Identification of fibronectin fragments that bind to carboxy-group-modified proteins

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Limited proteolysis of human plasma fibronectin with chymotrypsin, trypsin or thermolysin has been used to localize binding sites responsible for binding [Vuento, Korkolainen & Stenman (1982) *Biochem. J.* **205**, 303–311] of fibronectin to carboxy-group-modified proteins. These binding sites are different from those mediating binding of fibronectin to gelatin or heparin. They are located close to the C-terminus of the polypeptide chains of fibronectin, and apparently overlap with the C-terminal fibrin binding site.

Fibronectins are high-molecular-mass multi-functional proteins present in connective tissue and in body fluids (reviewed by Hynes & Yamada, 1982). Fibronectins bind various macromolecular ligands including collagen, fibrin and glycosaminoglycans (Hynes & Yamada, 1982). It has been shown that plasma fibronectin also binds to proteins modified with carboxy-group-specific reagents (Vuento *et al.*, 1982). We have suggested that this activity may reflect an ability of fibronectin to recognize and bind to denatured proteins. Such binding could be significant in terms of the proposed function (Saba & Jaffe, 1980) of plasma fibronectin as a non-specific opsonin.

Various binding activities of fibronectin have been previously localized in proteinase-resistant fragments of the protein [current models for fibronectin structure and for the relationship of various fragments, including those used in the present study, are outlined in papers by Sekiguchi & Hakomori (1983) and Ehrismann *et al.* (1982)]. In the present study we have used limited proteolytic fragmentation to localize binding site(s) responsible for the binding of fibronectin to carboxy-group-modified proteins. These sites appear to be different from the ones mediating the binding of fibronectin to gelatin or to heparin, and they are located close to the C-terminus of the polypeptide chains of fibronectin.

Experimental

Human plasma fibronectin was purified as described previously by using urea (Sekiguchi *et al.*,

1981) or arginine (Vuento & Vaheri, 1979) in elution of fibronectin from gelatin–Sephacrose. Purified fibronectin was digested with trypsin or with thermolysin as described previously (Sekiguchi & Hakomori, 1980; for details, see the Figure legends). Digestion of fibronectin with α -chymotrypsin was performed as described by Ehrismann *et al.* (1982), with fibronectin bound to gelatin–Sephacrose as a substrate for the proteinase. After washing out peptides released with chymotrypsin, a homogeneous gelatin-binding fragment with a molecular mass of 46 kDa was eluted from gelatin–Sephacrose with urea (Ehrismann *et al.*, 1982). Human IgG (Kabi, Stockholm, Sweden), human serum albumin (Sigma) and heparin (Medica, Helsinki, Finland) were coupled to CNBr-activated Sepharose 4B gel (Pharmacia). Sepharose-linked proteins were modified with water-soluble carbodiimide in the presence of glycine ethyl ester as described previously (Vuento *et al.*, 1982). Affinity chromatography of fibronectin fragments was performed at 22°C on columns (2 ml) of Sepharose-linked proteins or of Sepharose-linked heparin. The columns were prewashed with 8 M-urea/1 M-NaCl and equilibrated with buffer. Unless indicated otherwise, 50 mM-Tris/HCl (pH 7.5)/100 mM-NaCl was used. A portion (2 mg) of proteinase-digested fibronectin was applied, and the columns were washed with equilibrating buffer until the A_{280} of the eluate was less than 0.01. The columns were then eluted with 8 M-urea/1 M-NaCl in 10 mM-Tris/HCl, pH 7.5. Polyacrylamide-gel electrophoresis in the presence of SDS was performed in vertical gel slabs, the discontinuous buffer system of Laemmli (1970) being used.

Abbreviations used: IgG, immunoglobulin G; SDS, sodium dodecyl sulphate.

Results

Intact fibronectin and fibronectin fragments showed strong binding to columns of Sepharose-linked modified IgG. This binding was dependent on the buffer system used, being complete and non-selective at low ionic strength (e.g. in 10 mM-Tris/HCl, pH 7.5). Selective retardation of specific fibronectin fragments was achieved by adding NaCl to the buffers to a concentration of 50–100 mM. No binding of fibronectin fragments to non-modified IgG-Sepharose occurred under these conditions.

The peptides released from gelatin-bound fibronectin on treatment with chymotrypsin included three major ones with molecular-mass values of 180 kDa, 170 kDa and 150 kDa. In addition, a major peptide of 25 kDa was also observed (Fig. 1).

Fig. 1. SDS/polyacrylamide-gel electrophoresis of fibronectin fragments obtained by digestion with chymotrypsin

1, Gelatin-binding fragment; 2, fragments released from gelatin-bound fibronectin with chymotrypsin; 3, fraction of the fragments shown on lane 2 not retained on modified IgG-Sepharose; 4, fragments bound to and eluted from modified IgG-Sepharose; 5, fragments not retained on heparin-Sepharose; 6, fragments bound to, and eluted from, heparin-Sepharose. The migration positions of molecular-mass (m) standards are shown on the right.

The gelatin-binding fragment eluted from the column as a single polypeptide of 46 kDa. The 180 kDa and 170 kDa fragments showed extensive binding to modified IgG (Fig. 1), whereas only a small amount of the 150 kDa fragment and none of the 25 kDa fragment did bind. No binding was observed with the gelatin-binding fragment. Similar results were obtained with modified albumin-Sepharose (results not shown). The chymotryptic fragments binding to heparin-Sepharose included those of 180 kDa, 170 kDa and 150 kDa (Fig. 1).

Binding of tryptic fragments to modified IgG-Sepharose is shown in Fig. 2. Mild trypsin digestion cleaved fibronectin into four fragments (215 kDa, 185 kDa, 37 kDa and 32 kDa; Sekiguchi & Hakomori, 1983). The 32 kDa fragment, derived from the *N*-terminal region, did not bind to the modified protein, whereas the 37 kDa fragment, derived from the *C*-terminal region (Sekiguchi & Hakomori, 1983), was capable of binding to the modified IgG. The larger 215 kDa and 185 kDa fragments could also bind to the modified protein.

Fig. 3 shows binding of thermolysin fragments to a modified IgG-Sepharose column. Approx. 18% of the input fragments were retained on the column. It

Fig. 2. Binding of tryptic fragments to carboxy-group-modified IgG-Sepharose

Intact fibronectin was digested with trypsin (1 μ g/ml) at 22°C for 15 min. After inactivating the proteinase with soya-bean trypsin inhibitor (2 μ g/ml), the digest was fractionated on modified IgG-Sepharose. 1, Intact fibronectin; 2, tryptic digest; 3, fragments not bound to modified IgG; 4, fragments bound to modified IgG. The migration positions of molecular-mass (m) standards are shown on the right.

Fig. 3. Binding of thermolysin fragments to carboxy-group-modified IgG-Sepharose

(a) Chromatography of the thermolysin digest on modified IgG-Sepharose. Intact fibronectin was digested with thermolysin ($5 \mu\text{g/ml}$) at 22°C for 2 h in 10 mM-Tris/HCl, pH 7.6, containing 0.5 mM-EDTA, 2.5 mM- CaCl_2 and 70 mM-NaCl. After inactivation of the proteinase by the addition of 5 mM-EDTA, the digest was applied to a column (0.9 ml) of carboxy-group-modified IgG-Sepharose. The column was washed with 10 mM-Tris/HCl, pH 7.6, containing 0.5 mM-EDTA and 70 mM-NaCl to collect four fractions (3 ml), and then the bound fragments were eluted with the same buffer containing 1 M-NaCl and 8 M-urea. The first two non-binding fractions were separately analysed by

was also noted that a significant amount of fragments was recovered in the second non-binding fraction (Fig. 3a), indicating that these fragments interacted with the column with a low affinity. A separate analysis using polyacrylamide-gel electrophoresis of the first and second non-binding fractions as well as of the bound fragments (Fig. 3b) revealed that: (i) most of the 16 kDa fragment was recovered in the first non-binding fraction; (ii) the 44 kDa and 24 kDa fragments were recovered in both first and second non-binding fractions, but not in the bound fraction; (iii) most of the 22 kDa fragment was eluted in the first and second non-binding fractions, but a small portion of it was also recovered in the bound fraction; and (iv) only the 155 kDa/145 kDa fragments were stably adsorbed on the column, although a proportion of them was also eluted in the second non-binding fraction. More of the 155 kDa fragment than of the 145 kDa fragment was recovered in the bound fraction.

Discussion

The experimental technique used here has the advantage of giving direct information about binding properties of various proteolytic fragments. However, as the affinity matrixes used may function as ion-exchangers, attention must be paid to the experimental conditions under which the binding tests are performed. In the present experiments, electrostatic effects resulting in non-selective binding of fragments to Sepharose-linked modified proteins were evident if buffers of low ionic strength were used. Selective binding was achieved by increasing the ionic strength of buffers close to the physiological level. Under these conditions, the relative strength of binding of various fragments could be compared, and the fragments could be assigned as non-binding, weakly binding or strongly binding.

Three high-molecular-mass heparin-binding fragments (180 kDa, 170 kDa and 150 kDa) were released with chymotrypsin from human fibronectin bound to gelatin-Sepharose (Fig. 1). Under similar experimental conditions, horse fibronectin has been cleaved to heparin-binding fragments of 160 kDa

polyacrylamide-gel electrophoresis. Note that a significant amount of fragments was eluted in the second non-binding fraction, i.e., after washing with more than three column volumes of buffer. (b) SDS/polyacrylamide-gel electrophoresis of non-binding and bound fragments. 1, Thermolysin digest; 2, fragments from the first non-binding fraction; 3, fragments from the second non-binding fraction; 4, bound fragments. The migration positions of the molecular-mass (m) standards are shown on the right.

and 135kDa (Ehrismann *et al.*, 1982). The size differences between our fragments and those obtained from horse fibronectin may reflect some minor differences between the domain structures of human and horse fibronectins. On the basis of the known specificity of chymotryptic cleavage of fibronectin (Ehrismann *et al.*, 1982) we are assuming that our high-molecular-mass fragments are derived from the C-terminal end of fibronectin polypeptide chains, and that they do not contain the gelatin-binding region. No binding to modified IgG was observed with the 46kDa fragment, which indicates that the gelatin-binding domain is not involved. The ability of the 180kDa and 170kDa fragments to bind to modified IgG suggests that the binding site is located on a segment between gelatin-binding site and C-terminus. The weak binding of the 150kDa fragment suggests that: (i) this fragment may contain only weak (non-specific) binding sites and, more importantly, that (ii) strong binding sites are located at least a length of a 150kDa segment from the gelatin-binding site towards the C-terminus. Because the 150kDa fragment bound strongly to heparin-Sepharose but only weakly to modified IgG, it seems likely that heparin-binding sites are not involved in binding of fibronectin to modified proteins.

These results were confirmed and extended by using digestion with thermolysin or trypsin. The relatively strong binding to modified IgG of the 145kDa and 155kDa fragments (Fig. 3) supports the idea that the binding site(s) could be located near the C-terminus. The fibrin-binding 24kDa peptide, previously shown (Sekiguchi & Hakomori, 1983) to be located in the N-terminal region, and the neighbouring 44kDa gelatin-binding fragment did not bind, which supports the above conclusions based on chymotryptic peptides. The C-terminal 22kDa fragment (Sekiguchi & Hakomori, 1983) could bind only weakly, suggesting that it could perhaps partially overlap with the region containing the binding site.

Recent results suggest that the two polypeptide chains of hamster (Sekiguchi *et al.*, 1981) and human (Sekiguchi & Hakomori, 1983) fibronectins are not completely identical, but that these fibronectins are heterodimers. In human fibronectin, the

portion of the polypeptide chain that is different in the two subunits is located within the 37kDa tryptic fragment close to the C-terminal end of the chain. The binding of this fragment, in addition to the high-molecular-mass tryptic fragments, to modified IgG (Fig. 2) confirms the above conclusions about the location of the binding site. It is noteworthy that the 37kDa fragment also contains the C-terminal fibrin-binding site of fibronectin (Sekiguchi & Hakomori, 1983).

The present results show that the ability to bind to carboxy-group-modified proteins is retained in specific proteolytic fragments of fibronectin. This activity resides in a domain different from those mediating the binding of fibronectin to gelatin or heparin. However, the binding site for carboxy-group-modified proteins could be located at, or close to, a fibrin-binding site. These results suggest that interactions of fibronectin with fibrin and carboxy-group-modified proteins could be mediated by a common binding site. This supports the idea that such a binding site could be related to the opsonin activity of plasma fibronectin.

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