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 β 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
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 nonapeptide (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 occurring 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 (10FN-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

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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.

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 2n to reflect the presence of several identical copies of this K. The cleavage sites by leukocyte elastase are indicated by arrows.
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).

The rhesus Lp[a] and apo[a] isolation were carried out immediately after blood draw- ing with 2-mercaptoethanol; lane 3, molecular mass standards.

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

Lp[a] was isolated from rhesus plasma as described previously (16). 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 \( \rho \)-nitrophenol/s from N-t Btyloxy carbonyl-L-Ala \( \rho \)-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 ΔKIV6-8ΔPD) was prepared by digesting the 6 K expression plasmid with Clal and EcoRV (19). Subsequently, the 2.4 kb Clal- EcoRV insert was subjected to digestion with BamHI to remove the BamHI-BamHI internal DNA domains coding for apo[a] KIV-6 through -8. The 0.32 kb Clal- BamHI and the 1.03 kb BamHI- EcoRV DNA fragments were then ligated back into the 6 K expression plasmid that was digested with Clal and EcoRV (vector part). rIII (6K ΔKIV6-8ΔPD) (Fig. 3) was sequenced and transfected into human embryonic kidney 293 cells. The clone produced significant amounts of recombinant product (0.5–10 mg/l of the culture medium) that was purified by lysine-Sepharose chromatography.

Preparation of \( ^{10}\text{FN-III} \)

The recombinant product, \( ^{10}\text{FN-III} \), 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 \( ^{10}\text{FN-III} \) 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 Beck- man XL-A analytical ultracentrifuge. Approximately 0.4 mg/ml \( ^{10}\text{FN-III} \) 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
coated with 100 μl of either Fn or 14FN-III, each at 10 μg/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 14FN-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 14FN-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 14FN-III. Bound apo[a] was detected using a monospecific

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**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 ± SEM.
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 (ε280 = 1.31 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 ± 1.62 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 structurally 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 ± 0.26 nM and 10.25 ± 1.62 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 \(^{125}\)FN-III

In these studies, we coated the wells of the microtiter plates with \(^{125}\)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 \(^{125}\)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 μg/ml and then incubated the wells with solutions containing variable amounts (0–80 μg/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 \(^{125}\)FN-III. In addition, in a system in which apo[a] was coated on microtiter plates at 10 μg/ml or 45 nM, binding of either Fn or \(^{125}\)FN-III was not observed (data not shown).

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**TABLE 1. Summary of the binding parameter \( K_d \) of apo[a] and its fragments to immobilized Fn and \(^{125}\)FN-III**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fn</th>
<th>(^{125})FN-III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apo[a], 15 kringle IV</td>
<td>10.25 ± 1.62 (6)</td>
<td>1.75 ± 0.31 (3)</td>
</tr>
<tr>
<td>F2</td>
<td>28.75 ± 6.10 (4)</td>
<td>5.64 ± 0.46 (3)</td>
</tr>
<tr>
<td>F4</td>
<td>32.16 ± 1.91 (3)</td>
<td>15.16 ± 1.57 (4)</td>
</tr>
<tr>
<td>Rhesus apo[a]</td>
<td>7.72 ± 0.26 (3)</td>
<td>3.42 ± 0.62 (3)</td>
</tr>
</tbody>
</table>

apo[a], apolipoprotein [a]; Fn, fibronectin; \(^{125}\)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 ± SEM. The numbers of independent experiments conducted in triplicate are shown in parentheses.

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**Fig. 6.** Binding of human and rhesus (Rh) apo[a] and elastase fragments of human apo[a] to immobilized \(^{125}\)FN-III. Human apo[a], rhesus apo[a], F2, F4, and F1 at the indicated concentrations (0–125 nM) were incubated with immobilized \(^{125}\)FN-III for 2 h at 22°C. The data are means of three independent experiments for human and rhesus apo[a], F2, F1 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 ± SEM.
The structure of 10FN-III has been determined by NMR (24) and disulfide bonds (3, 4). Moreover, the three-dimensional structure of 10FN-III is best described as a β-sandwich with seven β-strands containing the integrin binding RGD sequence involved in cell adhesion (24). Among the already established properties of 10FN-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 10FN-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 10FN-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 10FN-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 10FN-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 10FN-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 10FN-III bind to apo[a] when immobilized (26, 27) and conformational dynamics (28) are also well established. The structure is best described as a β-sandwich with seven β-strands containing the integrin binding RGD sequence involved in cell adhesion (24). Among the already established properties of 10FN-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 10FN-III are responsible for apo[a] binding.

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The current studies also bring attention to the fact that both Fn and 10FN-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 10FN-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 10FN-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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