution, *Biochemistry 40*, 12515−12523.
32. Rouy, D., Koschinsky, M. L., Fleury, V., Chapman, J., and Angles-Cano, E. (1992) Apolipoprotein(a) and plasminogen interactions with fibrin: A study with recombinant apolipoprotein(a) and isolated plasminogen fragments, *Biochemistry 31*, 6333−6339.
33. Sha, J., McCullough, B., Hart, E., Nassir, F., Davidson, N. O., and Hoover-Plow, J. (2005) Apo(a) promotes thrombosis in a vascular injury model by a mechanism independent of plasminogen, *J. Thromb. Haemostasis 3*, 2281−2289.
34. Procyk, R., Kudryk, B., Callender, S., and Blomback, B. (1991) Accessibility of epitopes on fibrin clots and fibrinogen gels, *Blood 77*, 1469−1475.
35. Ugarova, T. P., Budzynski, A. Z., Shattil, S. J., Ruggeri, Z. M., Ginsberg, M. H., and Plow, E. F. (1993) Conformational changes in fibrinogen elicited by its interaction with platelet membrane glycoprotein GPIIb-IIIa, *J. Biol. Chem. 268*, 21080−21087.
36. Moskowitz, K. A., Kudryk, B., and Coller, B. S. (1998) Fibrinogen coating density affects the conformation of immobilized fibrinogen: Implications for platelet adhesion and spreading, *Thromb. Haemostasis 79*, 824−831.
37. Hu, W. J., Eaton, J. W., Ugarova, T., and Tang, L. (2001) Molecular basis of biomaterial-mediated foreign body reactions, *Blood 98*, 1231−1238.
38. Lishko, V. K., Kudryk, B., Yakubenko, V. P., Yee, V. C., and Ugarova, T. P. (2002) Regulated unmasking of the cryptic binding site for integrin $\alpha_M \beta_2$ in the γC-domain of fibrinogen, *Biochemistry 41*, 12942−12951.
39. Yang, Z., Mochalkin, I., and Doolittle, R. F. (2000) A model of fibrin formation based on crystal structures of fibrinogen and fibrin fragments complexed with synthetic peptides, *Proc. Natl. Acad. Sci. U.S.A. 97*, 14156−14161.
40. Medved, L., Tsurupa, G., and Yakovlev, S. (2001) Conformational changes upon conversion of fibrinogen into fibrin. The mechanisms of exposure of cryptic sites, *Ann. N.Y. Acad. Sci. 936*, 185−204.
41. Weisel, J. W., and Medved, L. (2001) The structure and function of the αC domains of fibrinogen, *Ann. N.Y. Acad. Sci. 936*, 312−327.
42. DeLano, W. L. (2002) *PyMol*, DeLano Scientific, San Carlos, CA.

BI060526H