

# Elements in the C terminus of apolipoprotein [a] responsible for the binding to the tenth type III module of human fibronectin

Celina Edelstein,\* Mohammed Yousef,<sup>†</sup> and Angelo M. Scanu<sup>1,\*</sup>

Department of Medicine\* and Department of Biochemistry and Molecular Biology,<sup>†</sup> University of Chicago, Chicago, IL 60637

**Abstract** In previous studies, we showed that the C-terminal domain, F2, but not the N-terminal domain, F1, is responsible for the binding of apolipoprotein [a] (apo[a]) to human fibronectin (Fn). To pursue those observations, we prepared, by both elastase digestion and recombinant technology, subsets of F2 of a different length containing either kringle (K) V or the protease domain (PD). We also studied rhesus monkey apo[a], which is known to contain PD but not KV. In the case of Fn, we used both an intact product and its tenth type III module (<sup>10</sup>FN-III) expressed in *Escherichia coli*. The binding studies carried out on microtiter plates showed that the affinity of F2 for immobilized <sup>10</sup>FN-III was ~6-fold higher than that for Fn (dissociation constants =  $1.75 \pm 0.31$  nM and  $10.25 \pm 1.62$  nM, respectively). The binding was also exhibited by rhesus apo[a] and by an F2 subset containing the PD linked to an upstream microdomain comprising KIV-8 to KIV-10 and KV, inactive by itself. Competition experiments on microtiter plates showed that both Fn and <sup>10</sup>FN-III, when in solution, are incompetent to bind F2. Together, our results indicate that F2 binds to immobilized <sup>10</sup>FN-III more efficiently than whole Fn and that the binding can be sustained by truncated forms of F2 that contain the catalytically inactive PD linked to an upstream four K microdomain.—Edelstein, C., M. Yousef, and A. M. Scanu. Elements in the C terminus of apolipoprotein [a] responsible for the binding to the tenth type III module of human fibronectin. *J. Lipid Res.* 2005. 46: 2673–2680.

**Supplementary key words** lipoprotein [a] • kringle V • protease domain • RGD motif

Fibronectin (Fn) is a large (450 kDa) multidomain protein that interacts with a variety of macromolecules, including components of the cytoskeleton and the extracellular matrix and cell surface receptors particularly of fibroblasts, neurons, macrophages, and bacteria (1). Fn occurs

in two main forms: one is insoluble, exhibiting adhesive properties, and is synthesized by fibroblasts, chondrocytes, endothelial cells, epithelial cells, and macrophages; the other is soluble, a heterodimer present in the plasma, and is synthesized by hepatocytes. In both forms there are three internally homologous repeats, called modules (types I, II, and III), that are readily separable by the action of proteolytic enzymes and exhibiting various binding motifs (2). In human soluble Fn, there are 12 type I, 2 type II, and 15 type III modules, each module representing independently folded units containing mostly  $\beta$  sheets and turns (Fig. 1). The first two modules contain four conserved cysteines comprising two disulfide bonds that are critical for module stability and function. In turn, type III modules are devoid of disulfide bonds because the two unpaired cysteines are buried (3, 4). Although Fn is present in early atherosclerotic lesions and in atherosclerotic plaques, its overall role in the atherosclerotic process is unclear (5).

Salonen et al. (6) were the first to show that lipoprotein [a] (Lp[a]) binds, via apolipoprotein [a] (apo[a]), to immobilized Fn using preparations isolated from human plasma. Moreover, by studying thermolysin digests of Fn, they observed that the strongest binding involved the C-terminal heparin binding 29 kDa fragment. Binding of apo[a] to immobilized Fn was also reported by van der Hoek et al. (7), who used Fn isolated from human plasma and a recombinant apo[a]. The binding on Fn was located to a 12 amino acid sequence in the N-terminal region of the overlapping 29–38 kDa thermolysin fragments. These two previous studies did not rule out the possibility that in its immobilized form, Fn has additional binding sites for apo[a]. Moreover, they did not provide information on the

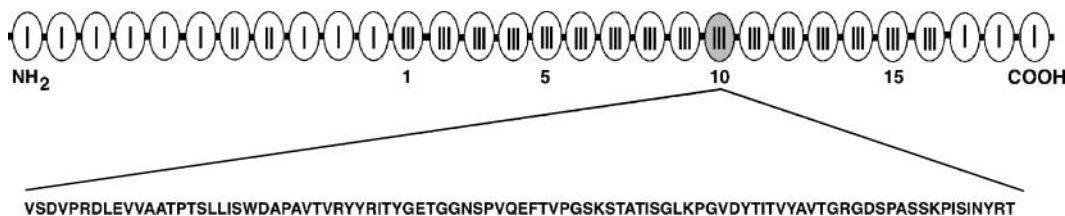
Abbreviations: apo[a], apolipoprotein [a]; EACA,  $\epsilon$ -aminocaproic acid; Fn, fibronectin; <sup>10</sup>FN-III, tenth type III module of fibronectin; K, kringle;  $K_d$ , dissociation constant; Lp[a], lipoprotein [a]; PD, protease domain of apolipoprotein [a].

<sup>1</sup> To whom correspondence should be addressed.  
e-mail: ascanu@medicine.bsd.uchicago.edu

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**Fig. 1.** Domain structure of human plasma fibronectin (Fn). The complete Fn chain represents domains that have internal homology (types I, II, and III) with nonhomologous connecting strands (solid horizontal lines). The numbers beneath the type III domains indicate unit numbers. Unit 10 of domain III is shaded and its sequence is shown.

site(s) on apo[a] involved in the binding. In this context, we previously showed that the C-terminal domain of apo[a], referred to as F2, is critical for the binding of apo[a] to Fn (8). This domain comprises kringle (K) IV types 5–10, one copy of KV, and the protease domain (PD) (9, 10) (**Fig. 2**). This PD has a high degree of homology with that of plasminogen; however, unlike the latter, it is inactive as a result of the presence of serine instead of arginine at the site of activation by tissue plasminogen activator (11) and possibly because of the influence of the deletion of a napeptide (present in plasminogen) between residues 4,483 and 4,491 of the apo[a] sequence (12). The significance of this inactive protease in apo[a] has not been determined, and questions have been raised regarding possible enzyme-unrelated activities.

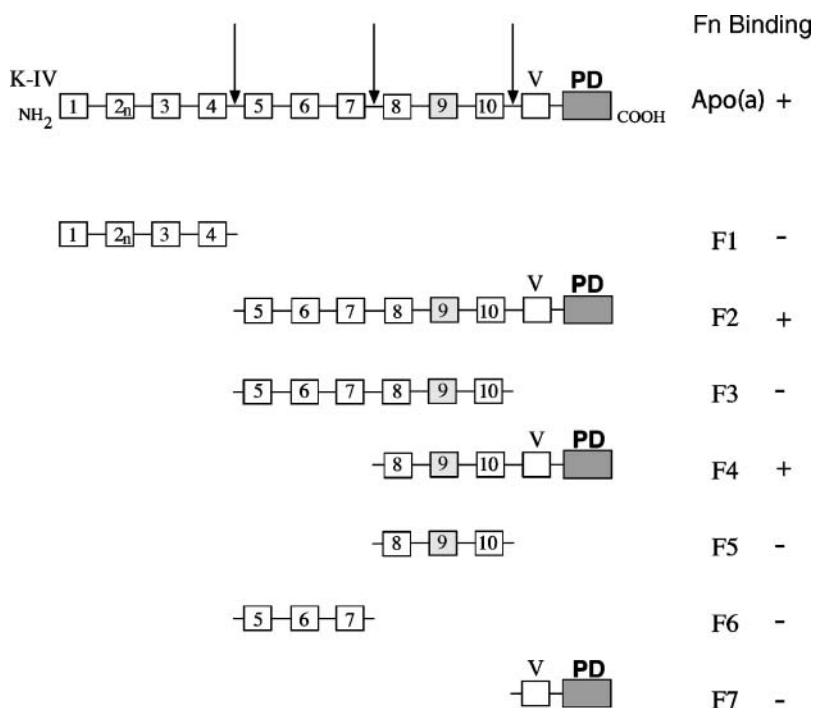
The current studies were carried out to define the elements in F2 responsible for the binding of apo[a] to Fn. For this purpose, we used a series of truncated products obtained by either proteolytic digestion of naturally occur-

ring human apo[a] or recombinant technology, an approach that we have found useful in investigating the complex structure-function relationships in Lp[a] (13). In our binding systems, besides intact Fn, we also used the tenth type III module (<sup>10</sup>FN-III) that in preliminary studies in human carotid artery plaques was found to be associated with apo[a] (A. M. Scanu et al., unpublished observation). Here, we provide evidence that this module, known to participate in a number of cell adhesion events, is involved in the binding of Fn to human apo[a] and that in the latter case, the PD is a necessary but not sufficient element in the binding.

## METHODS

### Materials

Fn was purchased from Enzyme Research Laboratories (South Bend, IN). Human leukocyte elastase (EC 3.4.21.37), BSA, Tween



**Fig. 2.** Schemes of apolipoprotein [a] (apo[a]) fragments. Apo[a] is represented by kringle (K) IV repeats numbered 1–10, one KV, and a protease domain (PD) indicated by gray squares. KIV-2 is indicated as 2<sub>n</sub> to reflect the presence of several identical copies of this K. The cleavage sites by leukocyte elastase are indicated by arrows.

20, 2-mercaptoethanol, SDS, diisopropylfluorophosphate,  $\epsilon$ -aminocaproic acid (EACA), goat anti-rabbit and anti-mouse IgG alkaline phosphatase conjugates, rabbit affinity-purified anti-human Fn, and *p*-nitrophenyl phosphate were all purchased from Sigma (St. Louis, MO). Kallikrein inactivator was purchased from Calbiochem (San Diego, CA). Rabbit affinity-purified antibodies to apo[a] were prepared as described previously (14). Anti-apo[a] did not react against either LDL or plasminogen. A monoclonal antibody against a recombinant human apo[a] KV was a gift from Abbott Laboratories (Abbott Park, IL), and its specificity was verified in our laboratory. All chemicals were of reagent grade.

### Human donors and Lp[a] and apo[a] preparations

The subjects used for the preparation of Lp[a] were healthy donors with plasma Lp[a] protein levels in the range of 15–43 mg/dl with a known apo[a] phenotype. Their plasma was obtained by plasmapheresis performed in the blood bank at the University of Chicago. All of the subjects in the study gave written informed consent according to protocols approved by the Institutional Review Board of the University of Chicago. The steps for Lp[a] isolation were carried out immediately after blood drawing using a combination of ultracentrifugation and lysine-Sepharose affinity chromatography as described previously (15). For the current study, we used Lp[a] having 15 type IV Ks. In those subjects having two apo[a] isoforms, the Lp[a] species containing the desired isoform was separated from the other by CsCl density gradient ultracentrifugation as described (15). Apo[a] was isolated from Lp[a] essentially as described by Edelstein et al. (16) in the presence of 1.25 mM dithioerythritol.

### Apo[a] phenotyping

Apo[a] phenotyping was performed on plasma samples by SDS-PAGE followed by immunoblotting using a monospecific anti-human apo[a] antibody. The mobility of the individual apo[a] bands was compared with that of isolated apo[a] isoforms of known molecular weights, and the number of Ks was compared with those provided by Dr. Angles-Cano (Institut National de la Santé et de la Recherche Médicale Unit 143, Paris, France).

### Isolation of rhesus Lp[a] and apo[a]

Lp[a] was isolated from rhesus plasma as described previously (17). Apo[a] was separated from Lp[a] by the same techniques used for the human Lp[a] and had an apparent mass of 325 kDa. We have shown previously that both products are recognized by the human antibodies (17).

### Isolation and purification of human apo[a] fragments

A schematic representation of the apo[a] fragments used in our studies is shown in Fig. 2. Human leukocyte elastase (1 unit = 1 nm *p*-nitrophenol/s from N-1 T-butyloxycarbonyl-L-Ala *p*-nitrophenol ester) was diluted 1,000-fold in 50 mM Tris-HCl, 0.1 M NaCl, pH 8.0. One microliter of the diluted enzyme was incubated per 15  $\mu$ g of apo[a] at 37°C for 30 min. The reaction was terminated with 5 mM diisopropylfluorophosphate for 20 min at 22°C. The purification of the fragments was carried out as described previously, and each fragment gave a single band on SDS-PAGE (18). The fragments behaved as monomers when examined by sedimentation velocity in the analytical ultracentrifuge.

### Generation of rIII

The rIII recombinant protein lacking KIV-6, -7, -8, and PD (6K $\Delta$ KIV6-8 $\Delta$ PD) was prepared by digesting the 6 K expression plasmid with *Cla*I and *Eco*RV (19). Subsequently, the 2.4 kb *Cla*I-*Eco*RV insert was subjected to digestion with *Bam*HI to remove the *Bam*HI-*Bam*HI internal DNA domains coding for apo[a] KIV-6



**Fig. 3.** Scheme of the rIII recombinant protein. rIII contains a signal sequence (S) followed by a fusion of KIV-1 and KIV-5 (consisting of the first 30 amino acids of KIV-1 and 55 amino acids of KIV-5), KIV-9, KIV-10, and a single KV but is lacking the PD. The K numbers correspond to those indicated for apo[a] in Fig. 2.

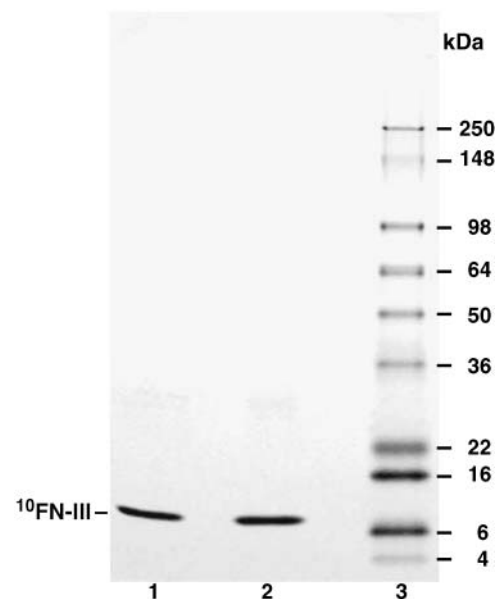
through -8. The 0.32 kb *Cla*I-*Bam*HI and the 1.03 kb *Bam*HI-*Eco*RV DNA fragments were then ligated back into the 6 K expression plasmid that was digested with *Cla*I and *Eco*RV (vector part). rIII (6K $\Delta$ KIV6-8 $\Delta$ PD) (Fig. 3) was sequenced and transfected into human embryonic kidney 293 cells. The clone produced significant amounts of recombinant product (0.5–10 mg/1 of the culture medium) that was purified by lysine-Sepharose chromatography.

### Preparation of <sup>10</sup>Fn-III

The recombinant product, <sup>10</sup>FN-III, was the tenth unit of the human Fn type III domain containing 96 amino acids (Fig. 1). The steps involved in the gene construction, phage display, expression in *Escherichia coli*, and purification of <sup>10</sup>FN-III from cell culture medium were described previously (20). By SDS-PAGE, it migrated as a single band in the expected position (Fig. 4). Sedimentation velocity experiments were conducted using the Beckman XL-A analytical ultracentrifuge. Approximately 0.4 mg/ml <sup>10</sup>FN-III in TBS was placed in a two sector centerpiece and centrifuged at 60,000 rpm for 15 h at 22°C. The data were analyzed using SEDFIT (21). A single symmetrical peak was observed with an apparent molecular weight of 10,400.

### Binding experiments on microtiter plates

All binding experiments were conducted at room temperature. Microtiter plates (Beckman Instruments, Fullerton, CA) were



**Fig. 4.** SDS-PAGE of the tenth type III module of fibronectin (<sup>10</sup>FN-III). <sup>10</sup>FN-III was run on 4–20% SDS-PAGE under reducing and nonreducing conditions, and the gel was stained with Coomassie blue. Lane 1, <sup>10</sup>FN-III, nonreduced; lane 2, <sup>10</sup>FN-III, reduced with 2-mercaptoethanol; lane 3, molecular mass standards.

coated with 100  $\mu$ l of either Fn or  $^{10}$ FN-III, each at 10  $\mu$ g/ml, in TBS buffer (50 mM Tris-HCl, pH 7.5, 0.15 M NaCl) for 24 h. Nonspecific binding sites were blocked with 1% BSA in TBS for 1.5 h. After three washes with TBST (TBS supplemented with 0.1% BSA and 0.02% Tween 20), various concentrations of either apo[a] or apo[a] fragments were added to the wells in TBS buffer and incubated for 2 h. After incubation, the wells were washed three times with TBST. The bound protein was detected using a monospecific polyclonal anti-apo[a] antibody in TBST for 1 h. In the specific case of fragment F7 (KV-PD), a monoclonal anti-KV antibody was used because this fragment was not recognized by our anti-apo[a] antibody. At this time, the wells were washed three times with TBST, and goat anti-rabbit IgG, or in the case of KV-PD, anti-mouse IgG, both conjugated to alkaline phosphatase, were added for 1 h. After washing with TBST, *p*-nitrophenyl phosphate (1 mg/ml in diethanolamine buffer, pH 9.8) was added, and the color development was followed at 405 nm on a Versamax microplate reader (Molecular Devices, Sunnyvale, CA). In another set of experiments, we coated the wells with apo[a] and after washing the excess we examined the binding of either Fn or  $^{10}$ FN-III using a polyclonal antibody specific for human Fn. Analysis of the binding data was performed on the assumption of single-site binding. Dissociation constants ( $K_d$ ) were derived using the program Origin version 7.03 (Origin Lab Co.,

Northampton, MA) fitting the data to a one-site model represented by the equation

$$[Y] = \frac{B[X]}{(K + [X])}$$

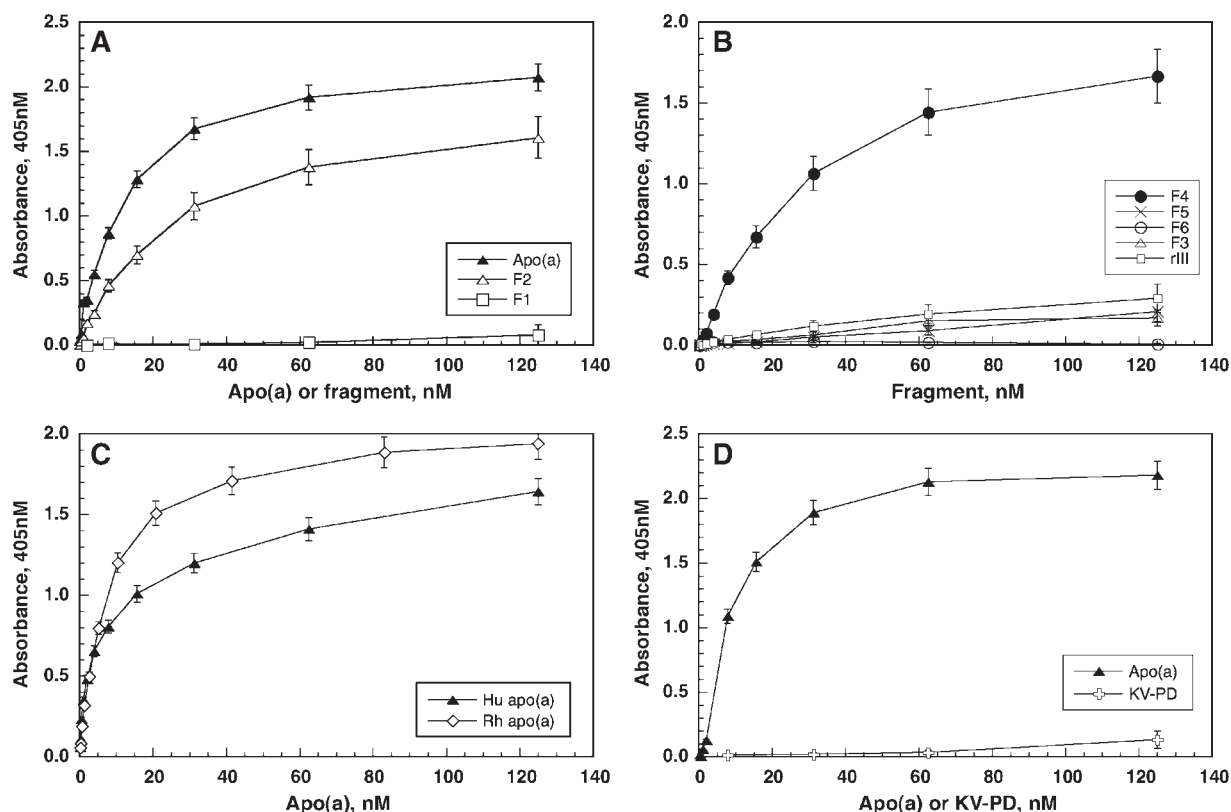
where [Y] represents the absorbance at 405 nm, which is proportional to the amount of ligand bound; [X] represents the concentration of free ligand; B represents the maximum absorbance at saturation; and K represents the association constant.

### Factors affecting binding

In some experiments, a constant amount (50 nM) of either apo[a] or fragments was incubated with immobilized Fn in the presence of various concentrations of EACA (0–200 mM) or NaCl (0–2 M). After incubation for 1 h, the bound protein was detected with anti-apo[a] antibody or anti-KV antibody, as described above, depending on the protein under study.

### Competition studies

A range of concentrations of either  $^{10}$ FN-III or Fn were mixed with 50 nM apo[a] or in some cases a range of apo[a] concentrations and incubated in wells of microtiter plates coated with Fn or  $^{10}$ FN-III. Bound apo[a] was detected using a monospecific



**Fig. 5.** Binding to immobilized Fn. A: Apo[a], F1, and F2 at the indicated concentrations (0–125 nM) were incubated with immobilized Fn for 2 h at 22°C. The data represent means of six independent experiments for apo[a] and four experiments for F1 and F2, all carried out in triplicate. B: Elastase-generated apo[a] fragments F3, F4, F5, and F6 and the recombinant protein rIII were incubated with immobilized Fn at the indicated concentrations (0–125 nM) for 2 h at 22°C. The data represent means of three independent experiments carried out in triplicate. C: Human (Hu) apo[a] and rhesus (Rh) apo[a] at the indicated concentrations (0–125 nM) were incubated with immobilized Fn for 2 h at 22°C. The data represent means of three independent experiments carried out in triplicate. D: Apo[a] and the elastase-generated fragment KV-PD at the indicated concentrations (0–125 nM) were incubated with immobilized Fn for 2 h at 22°C, and in this case, the extent of binding was detected by the addition of monoclonal antibody specific for KV. The data represent means of six independent experiments carried out in triplicate. In all experiments, the extent of binding was followed by the absorbance at 405 nm as described in Methods. The data presented are means  $\pm$  SEM.



anti-apo[a] antibody. The secondary antibody and color development reaction were as described above.

## Quantitative analyses

Lp[a] protein was quantified by a sandwich ELISA as described previously (14). The concentration of apo[a] was determined either by ELISA or using an extinction coefficient ( $\epsilon_{278} = 1.31 \text{ ml/mg/cm}$ ) established previously (22) for apo[a]. Protein concentrations of the fragments were determined by the Bio-Rad DC protein assay.

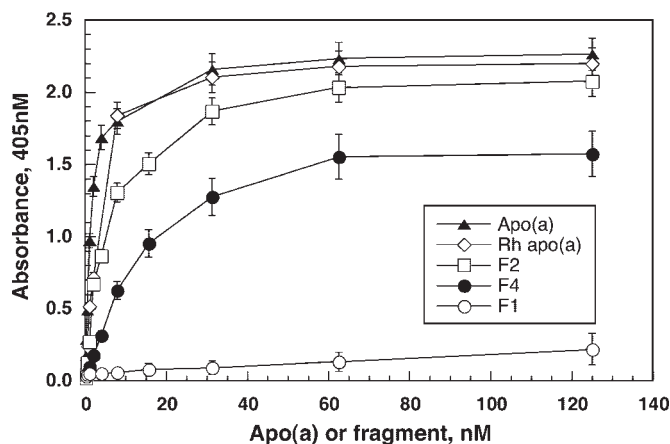
## RESULTS

### Binding studies with immobilized Fn: studies on apo[a] and its elastase-digested fragments

As shown in **Fig. 5A**, apo[a] bound in a saturable manner. The apparent  $K_d$  value was  $10.25 \pm 1.62 \text{ nM}$  (**Table 1**). To determine which portion of apo[a] was responsible for this binding, we examined F1 and F2, the N- and C-terminal fragments, respectively, generated by limited digestion of apo[a] with leukocyte elastase (**Fig. 2**). F2 bound in a saturable manner, whereas F1 exhibited no detectable binding (**Fig. 5**, **Table 1**). Concentrations of EACA up to 200 mM or NaCl up to 2 M had no effect on the binding of either apo[a] or F2 (data not shown), suggesting a hydrophobic mode of interaction. These results indicated that F2 has the necessary elements for binding to Fn and that this binding was stronger when F2 was an integral part of apo[a], suggesting an enhancing effect by the attached F1.

To define the region within F2 responsible for the binding to Fn, we studied the elastase digests of apo[a] shown in **Fig. 2**. Only F4 (KIV-8, -9, -10, KV, and PD) bound, whereas F3 (KIV-5, -6, -7, -8, -9, -10), F5 (KIV-8, -9, -10), and F6 (KIV-5, -6, -7) were inactive (**Fig. 5B**). These results suggested that either KV or PD or both were participants in the binding. To resolve this issue, we used the recombinant protein rIII, containing KIV-9, -10, and KV but not the PD. We found that rIII exhibited no binding (**Fig. 5B**), suggesting that the PD is important for the binding of apo[a] to Fn.

To corroborate this conclusion, we used apo[a] isolated from rhesus Lp[a]. This nonhuman primate apo[a] is struc-



**Fig. 6.** Binding of human and rhesus (Rh) apo[a] and elastase fragments of human apo[a] to immobilized  $^{10}\text{FN-III}$ . Human apo[a], rhesus apo[a], F2, F4, and F1 at the indicated concentrations (0–125 nM) were incubated with immobilized  $^{10}\text{FN-III}$  for 2 h at  $22^\circ\text{C}$ . The data are means of three independent experiments for human and rhesus apo[a], F2, and F4 and four experiments for F4, all carried out in triplicate. The extent of apo[a] binding was followed by the absorbance at 405 nm as described in Methods. The data presented are means  $\pm$  SEM.

turally similar to human apo[a] but lacks KV. As shown in **Fig. 5C**, the binding of this apo[a] to Fn was comparable to that of its human counterpart, with apparent  $K_d$  values of  $7.72 \pm 0.26 \text{ nM}$  and  $10.25 \pm 1.62 \text{ nM}$ , respectively (**Table 1**).

We next tested the small fragment, F7 (KV-PD), for its ability to bind to Fn. For detection, we used, as a primary antibody, a monoclonal antibody directed against KV after establishing that it was suited to recognize both KV-PD and apo[a]. We observed binding with apo[a] but not with KV-PD (**Fig. 5D**). Together, all of the information gathered provided evidence that the PD required upstream sequences longer than KV for Fn binding.

### Binding studies with immobilized $^{10}\text{FN-III}$

In these studies, we coated the wells of the microtiter plates with  $^{10}\text{FN-III}$ . As shown in **Fig. 6**, human apo[a], rhesus apo[a], F2, and F4 exhibited binding, whereas F1 did not. In all cases, the binding affinity to  $^{10}\text{FN-III}$  was markedly higher than that exhibited by Fn. This was particularly true for apo[a] and F2, which exhibited a 5- to 6-fold difference from the data obtained with Fn (**Table 1**).

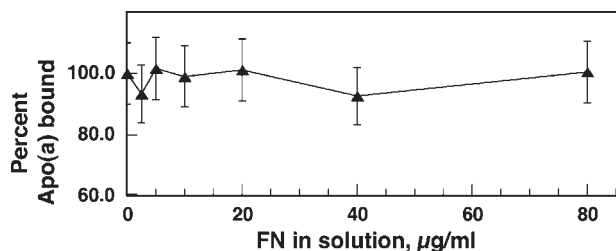
### Competition binding studies

To assess the ability of apo[a] to bind Fn in solution, we coated the microtiter plates with Fn at  $10 \mu\text{g/ml}$  and then incubated the wells with solutions containing variable amounts (0–80  $\mu\text{g/ml}$ ) of Fn and a constant amount (50 nM) of apo[a]. As shown in **Fig. 7**, when in the solution phase, Fn was unable to compete for the binding of apo[a]. Similar results were observed for  $^{10}\text{FN-III}$ . In addition, in a system in which apo[a] was coated on microtiter plates at  $10 \mu\text{g/ml}$  or 45 nM, binding of either Fn or  $^{10}\text{FN-III}$  was not observed (data not shown).

**TABLE 1.** Summary of the binding parameter  $K_d$  of apo[a] and fragments to immobilized Fn and  $^{10}\text{FN-III}$

Sample	Fn	
	nM	
Apo[a], 15 kringle IV	$10.25 \pm 1.62$ (6)	$1.75 \pm 0.31$ (3)
F2	$28.75 \pm 6.10$ (4)	$5.64 \pm 0.46$ (3)
F4	$32.16 \pm 1.91$ (3)	$15.16 \pm 1.57$ (4)
Rhesus apo[a]	$7.72 \pm 0.26$ (3)	$3.42 \pm 0.62$ (3)

apo[a], apolipoprotein [a]; Fn, fibronectin;  $^{10}\text{FN-III}$ , tenth type III module of fibronectin;  $K_d$ , dissociation constant.  $K_d$  was calculated by fitting the data from replicate experiments globally to a one-site model as described in Methods. The data shown are means  $\pm$  SEM. The numbers of independent experiments conducted in triplicate are shown in parentheses.



**Fig. 7.** Competitive binding of apo[a] and Fn. Fn was immobilized on microtiter plates and incubated with apo[a] at a constant concentration of 50 nM and various concentrations of Fn (0–80 µg/ml) in solution. The extent of binding of apo[a] was followed by the absorbance at 405 nm as described in Methods. The data are means of three independent experiments carried out in triplicate. The percentage of apo[a] that bound was calculated using the absorbance at a specified concentration of Fn divided by that without the addition of Fn and multiplied by 100. The data presented are means  $\pm$  SEM.

## DISCUSSION

The current studies have identified five elements in the C terminus of F2 that together were required for the binding of human apo[a] to immobilized Fn. Of these, the PD proved to be necessary but not sufficient for binding, given the need for the three upstream Ks, KIV-8 to -10, which by themselves were functionally inert. This conclusion is based on the combined results summarized in Fig. 5 obtained by studying proteolytic digests of apo[a] and recombinant products. KV was not required based on the results with the truncated products and those for rhesus monkey apo[a], which is known to contain PD but not KV (23), that proved to be as effective as a human counterpart in binding Fn. Our conclusion is also in keeping with the lack of binding exhibited by the small KV-PD unit. With reference to the type of binding, the lack of effect by EACA suggests a lysine-independent mode despite the fact that the PD, a 236 amino acid residue structure, contains 14 of the 23 lysines present in the whole apo[a]. Of note, the PD, although containing the histidine/asparagine/serine triad, a requisite for serine protease activity, is catalytically inactive because the arginine required for cleavage by tissue plasminogen activator is replaced by serine (11) and possibly also because of conformational issues relating to the nine amino acid deletion in the PD (12). It is unclear whether this catalytic inactivity has meaning regarding Fn binding. In this regard, plasminogen, which is highly homologous to apo[a] and has a catalytically active PD (11), has been shown to bind to Fn via a lysine-dependent mechanism (6, 7), indicating a mode of Fn binding divergent from that of apo[a].

The other novel finding of our work is the identification of the tenth type III module, referred to as <sup>10</sup>FN-III, as a major site on Fn involved in apo[a] binding. This is an interesting model from a structural standpoint in that it represents a relatively small monomeric unit, one of the few members of the immunoglobulin superfamily with no disulfide bonds (3, 4). Moreover, the three-dimensional structure of <sup>10</sup>FN-III has been determined by NMR (24) and X-ray crystallography (25), and its stability and folding


(26, 27) and conformational dynamics (28) are also well established. The structure is best described as a  $\beta$ -sandwich with seven  $\beta$ -strands containing the integrin binding RGD sequence involved in cell adhesion (24). Among the already established properties of <sup>10</sup>FN-III, we now show that it also binds to critical elements of the C-terminal domain of apo[a]. It remains to be established which site(s) on <sup>10</sup>FN-III are responsible for apo[a] binding.

In previous studies, van der Hoek et al. (7) identified a 12 amino acid sequence in Fn involved in the binding to a recombinant form of apo[a]. As for <sup>10</sup>FN-III, the binding was not lysine-mediated and was unaffected by high salt concentrations. According to the published data on Fn, the sequence reported by van der Hoek et al. (7) is located downstream of <sup>10</sup>FN-III in the junction between the 11th and 12th modules (29). This site was identified by submitting soluble Fn to thermolysin digestion followed by a trypsin step resulting in the unmasking of a site buried in undigested Fn. It is difficult to compare those data with ours because the authors neither provided quantitative binding parameters nor identified the elements in the recombinant apo[a] used. Overall, we believe that our current results make a strong case for <sup>10</sup>FN-III being a major site for apo[a] binding. This conclusion is corroborated by the observation that the binding affinity of this module was 6-fold higher than that exhibited by the whole Fn, suggesting that in the intact molecule the binding site on <sup>10</sup>FN-III is partially buried.

Past studies from this laboratory have underscored the value of proteolytic dissection in the investigation of the structure and biology of Lp[a] (13, 22). Since then, the use of proteolytically derived fragments alone or in combination with recombinant products has provided and is continuing to provide evidence for the structural heterogeneity of apo[a] associated with a functional diversity in which Ks play an important role. For instance, KIV-9, which is known to be engaged in disulfide linkage with the C-terminal domain of apolipoprotein B-100 (11), was implicated recently in the stimulating action of apo[a] on the migration and proliferation of vascular smooth muscle cells (30). KIV-10 contains the high-affinity lysine binding site important for critical apo[a] functions (31), whereas KV-5 to -8 contain some lysine residues that are linked covalently to oxidized phospholipids that appear to impart a proinflammatory function to apo[a] (19). Moreover, a recombinant form of KV has been reported to exhibit an antiangiogenic function (32). Very recently in mouse models, the KIV-5 to -8 peptide was implicated in the delayed chylomicron remnant removal from the plasma and also immunochemically identified in the atherosclerotic area of the aortic root examined (33). The PD has received relatively little attention, being functionally inert from an enzymatic standpoint, although recently it was shown to be one of the elements involved in the binding of human apo[a] to fibrinogen (34). Our current studies now provide evidence that in apo[a], the PD plays a role in the binding of human apo[a] to Fn, but only in cooperation with at least four Ks located upstream.

The current studies also bring attention to the fact that both Fn and <sup>10</sup>FN-III bind to apo[a] when immobilized

but not in solution. This observation, which in the case of Fn is in keeping with previous studies by Salonen et al. (6), is not unique to the Fn-apo[a] system, because immobilization of Fn has been shown to be required for the binding of Fn to plasminogen, tissue-type plasminogen activator (35), and acute-phase C-reactive protein (36). In this regard, recent studies have shown that Fn undergoes a conformational transition from a closed form to an open form when moving from a solution to a solid phase (37, 38). Noteworthy, this notion explains why Fn and apo[a] do not interact with each other in the circulating plasma.

Regarding the biological relevance of the current findings, we have previously shown that apo[a], via F2, binds to fibrinogen and to the protein core of the proteoglycans decorin and biglycan (8); in addition, we confirmed the critical role of F2 in this binding by subjecting it to limited proteolysis (either pancreatic elastase or metalloproteinase-12), and apo[a] bound to either decorin, biglycan, or Fn immobilized onto microtiter plates (39). Moreover, we have observed that F2 undergoes further fragmentation under more extensive proteolytic conditions (22). Together, these results, in keeping with the lipoprotein retention hypothesis (40), suggest that the trapping by the vascular extracellular matrix elements favors the fragmentation of apo[a], the extent of which is dependent on the activity of the proteolytic enzymes, an expression of the chronic inflammatory milieu of the vessel wall. In this context, we recently provided evidence for an association between proteolytic activity and inflammation in plaques from endarterectomy segments of human carotid arteries and also showed the presence of fragments of apo[a], decorin, biglycan, versican (41, 42), and, more recently, Fn (A.M. Scanu et al., unpublished observations). In the latter case, we have identified the <sup>10</sup>FN-III module associated with apo[a] using both immunohistochemical and immunoprecipitation techniques. The notion emerging from these findings is that in the inflammatory microenvironment of the atheromatous plaque, fragments of blood-derived lipoproteins become linked to elements of the extracellular matrix, generating entities likely exhibiting unique functions. For instance, apo[a] binding may affect the <sup>10</sup>FN-III-mediated interactions of Fn regarding the atherosclerotic process; in turn, immobilized apo[a] would become more amenable to the action of proteolytic enzymes, generating some potentially bioactive fragments. 

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## REFERENCES

- Schwarzbauer, J. E. 1991. Alternative splicing of fibronectin: three variants, three functions. *Bioassays*. **13**: 527–533.
- Hynes, R. O. 1985. The molecular biology of fibronectin. *Annu. Rev. Cell Biol.* **1**: 67–85.
- Baron, M., D. G. Norman, and I. D. Campbell. 1991. Protein modules. *Trends Biochem. Sci.* **16**: 13–17.
- Potts, J. R., and I. D. Campbell. 1994. Fibronectin structure and assembly. *Curr. Opin. Cell Biol.* **6**: 648–655.
- Wight, T. N. 2005. The Vascular Extracellular Matrix. In *Atherothrombosis and Coronary Heart Disease*. V. Fuster, E. J. Topol, and E. G. Nabel, editors. Lippincott Williams & Williams, Philadelphia, PA. 421–437.
- Salonen, E.-M., M. Jauhiainen, L. Zardi, A. Vaheri, and C. Ehnholm. 1989. Lipoprotein(a) binds to fibronectin and has serine proteinase activity capable of cleaving it. *EMBO J.* **8**: 4035–4040.
- van der Hoek, Y. Y., W. Sangrar, G. P. Cote, J. P. Kastelein, and M. L. Koschinsky. 1994. Binding of recombinant apolipoprotein(a) to extracellular matrix proteins. *Arterioscler. Thromb.* **14**: 1792–1798.
- Scanu, A. M., C. Edelstein, and O. Klezovitch. 2000. Dominant role of the C-terminal domain of apolipoprotein(a) to the protein core of proteoglycans and other members of the extracellular matrix. *Trends Cardiovasc. Med.* **9**: 196–200.
- Edelstein, C., J. A. Italia, O. Klezovitch, and A. M. Scanu. 1996. Functional and metabolic differences between elastase-generated fragments of human lipoprotein[a] and apolipoprotein[a]. *J. Lipid Res.* **37**: 1786–1801.
- Scanu, A. M., and C. Edelstein. 1995. Kringle-dependent structural and functional polymorphism of apolipoprotein (a). *Biochim. Biophys. Acta*. **1256**: 1–12.
- McLean, J. W., J. E. Tomlinson, W. Kuang, D. L. Eaton, E. Y. Chen, G. M. Fless, A. M. Scanu, and R. M. Lawn. 1987. cDNA sequence of human apolipoprotein(a) is homologous to plasminogen. *Nature*. **330**: 132–137.
- Gabel, B. R., and M. L. Koschinsky. 1995. Analysis of the proteolytic activity of a recombinant form of apolipoprotein(a). *Biochemistry*. **34**: 15777–15784.
- Scanu, A. M., and C. Edelstein. 1997. Learning about the structure and biology of human lipoprotein [a] through dissection by enzymes of the elastase family: facts and speculations. *J. Lipid Res.* **38**: 2193–2206.
- Fless, G. M., M. L. Snyder, and A. M. Scanu. 1989. Enzyme-linked immunoassay for Lp[a]. *J. Lipid Res.* **30**: 651–662.
- Fless, G. M., M. L. Snyder, J. W. J. Furbee, M. T. Garcia-Hedo, and R. Mora. 1994. Subunit composition of lipoprotein(a) protein. *Biochemistry*. **33**: 13492–13501.
- Edelstein, C., M. Mandala, D. Pfaffinger, and A. M. Scanu. 1995. Determinants of lipoprotein(a) assembly: a study of wild-type and mutant apolipoprotein(a) phenotypes isolated from human and rhesus monkey lipoprotein(a) under mild reductive conditions. *Biochemistry*. **34**: 16483–16492.
- Scanu, A. M., L. A. Miles, G. M. Fless, D. Pfaffinger, J. Eisenbart, E. Jackson, J. L. Hooverplow, T. Brunck, and E. F. Plow. 1993. Rhesus monkey lipoprotein(a) binds to lysine Sepharose and U937 monocyte cells less efficiently than human lipoprotein(a). Evidence for the dominant role of kringle 4(37). *J. Clin. Invest.* **91**: 283–291.
- Klezovitch, O., C. Edelstein, and A. M. Scanu. 2001. Stimulation of interleukin-8 production in human THP-1 macrophages by apolipoprotein(a): evidence for a critical involvement of elements of its C-terminal domain. *J. Biol. Chem.* **276**: 46864–46869.
- Edelstein, C., D. Pfaffinger, J. Hinman, E. Miller, G. Lipkind, S. Tsimikas, C. Bergmark, G. S. Getz, J. L. Witztum, and A. M. Scanu. 2003. Lysine-phosphatidylcholine adducts in kringle V impart unique immunological and potential pro-inflammatory properties to human apolipoprotein(a). *J. Biol. Chem.* **278**: 52841–52847.
- Koide, A., C. W. Bailey, X. Huang, and S. Koide. 1998. The fibronectin type III domain as a scaffold for novel binding proteins. *J. Mol. Biol.* **284**: 1141–1151.
- Schuck, P. 2000. Size-distribution analysis of macromolecules by sedimentation velocity ultracentrifugation and Lamm equation modeling. *Biophys. J.* **78**: 1606–1619.
- Edelstein, C., J. I. Italia, and A. M. Scanu. 1997. Polymorphonuclear cells isolated from human peripheral blood cleave lipoprotein(a) and apolipoprotein(a) at multiple interkringle sites via the enzyme elastase: generation of mini Lp(a) particles and apo(a) fragments. *J. Biol. Chem.* **272**: 11079–11087.
- Tomlinson, J., J. McLean, and R. Lawn. 1989. Rhesus monkey apolipoprotein(a). *J. Biol. Chem.* **264**: 5957–5965.
- Main, A. L., T. S. Harvey, M. Baron, J. Boyd, and I. D. Campbell. 1992. The three dimensional structure of the tenth type III mod-



- ule of fibronectin: an insight into RGD-mediated interactions. *Cell*. **71**: 671–678.
25. Dickinson, C. D., B. Veerapandian, X-P. Dai, R. C. Hamlin, N-H. Xuong, E. Ruoslahti, and K. R. Ely. 1994. Crystal structure of the tenth type III cell adhesion module of human fibronectin. *J. Mol. Biol.* **236**: 1079–1092.
  26. Plaxco, K. W., C. Spitzfaden, I. D. Campbell, and C. M. Dobson. 1996. Rapid refolding of proline-rich beta-sheet fibronectin type III module. *Proc. Natl. Acad. Sci. USA*. **93**: 10703–10706.
  27. Plaxco, K. W., C. Spitzfaden, I. D. Campbell, and C. M. Dobson. 1997. A comparison of the folding kinetics and thermodynamics of two homologous fibronectin type III modules. *J. Mol. Biol.* **270**: 763–770.
  28. Carr, P. A., H. P. Erickson, and A. G. R. Palmer. 1997. Backbone dynamics of homologous fibronectin type III cell adhesion domains from fibronectin and tenascin. *Structure*. **5**: 949–959.
  29. Kornblihtt, A. R., K. Umezawa, K. Vibe-Pedersen, and F. E. Baralle. 1985. Primary structure of human fibronectin: differential splicing may generate at least 10 polypeptides from a single gene. *EMBO J.* **4**: 1755–1759.
  30. O'Neil, C. H., M. B. Boffa, M. A. Hancock, J. G. Pickering, and M. L. Koschinsky. 2004. Stimulation of vascular smooth cell proliferation and migration by apolipoprotein(a) is dependent on inhibition of transforming growth factor-beta activation and on the presence of kringle IV type 9. *J. Biol. Chem.* **279**: 55187–55195.
  31. Scanu, A. M., and G. M. Fless. 1990. Lipoprotein (a): heterogeneity and biological relevance. *J. Clin. Invest.* **85**: 1709–1715.
  32. Kim, J-S., H-K. Yu, J-H. Ahn, H-J. Lee, S-W. Hong, H-H. Jung, S-I. Chang, Y-K. Hong, Y-A. Joe, S-M. Byun, et al. 2004. Human apolipoprotein(a) kringle V inhibits angiogenesis in vitro and in vivo by interfering with the activation of focal adhesion kinases. *Biochem. Biophys. Res. Commun.* **313**: 534–540.
  33. Devlin, C. M., S-J. Lee, G. Kuriakose, C. Spencer, L. Becker, I. Grosskopf, C. Ko, L-S. Huang, M. Koschinsky, A. D. Cooper, et al. 2005. An apolipoprotein(a) peptide delays chylomicron remnant clearance and increases plasma remnant lipoproteins and atherosclerosis in vivo. *Arterioscler. Thromb. Vasc. Biol.* **25**: 1704–1710.
  34. Xue, S., E. L. Madison, and L. A. Miles. 2001. The Kringle V-protease domain is a fibrinogen binding region within human apo(a). *Thromb. Haemost.* **86**: 1229–1237.
  35. Salonen, E-M., O. Saksela, T. Vartio, A. Vaheri, L. S. Nielsen, and J. Zeuthen. 1985. Plasminogen and tissue-type plasminogen activator binds to immobilized fibronectin. *J. Biol. Chem.* **260**: 12302–12307.
  36. Salonen, E-M., T. Vartio, K. Hedman, and A. Vaheri. 1984. Binding of fibronectin to the acute phase-reactant C-reactive protein. *J. Biol. Chem.* **259**: 1496–1514.
  37. Halter, M., M. Antia, and V. Vogel. 2005. Fibronectin conformational changes induced by adsorption to liposomes. *J. Control. Release*. **101**: 209–222.
  38. Kowalczyńska, H. M., M. Norwak-Wyrzykowska, R. Kolos, J. Dobkowski, and J. Kamiński. 2005. Fibronectin adsorption and arrangement on copolymer surfaces and their significance in cell adhesion. *J. Biomed. Mater. Res.* **72A**: 228–236.
  39. Scanu, A. M., and S. Chellamma. 2004. Cleavage of human apolipoprotein(a) coated on the surface of constituents of the vascular extracellular matrix by pancreatic elastase and metalloproteinase-12. Different partitions between cleaved N-terminal and C-terminal domains. *Vascular Disease Prevention*. **1**: 59–63.
  40. Williams, K. J., and I. Tabas. 1995. The response-to retention hypothesis of early atherogenesis. *Arterioscler. Thromb. Vasc. Biol.* **15**: 551–561.
  41. Formato, M., M. Farina, R. Spirito, M. Maggioni, A. Guarino, G. Cherchi, P. Biglioli, C. Edelstein, and A. M. Scanu. 2004. Evidence for a proinflammatory and proteolytic environment in plaques from endarterectomy segments of human carotid arteries. *Arterioscler. Thromb. Vasc. Biol.* **24**: 129–135.
  42. Fortunato, J. E., H. S. Bassiouny, R. H. Song, H. Kocharian, S. Glagov, C. Edelstein, and A. M. Scanu. 2000. Apolipoprotein(a) fragments in relation to human carotid plaque instability. *J. Vasc. Surg.* **32**: 555–563.