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FIBRONECTIN BINDS TO AMYLOID P COMPONENT. LOCALIZATION OF THE BINDING SITE TO THE 31,000 DALTON C-TERMINAL DOMAIN

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Fibronectin has been shown to play an important role in reticuloendothelial system functioning as well as in neutrophil and fibroblast migration to tissue injury sites. Fibronectin binds several macromolecules including components of the acute phase response. We have studied the interaction of fibronectin with the amyloid P component (AP). This glycoprotein, closely related to C-reactive protein, is deposited together with amyloid fibrils and is also a normal constituent of human tissues. AP-Sepharose affinity columns were used to determine the binding of fibronectin, its whole tryptic digest, and isolated fragments; fibronectin was retained by immobilized AP in a molar ratio fibronectin:AP of 1:5.8. In this paper we localized the binding site for AP in a tryptic 31 kDa fragment, near the C-terminal end of the fibronectin molecule. A shorter fragment of 22 kDa starting at position 82 of the 31 kDa domain and containing all the disulfide bridges present in the 31 kDa domain did not bind to AP; therefore the active site appears to be located within the 81 N-terminal residues of the 31 kDa fragment. To further support this conclusion, reduction and alkylation of either fibronectin or the 31 kDa fragment had no effect on their binding properties.

Fibronectin (Fn) is an adhesive glycoprotein present in soluble form in plasma and other body fluids and in an insoluble form in interstitial connective tissues and in association with basement membranes. Plasma Fn is a macromolecular dimer of disulfide bonded subunits each of Mr 220,000, which are susceptible to proteolytic fragmentation.

The biological functions of this protein include a role in cellular adhesion, embryogenesis and development, wound healing and reticuloendothelial system clearance (1-5). Fn also mediates the migration of certain cell types (neutrophils, fibroblasts, and monocytes) to injury sites where the protein is believed to act as a support for the formation of new tissue.

Fn is able to bind other macromolecules including collagen, heparin, fibrin and fibrinogen, C1q, actin and DNA (6-17). Recently, it has been shown that Fn interacts with components of the acute phase response such as C-reactive protein

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(CRP)(18) and the serum form of the amyloid P component (SAP)(19). Both CRP and SAP belong to the pentaxin family of proteins; they are characterized by a similar arrangement of their non-covalently associated subunits in an annular disk-like configuration; CRP and SAP have a 52% homology in their primary structure (20).

Most of the binding sites of the Fn molecule have been localized in specific globular domains. The region of Fn responsible for the binding to CRP was recently located towards the carboxy-terminal end of the molecule (18). However, the position of the binding site for SAP is not known.

We have studied the interaction between Fn and the tissue form of amyloid P component (AP). AP is found in most types of amyloid deposits and is also a normal component of basement membranes and of elastic fibers. AP is indistinguishable from SAP in antigenicity, molecular weight and appearance in the electron microscope (19). Reports on the complete amino acid sequence of AP (20) as well as on the nucleotide cDNA sequence of SAP (21), have shown these two molecules to be identical.

In this paper we show that Fn is able to bind to the tissue form of the amyloid P component (AP); we have localized this binding site within a fragment located at the C-terminal region of the Fn molecule. Although the significance of the interaction between these two proteins is not well understood it is likely to be important in the repair of tissue after injury and in inflammatory processes.

MATERIALS AND METHODS

Isolation of Human Fn

Fn was isolated from pooled human plasma by affinity chromatography on gelatin-Sepharose 4B (7).

Isolation of Fn Tryptic Fragments

Purified Fn (1-2 mg/ml) in 25 mM Tris, 50 mM NaCl, 0.5 mM Na_2EDTA, pH 7.6 buffer (25 mM Tris buffer) was digested with L-1-tosylamido-2-phenylethyl chlomethyl ketone trypsin (Worthington) in two ways: 1) enzyme/substrate ratio of 1:1000 (w/w), 15 min., room temperature (isolation of 31-, 29- and 22 kDa fragments and binding experiments with the whole digest on AP-Sepharose); and 2) enzyme/substrate ratio of 1:100 (w/w), 6 hr., 37°C (isolation of 30 kDa fragment). In both cases the digestion was stopped with 10^{-4}M phenylmethylsulfonyl fluoride (Sigma Chem.) and 10^{-3} mM diisopropyl fluorophosphate (Sigma Chem). For the isolation of the 31-, 20- and 22 kDa fragments, the whole tryptic digest was applied to a gelatin-Sepharose column and the unbound material passed through a heparin-Sepharose column. The material that did not bind to either column was further purified by DEAE cellulose (22,23). The 31- and 22 kDa fragments were eluted with 0.1M NaCl and separated by HPLC. The 29 kDa fragment was eluted from the heparin-Sepharose column with 0.1M NaCl. For the isolation of the 30 kDa gelatin binding domain, the tryptic digest was applied to a gelatin-Sepharose column and the bound material eluted with 4M urea; the 30 kDa fragment was repurified on a Sephacryl S-200 column (24).

Reduction and Alkylation of Fn and the 31 kDa Tryptic Fragment

Intact Fn or 31 kDa fragment in 10 mM Tris, 0.15M NaCl, 2 mM $CaCl_2$, pH 8.0 buffer (Tris-Ca buffer) was incubated with 5 mM DTT for 1 hr at room temperature in a nitrogen atmosphere. Alkylation was performed by making the solution 12.5 mM in ^{14}C Iodoacetic acid (N. Eng. Nuclear), 0.7 mCi/mmol. Complete reduction and alkylation of the 31 kDa fragment, resulted when the same procedure was followed except that the fragment was solubilized in 0.6M Tris, 0.001M EDTA, 6M Guanidine, pH 8.2 buffer. Partially reduced and alkylated Fn and 31 kDa fragment, as well as the completely reduced and alkylated 31 kDa fragment were dialyzed against Tris-Ca buffer before being applied to the AP-Sepharose column.

Isolation of AP

AP was isolated from the spleen of a patient (Mul) with primary amyloidosis as previousl described (25,26). The saline supernatants were concentrated by partial lyophilization and dialyzed three days against Tris-Ca buffer. This fraction was mixed with an equal volume of Sepharose 4B (Pharmacia Chem.), previously equilibrated in the same buffer. After stirring overnight at 4°C, the Sepharose was washed with Tris-Ca buffer until the A_{280} was zero. AP was then eluted with 10 mM Tris, 0.15M NaCl, 0.001M Na_2EDTA, pH 8.0. The presence of AP in this eluate was confirmed by immunodiffusion against rabbit anti-P component (Accurate Chem.) and its purity determined by SDS-PAGE gels.

Immobilized AP

AP was dialyzed for three days at 4°C against 0.1M NaHCO3, 0.5M NaCl, pH = 8.3 before coupling to CNBr-activated Sepharose 4B (Pharmacia Chem.). Two columns were prepared containing 5 and 10 mg/ml beads respectively. After the coupling reaction was completed, the remaining active groups were blocked with 1M ethanolamine, and AP-Sepharose was subsequently washed with coupling buffer, Tris-Ca buffer and 10 mM Tris, 0.15M NaCl, 0.05M EDTA (Tris-EDTA) buffer. The final volume of both columns was 1 ml. Cont ol beads were prepared by blocking the active groups on CNBr-Sepharose with 1M ethanolamine in the absence of protein.

Affinity Chromatography Experiments

All tests were performed at 4°C. The AP-Sepharose beads were always pre-equilibrated in Tris-Ca buffer; Fn (0.5 - 1 mg/ml) and its fragments were dialyzed into this buffer before being applied to the columns. Fn, its whole tryptic digest and the isolated fragments were passed over the affinity column, which was then washed with Tris-Ca buffer until no further material absorbing at 280 nm was detected. Bound material was eluted with Tris-EDTA buffer. Starting material and bound and unbound fractions were compared by SDS-PAGE.

SDS-PAGE

SDS-PAGE of Fn, its proteolytic fragments and materials bound and unbound to AP-Sepharose 4B were carried out in slab gels as described (27). Molecular weights were determined by comparison with known standards on Commassie Blue stained gels (28).

Immunoblotting Experiments

Following SDS-PAGE, AP was transferred onto a nitrocellulose membrane (0.45 μm, Bio-Rad) using a trans-blot cell (Bio-Rad). The transfer was performed at room temperature for 16 hr at 150 mA, using 25 mM Tris/192 mM glycine buffer, pH 8.3, containing 20% methanol (v/v)(29). Active sites were blocked with 3% BSA in Tris-Ca buffer, and the membrane was incubated with Fn or the isolated fragments for 6 hr at 4°C. After extensive washing, the membrane was allowed to react with rabbit anti-Fn antiserum (E.Y Lab.) and goat anti-rabbit IgG conjugated with peroxidase (E.Y Lab.). The reaction was developed using 3-3' diaminobenzidine (Sigma Chem.) and H_2O_2. Fibronectin was replaced by ovalbumin in one of the lanes and used as a negative control.

RESULTS

Binding of Fn to AP-Sepharose Columns

Depending on the amount of AP linked to Sepharose, the percentage of Fn bound to the columns varied from 29% (5 mg/ml beads) to 45% (10 mg/ml beads) (Table I). The

TABLE I. Binding of Fibronectin and Its Tryptic Fragments to AP-Sepharose

	Protein eluted with EDTA[a]							
	Fn	Tryptic Digest	31K	30K	29K	22K	Fn R/A	31K R/A
AP-Sepharose[b]	29	13.5	ND	ND	ND	ND	26	ND
AP-Sepharose[c]	45	20	35	4	8	9	ND	35
Sepharose[d]	7	2	8	5	5	9	ND	ND

[a] Values expressed as % of protein applied; average of four experiments.
[b] 5 mg/ml beads.
[c] 10 mg/ml beads.
[d] No protein coupled.

absolute amounts of Fn bound by the AP-Sepharose columns were 0.3 mg Fn/mg AP (column #1) and 0.27 mg Fn/mg AP (column #2) corresponding to a molar ratio Fn:AP of 1:5.8 for column #1 and 1:6.5 for column #2.

Binding of Fn Tryptic Digest to AP Columns

We have previously reported that trypsin digestion of Fn under limited conditions results in the production of four major fragments, namely 200-, 180-, 31-, and 29 kDa (Fig. 2A lane 2). When the complete digest was applied to the AP columns, the percentage of bound protein was 13.5% and 20% for columns #1 and #2 respectively (Table I). SDS-PAGE analysis of these fractions (Fig. 2A) showed that the 200-, 180-, and 31 kDa were able to bind to the AP column (lane 4), while the 29 kDa fragment was not (lane 3). The unbound fraction contained all the 29 kDa fragment as well as part of the 200- and 180 kDa while the amount of the 31 kDa fragment was highly diminished. Repassing of the unbound fraction over the same column did not result in further binding of these three fragments.

Binding of Isolated Fragments to AP-Sepharose

Figure 1 shows the isolated tryptic fragments that were tested for their binding capacity to AP-Sepharose. The 31- and 29 kDa fragments have been extensively characterized by us (23-24); the 22 kDa fragment was produced by degradation of the 31 kDa during isolation procedures, and easily separated by HPLC. Determination of the N-terminal amino acid sequence of this fragment showed that it started at

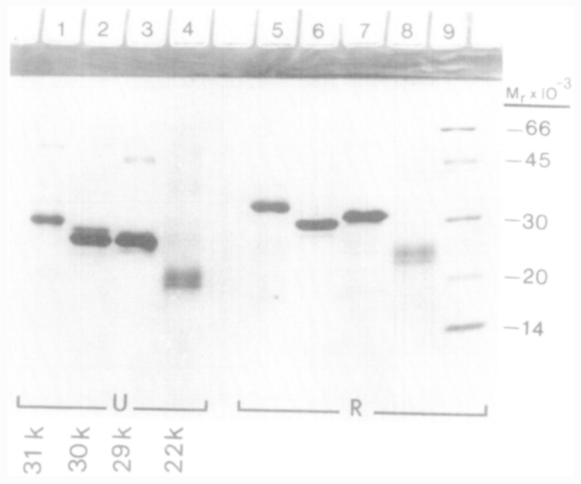

Figure 1. Fibronectin fragments tested for their binding capacity to AP-Sepharose. Lanes 1 and 5: 31 kDa; lanes 2 and 6: 30 kDa (gelatin-binding domain); lanes 3 and 7: 29 kDa (N-terminal domain); lanes 4 and 8: 22 kDa; lane 9: molecular weight markers consisting of phosphorylase b (96,000), bovine serum albumin (66,000), ovalbumin (45,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,000) and lactalbumin (14,000).

position 82 of the 31 kDa domain (Garcia-Pardo, unpublished results), and therefore contains all the disulfide bridges present in the 31 kDa fragment (24). Table I

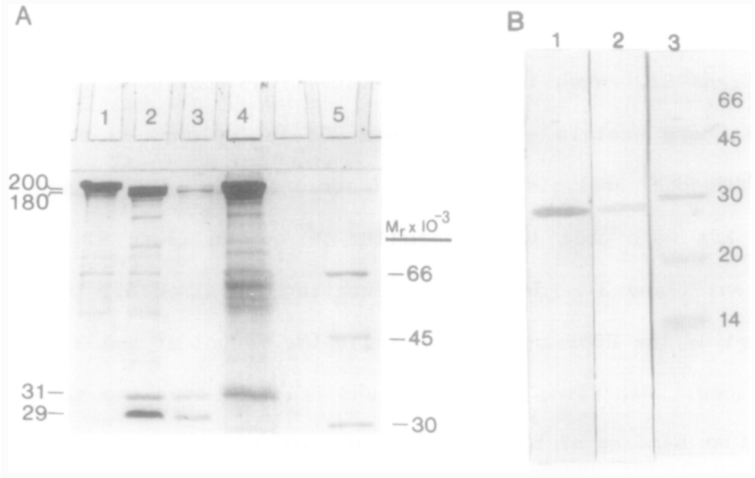

Figure 2. Binding of Fn and its tryptic fragments to immobilized AP. (A) Affinity chromatography on AP-Sepharose. Lane 1: intact Fn; lane 2: whole tryptic digest (composed mainly of 200-, 180-, 31- and 29 kDa fragments); lane 3: material unbound to AP-Sepharose eluted with 10 mM Tris, 0.15M NaCl, 2 mM $CaCl_2$, pH 8.0; lane 4: fragments bound to AP-Sepharose eluted with the same buffer containing 5 mM EDTA instead of calcium; lane 5: molecular weight markers same as in figure 1. All samples reduced. (B) Western blot of AP incubated with Fn and the 31 kDa fragment. Lane 1: AP incubated with Fn, anti-Fn, and peroxidase conjugated anti-rabbit Western blot IgG; lane 2: AP incubated with 31 kDa fragment, developed as in lane 1; lane 3: molecular weight markers stained with Coomassie Blue.

shows the percentage of these fragments that were retained on AP-Sepharose; 35% of the applied 31 kDa bound to the column while only background levels of the 30-, 29-, and 22 kDa fragments were retained.

Effect of Reduction and Alkylation on the Binding to AP

Partial reduction and alkylation of Fn molecule did not affect the amount bound by AP-Sepharose (Table I). Similarly when the 31 kDa fragment was either partially or completely reduced and alkylated, no decrease in the percentage of material bound to AP-Sepharose was observed (Table I).

Binding of Fn and Its Isolated Tryptic Peptides to AP Transferred Onto Nitrocellulose

As shown in figure 2B, the AP immobilized on the nitrocellulose membrane gave a strong reaction when incubated stepwise with Fn, rabbit anti-Fn antiserum and goat anti-rabbit IgG peroxidase conjugated (lane 1). The same results were obtained with the 31 kDa domain (lane 2), while the 30-, 29-, and 22 kDa peptides gave no reaction; reduction and alkylation of AP had no effect on the binding to Fn or 31 kDa (not shown).

DISCUSSION

Fn and AP are molecules characterized by their binding properties. None of the interactions with other macromolecules described for human Fn require calcium or any other divalent cations. In contrast, all the binding properties reported for AP/SAP depend on calcium ions; these include the interaction with amyloid fibrils (30), agarose (31) C4 binding protein and Fn (19).

The amount of soluble Fn bound to immobilized AP is in agreement with the values reported for SAP in previous work (19) and very similar to the results obtained with CRP (18). The molar ratio Fn:CRP described by these authors was 1:5.8, exactly the same value that we obtained when 5 mg of AP is coupled to 1 ml of beads. The significance of this value is difficult to interpret, because it is not known what percentage of the AP coupled to Sepharose is actually active.

Some of the binding sites of the Fn molecule have been localized in specific domains within the molecule. We therefore attempted to characterize the region of Fn responsible for the interaction with AP/SAP. When the whole tryptic digest of Fn

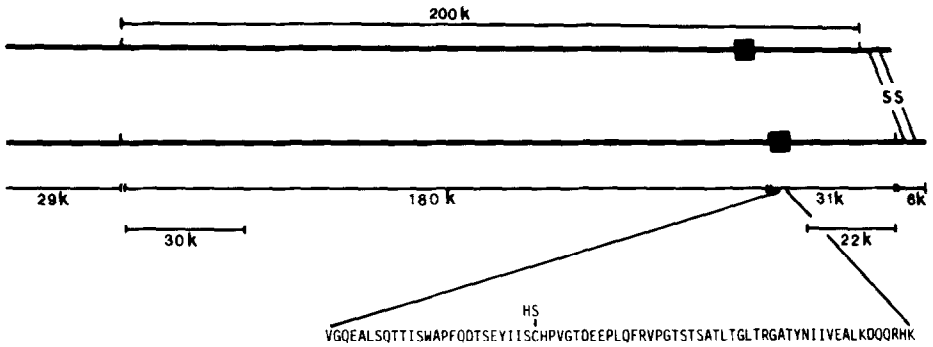

Figure 3. Model of plasma fibronectin showing the position of the tryptic fragments. The black box marks the location of the binding site for amyloid P component. The amino acid sequence of the residues involved in the binding is indicated (23).

was applied to AP-Sepharose columns, the 31 kDa fragment as well as the larger fragments of 200- and 180 kDa were retained. This indicated that at least one binding site for AP was located within the 31 kDa fragment. Confirmation of this result was obtained when the purified 31 kDa domain was passed through the same column and 35% was retained (Table I). In contrast, neither the 29 kDa fragment, which corresponds to the N-terminal domain of Fn, nor the 30 kDa gelatin-binding domain, bound to AP. We can therefore conclude that the N-terminal end of the Fn molecule represented by the 29- and 30 kDa domains is not involved in the AP binding.

Further localization of the AP binding site in the 31 kDa domain was achieved by testing the 22 kDa fragment for AP binding capacity. As mentioned before, the 22 kDa fragment starts at position 82 of the 31 kDa fragment and contains the seven intrachain disulfide bridges present in the 31 kDa domain (23). The 22 kDa fragment failed to bind to AP-Sepharose therefore mapping the binding site within the first 81 residues of the 31 kDa domain. Furthermore, we have recently shown (23) that the first 10 amino acid residues of this domain are part of a segment which might be present in the 180 kDa but not in the 200 kDa fragment (32). Since the 200 kDa binds to AP we can postulate that the binding site may be located between residues 11 and 81 of the 31 kDa fragment. Since the 180 kDa also binds to AP-Sepharose (see Fig. 2A, lane 4) the existence of another binding site in a different region of fibronectin cannot be ruled out. Figure 3 shows an schematic representation of the fragments used in this study and the location of the AP binding site.

The only well established reactivity of AP in vivo is the demonstration that it binds to amyloid fibrils (30). SAP is a plasma glycoprotein and a normal constituent of connective tissue, basement membranes and elastic fibers throughout the body. Fn is a glycoprotein also found circulating in plasma, in connective tissue, on the surface of many cell lines and at inflammation sites. Although the distribution of both proteins is not always coincidental, it is possible that a biological interaction between them during inflammation and/or injury could occur at sites where they are in close proximity such as capillary walls.

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