Association of fibronectin with carboxy-group-modified proteins *in vitro*

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Treatment of human immunoglobulin G, albumin and fibronectin with water-soluble carbodi-imide at pH 4.75 in the presence of glycine ethyl ester resulted in an avid binding of ¹²⁵I-labelled native fibrinectin to the modified proteins. Succinylation, reduction and alkylation or heat-denaturation had no such effect. In affinity chromatography under physiological conditions, serum was depleted of fibronectin when run through columns of the carbodi-imide-treated proteins coupled to agarose. Fractions eluted from such columns with urea were enriched in fibronectin. The binding of radiolabelled fibronectin to the carbodi-imide-treated proteins was inhibited by unlabelled fibronectin in relatively low concentrations, but also by albumin in higher concentrations. Heat-denatured albumin inhibited at concentrations approx. 10–30 times lower than native albumin. The binding reaction had a pH optimum of 6–8. It was inhibited at high ionic strength and in the presence of urea. Anionic detergents inhibited at millimolar concentrations, but non-ionic detergents did not inhibit the binding reaction. The results were interpreted as showing that: (1) fibronectin is capable of binding to itself, to immunoglobulin G and to albumin after a reduction of the negative surface charge of these proteins, and may have a general ability to bind such modified proteins; (2) this binding can take place under physiological conditions; (3) carboxy-group-modified proteins selectively bind fibronectin from serum. This novel binding phenomenon could be important in terms of the opsonin function of circulatory fibronectin. We propose that fibronectin may recognize modified (denatured) proteins and mediate their uptake by the reticuloendothelial system.

Fibronectin is a high-molecular-weight glycoprotein that is present in a soluble form in connective tissue and on the surface of cells grown *in vitro*, but also as a soluble protein in blood [for fairly recent reviews, see Mosher (1980) and Ruoslahti *et al.* (1981)]. A characteristic property of fibronectin is that it binds to a variety of macromolecular ligands. These include proteins such as collagen (Engvall & Ruoslahti, 1977), fibrin (Stemberger & Hörmann, 1976), actin (Keski-Oja *et al.*, 1980), acetylcholinesterase (Emmerling *et al.*, 1981), complement factor C1q (Menzel *et al.*, 1981) and serum amyloid P component (de Beer *et al.*, 1981). Fibronectin also has a tendency for self-association (Vuento *et al.*, 1980a). The reported non-protein ligands of fibronectin include glycosaminoglycans (Yamada *et al.*, 1980), deoxyribonucleic acid (Zardi *et al.*, 1979) and polyamines (Vuento & Vaheri, 1978). Fibronectin also displays haemagglutinin activity (Yamada *et al.*, 1975; Vuento, 1979), which may be mediated by binding to carbohydrate moieties

(Vuento & Salonen, 1981). Binding of fibronectin to bacteria has been reported (Kuusela, 1978).

At least some of these interactions are considered to be physiologically important. The interactions of fibronectin with itself and with collagen, glycosaminoglycans and cell surfaces have been suggested to play a role in the organization of the extracellular macromolecular matrix in cultures of cells grown *in vitro* and in connective tissue (reviewed by Ruoslahti *et al.*, 1981). Experimental and clinical evidence indicates that circulatory fibronectin serves as an opsonizing protein, facilitating removal of various harmful materials from circulation by phagocytic cells (reviewed by Saba & Jaffe, 1980). For this opsonizing function, binding of fibronectin to fibrin, collagen, actin and C1q could be important.

We describe here yet another binding reaction of fibronectin. Fibronectin binds avidly and specifically to itself, to immunoglobulin G and to albumin when these proteins are treated with reagents that specifically modify carboxy groups. We propose that

fibronectin has a general property of recognizing and binding to modified (denatured) proteins. This could be significant in terms of the opsonin function of fibronectin. These results may also explain at least part of the diversity of the previously recognized binding reactions of fibronectin.

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Materials and methods

Purification of fibronectin

Fibronectin was purified from citrated human plasma as described previously (Vuento & Vaheri, 1979). The purity of the material was controlled by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate (see below). Concentrations of solutions of purified fibronectin were determined by photometry, assuming $A_{280.1\text{cm}}^{1\%} = 12.8$ (Mosesson *et al.*, 1975). Other protein determinations were carried out by the method of Lowry *et al.* (1951).

Radiolabelling of fibronectin

Fibronectin was labelled with ^{125}I by lactoperoxidase-catalysed iodination (Marchalonis, 1969). The concentrations of the reagents were adjusted to give an incorporation of one atom of iodine per subunit of fibronectin. The degree of incorporation of ^{125}I into fibronectin was determined by gel chromatography on a column (0.5 cm $\times$ 19 cm) of Bio-Gel P-2 (Bio-Rad, Richmond, CA, U.S.A.) equilibrated with 10 mM-Tris/HCl (pH 7.5)/0.9% NaCl/0.02% NaN_3 /bovine serum albumin (5 mg/ml). The column was calibrated with Blue Dextran (Pharmacia, Uppsala, Sweden) and with potassium chromate. Usually all of the applied radioactivity was eluted in the void volume, indicating complete incorporation of ^{125}I into fibronectin. The specific radioactivity of the labelled protein was calculated from the degree of incorporation and from the amounts of ^{125}I and of fibronectin present in the reaction mixture. The specific radioactivity was approx. $10\mu\text{Ci}/\mu\text{g}$. The labelled protein was stored at $+4^\circ\text{C}$ in the above buffer.

Coupling of proteins to agarose

Sepharose 4B (Pharmacia, Uppsala, Sweden) was activated with CNBr by the method described for 'intermediately activated 1–8% agarose gels' by Porath & Kristiansen (1975). The coupling reaction was performed in 0.5 M-NaCl/0.1 M- NaHCO_3 . Approx. 5 mg of protein were used/ml of the activated gel. The gel suspension was mixed by magnetic stirring for 16 h at room temperature, after

which the gel was first washed with the coupling buffer (500 ml/g of gel) and then with Tris-buffered saline [10 mM-Tris/HCl (pH 7.5)/0.9% NaCl/0.02% NaN_3] (500 ml/ml of gel). The amount of protein bound to the gel was calculated from the difference in protein concentrations between the original protein solution and the supernatant remaining after the coupling reaction was completed. Many of the experiments described below were carried out with immobilized proteins further modified chemically. In these instances, suspensions of protein-Sepharose were treated with the modifying reagents, which were then removed by washing the gels with the above Tris buffer.

Modification of carboxy groups

Carboxy groups were modified with 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide (Sigma, St. Louis, MO, U.S.A.) at pH 4.75 in the presence of ethylenediamine or glycine ethyl ester (Carraway & Koshland, 1972). Sepharose gels containing the immobilized proteins were washed with 0.9% NaCl, and the suspension was adjusted to pH 4.75 and to a 1 M concentration of the nucleophile. The mixture was stirred on a magnetic stirrer. Carbodi-imide was added to a final concentration of 0.1 M, and the pH was kept at 4.75 by adding dilute HCl. After an incubation time of 1 h, the reagents were removed by washing the gel with Tris-buffered saline. Soluble proteins were modified under similar conditions by using a protein concentration of 1 mg/ml. The reagents were removed by dialysis against Tris-buffered saline.

Modification of amino groups by succinylation

Immobilized proteins were treated with succinic anhydride in order to modify free amino groups (Klotz, 1967). A 1 ml portion of the gel was suspended in 10 ml of 0.9% NaCl and stirred with a magnetic stirrer. Solid succinic anhydride (10 mg) was added in small portions during 1 h, and pH was kept at 7.0–8.0 by adding 0.2 M- Na_2CO_3 . The reagents were removed by washing the gels as described above.

Reduction and carboxymethylation

For reduction and alkylation, the immobilized proteins (1 ml) were suspended in 10 ml of 8 M-urea in 50 mM-Tris/HCl, pH 7.5, containing 1 mM-EDTA. The suspension was stirred with a magnetic stirrer. The reducing agent β -mercaptoethanol (30 μl) was added. After an incubation time of 2 h, 300 mg of iodoacetic acid were added, and the suspension was further incubated for 1 h. The reagents were removed by washing the gels.

Heat-denaturation

Immobilized proteins suspended in 0.9% NaCl

were incubated at 75°C for 30 min, and then rapidly cooled. Human serum albumin in solution was denatured at 75°C for 30 min at a concentration of 1 mg/ml. Albumin remained soluble during this treatment.

Solid-phase radioassay for binding of ^{125}I -labelled fibronectin to immobilized proteins

Binding of ^{125}I -labelled fibronectin to various proteins was studied using a solid-phase radioassay employing immobilized derivatives of the proteins. This type of assay has been previously used by us in studies on fibronectin–collagen interaction (Vuento *et al.*, 1982). Portions of the immobilized proteins (20 μl) were incubated in plastic tubes (7 mm $\times$ 50 mm) with labelled fibronectin. The volume of the incubation mixture was 1.0 ml. Bovine serum albumin was added to the various buffers used to a concentration of 1 or 5 mg/ml. This was necessary in order to prevent the binding of the labelled protein to tube walls (see Fig. 1 below). An incubation time of 16–20 h was sufficient for the binding reactions to reach equilibrium. After this incubation time, the gel was sedimented by centrifugation at 1000 *g* for 1 min and the supernatant was carefully removed by suction. The gel was rapidly washed once with 1 ml of buffer. The tubes with their contents were counted for radioactivity in a scintillation counter. Control incubations were carried out with plain Sepharose gel. In some experiments dealing with the effect of various reagents on binding, the results were calculated as inhibition of binding. This was done as follows. Background binding (the amount of radioactivity in c.p.m. bound to the control Sepharose 4B gel) was first subtracted from the data, and then the following equation was used:

$$\text{inhibition (\%)} = \frac{\text{c.p.m.}_{\text{max.}} - \text{c.p.m.}_{\text{sample}}}{\text{c.p.m.}_{\text{max.}}} \times 100$$

where c.p.m._{max.} refers to radioactivity bound to the gel when no inhibitory compounds were present.

Affinity chromatography

Pooled human serum was obtained from blood of ten healthy volunteers (blood was taken with informed consent). Affinity chromatography was performed on columns (5 ml; 1 cm $\times$ 6.5 cm) of agarose-coupled proteins. The columns were equilibrated with Tris-buffered saline. Samples of pooled serum (2.5 ml) were applied and the columns were eluted with the above buffer until the A_{280} of the eluting material was less than 0.01. The columns were then eluted with 8 M-urea in the above buffer. Fractions eluted with urea were dialysed against the above Tris-buffered saline. The protein concentration of the fractions was determined, and portions were further analysed by immunodiffusion analysis

and polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate.

Immunodiffusion

Immunodiffusion analysis was performed in 1% agarose buffered with 10 mM-Tris/HCl (pH 7.5)/0.9% NaCl/merthiolate (0.1 mg/ml). Antiserum to human plasma fibronectin used in the present study was described previously (Vuento *et al.*, 1980b).

Polyacrylamide-gel electrophoresis in dodecyl sulphate

Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate was performed in vertical gel slabs (6% acrylamide/0.2% bisacrylamide) with the discontinuous buffer system of Laemmli (1970). The proteins used for the calibration of the gels (all chemically reduced with β -mercaptoethanol) were: fibronectin (M_r 220 000), β -galactosidase (Worthington, Freehold, NJ, U.S.A.; M_r 130 000), phosphorylase *a* (Worthington; M_r 100 000), bovine serum albumin (Sigma; M_r 68 000), chicken ovalbumin (Sigma; M_r 43 000) and deoxyribonuclease I (Worthington; M_r 31 000). The gels were stained with Serva Blue R (Serva, Heidelberg, Germany).

Gel chromatography

Gel-chromatography experiments were carried out on a column (1 cm $\times$ 94 cm) of Sepharose 4B equilibrated with Tris-buffered saline. The column had a void volume of 24 ml as determined with Blue Dextran.

Reagents

Human serum albumin and human immunoglobulin G were from Kabi (Stockholm, Sweden). Possible contaminating fibronectin present in these preparations was removed by passing solutions of these proteins through columns of gelatin–agarose (Vuento *et al.*, 1980c). Soya-bean trypsin inhibitor was from Sigma. Human α_1 -acid glycoprotein was prepared from pooled serum as described by Whitehead & Sammons (1966). Triton X-100, Lubrol WX, Tween 20, Tween 80 and sodium deoxycholate were obtained from Sigma. Sodium dodecyl sulphate was obtained from Fluka (Switzerland) and used after recrystallization from ethanol. Other reagents were obtained from commercial suppliers and were of analytical grade.

Results

Binding of ^{125}I -labelled fibronectin to native fibronectin and to carboxy-group-modified proteins

The binding of ^{125}I -labelled fibronectin to various proteins coupled to agarose was studied by using the solid-phase radioassay. The immobilized proteins

tested included fibronectin itself, serum albumin, immunoglobulin G, α_1 -acid glycoprotein and soya-bean trypsin inhibitor (Tables 1–3). When the immobilized proteins were tested without further chemical modification, only fibronectin–Sephacrose was found to bind labelled fibronectin significantly. This self-association of fibronectin was abolished by heat-denaturation, by succinylation, and also weakened by reduction and alkylation (Table 1).

The binding of labelled fibronectin to fibronectin–Sephacrose was enhanced by treatment of the immobilized protein with carbodi-imide (Table 1). Also, immobilized immunoglobulin G and albumin bound avidly labelled fibronectin after this treatment (Tables 2 and 3).

In contrast with the above results, treatment of immobilized soya-bean trypsin inhibitor or α_1 -acid glycoprotein with carbodi-imide did not cause these proteins to bind labelled fibronectin significantly (Table 1). An incubation of the carbodi-imide-treated proteins with 0.5 M-hydroxylamine at pH 7.5 for 16 h had only a slight effect on the binding of labelled fibronectin to immunoglobulin G and to albumin, but no effect at all on binding to fibronectin (Tables 1–3).

Binding of 125 I-labelled fibronectin to carboxy-group-modified proteins: inhibition by fibronectin, immunoglobulin G, albumin and by some derivatives of these proteins

In the experiments with the solid-phase radioassay, bovine serum albumin was added to the incubation media to prevent binding of the labelled fibronectin to the walls of the plastic tubes. This effect is shown in Fig. 1, which also shows the effect of added albumin to the binding of labelled fibronectin to various carboxy-group-modified proteins. The binding of 125 I-labelled fibronectin to plastic tubes was decreased to a constant low level by addition of bovine serum albumin (1–5 mg/ml) to the buffers. Increasing concentrations of bovine albumin also inhibited the binding of labelled fibronectin to carboxy-group-modified proteins (Fig. 1). This effect was more marked with modified immunoglobulin G and albumin than with modified fibronectin.

In the experiments shown in Table 4, the incubation buffer contained 1 mg of bovine serum albumin/ml and various concentrations of unlabelled fibronectin, immunoglobulin G, human albumin and

Table 1. *Binding of 125 I-labelled fibronectin to native and chemically modified fibronectin coupled to Sepharose*
The assay was carried out by incubating labelled fibronectin (5 ng, 25 000 c.p.m.) with portions of the indicated gels in 50 mM-Tris/HCl, pH 7.5, containing 0.02% NaN₃ and 5 mg of bovine serum albumin/ml. The total volume of the incubation mixture was 1.0 ml. The volume of the gel was 20 μ l. In some experiments the gels containing immobilized proteins were diluted with 'plain' Sepharose 4B gel. The tubes were mixed by slow end-over-end rotation at room temperature. After an incubation time of 16 h, the gel beads were sedimented by centrifugation, washed, and analysed for bound radioactivity. The chemical-modification reactions are described in the Materials and methods section. The treatment of immobilized carboxy-group-modified fibronectin with hydroxylamine was performed at pH 7.5 and at room temperature with 0.5 M-hydroxylamine and an incubation time of 16 h. The fibronectin–Sephacrose gel and its modified derivatives contained 1.7 mg of bound fibronectin/ml of gel. The immobilized soya-bean trypsin inhibitor and α_1 -acid glycoprotein, which are included in the Table as controls, contained 0.4 and 0.1 mg of protein/ml of gel respectively. Abbreviation used: n.d., not determined.

Gel	Modification	Gel dilution (μ l) ...	125 I-labelled fibronectin bound (% of applied radioactivity)	
			1:20	1:1
A. Fibronectin–Sephacrose	(i) None		5	25
	(ii) Heat-denaturation		2	3
	(iii) Succinylation		2	6
	(iv) Reduction and alkylation		2	17
	(v) Incubation at pH 4.75 for 1 h		4	21
	(vi) Carbodi-imide at pH 4.75 with glycine ethyl ester		69	80
	(vii) Carbodi-imide as in (vi) above, then 0.5 M-hydroxylamine		n.d.	80
B. Sepharose 4B	None		n.d.	2
	Carbodi-imide as in A(vi) above		n.d.	2
C. Soya-bean trypsin inhibitor–Sephacrose	None		n.d.	2
	Carbodi-imide as in A(vi) above		n.d.	2
D. α_1 -Acid glycoprotein– Sephacrose	None		n.d.	2
	Carbodi-imide as in A(vi) above		n.d.	6

Table 2. *Binding of ^{125}I -labelled fibronectin to chemically modified immobilized immunoglobulin G*

The experiments were carried out as described in the Materials and methods section and in the legend to Table 1. The diluent buffer was 10 mM-Tris/HCl (pH 7.5)/0.9% NaCl/0.02% NaN_3 , containing 1 mg of bovine serum albumin/ml. The immunoglobulin G-Sepharose used contained 1.5 mg of bound protein/ml of gel. Abbreviation used: n.d., not determined.

Modification	Gel dilution (μl) ...	^{125}I -labelled fibronectin bound (% of applied radioactivity)	
		1:20	1:1
None		n.d.	7
Heat-denaturation		n.d.	9
Succinylation		n.d.	4
Reduction and alkylation		n.d.	9
Carbodi-imide at pH 4.75 with glycine ethyl ester		24	80
Carbodi-imide as above, then 0.5 M-hydroxylamine		n.d.	69

Table 3. *Binding of ^{125}I -labelled fibronectin to chemically modified immobilized human serum albumin*

The assay was carried out as described in the Materials and methods section and in the legend to Table 1. The diluent buffer was 10 mM-Tris/HCl (pH 7.5)/0.9% NaCl/0.02% NaN_3 , containing 1 mg of bovine serum albumin/ml. The albumin-Sepharose used in these experiments contained 0.6 mg of bound albumin/ml of gel. Abbreviation used: n.d., not determined.

Modification	Gel dilution (μl) ...	^{125}I -labelled fibronectin bound (% of applied radioactivity)	
		1:20	1:1
None		n.d.	4
Heat-denaturation		n.d.	6
Succinylation		n.d.	3
Reduction and alkylation		n.d.	4
Carbodi-imide at pH 4.75 with glycine ethyl ester		1	45
Carbodi-imide as above, then 0.5 M-hydroxylamine		n.d.	31

derivatives of these proteins. Fibronectin strongly inhibited the binding of ^{125}I -labelled fibronectin to carboxy-group-modified proteins, giving a 50% inhibition at a concentration of 10 $\mu\text{g}/\text{ml}$ with carboxy-group-modified fibronectin-Sepharose and immunoglobulin G-Sepharose or 60 $\mu\text{g}/\text{ml}$ with carboxy-group-modified albumin-Sepharose. Immunoglobulin G inhibited the binding reactions only at concentrations in excess of 10 mg/ml. However, carboxy-group-modified soluble immunoglobulin G inhibited strongly. A 50% inhibition was obtained with 50 μg of carboxy-group-modified immunoglobulin G/ml. Native human serum albumin was a more potent inhibitor in the assay than immunoglobulin G, giving a 50% inhibition in concentrations of approx. 2 mg/ml (with carboxy-group-modified fibronectin-Sepharose and albumin-Sepharose) or 0.8 mg/ml (with carboxy-group-modified immunoglobulin G-Sepharose). It appears that human albumin was more inhibitory than bovine albumin (Fig. 1). This inhibitory activity of albumin was enhanced by heat-denaturation of albumin or by

carboxy-group modification of the soluble protein. Thus heat-denatured albumin gave a 50% inhibition in concentrations approx. 10 times lower than those of native albumin (Table 4).

Affinity chromatography of human serum on columns of immobilized carboxy-group-modified fibronectin, immunoglobulin G and albumin

After observing the inhibitory effect of albumin in the radioassay experiments (Fig. 1, Table 4) we decided to test whether the binding of fibronectin to carboxy-group-modified proteins could take place under physiological conditions, that is, in the presence of albumin and other serum proteins, and at physiological pH and ionic strength. We thought it equally worthwhile to study whether or not fibronectin was specifically bound from serum by the carboxy-group-modified proteins. In affinity chromatography of human serum on carboxy-group-modified fibronectin-Sepharose, fibronectin was completely bound from serum to the column, as judged by immunodiffusion analysis (Fig. 2).

Fig. 1. Effect of bovine serum albumin on the binding of ^{125}I -labelled fibronectin to plastic tubes and to carboxy-group-modified Sepharose-linked proteins

The experiment was carried out as described in the Materials and methods section. The diluent buffer used was 10 mM-Tris/HCl (pH 7.5)/0.9% NaCl/0.02% NaN_3 , to which bovine serum albumin had been added to the indicated concentrations. The curves shown indicate the binding of labelled fibronectin, expressed as a percentage of applied radioactivity, to plastic tubes (○), carboxy-group-modified fibronectin-Sepharose (Δ), carboxy-group-modified immunoglobulin G-Sepharose (▽) and carboxy-group-modified albumin-Sepharose (□).

Immunoreactive fibronectin was present in the fractions eluted from the column with urea (Fig. 2). Similar results were obtained with carboxy-group-modified immunoglobulin G-Sepharose (results not shown). Carboxy-group-modified albumin also bound fibronectin from serum, but the binding was not complete under the conditions used in this experiment. Protein determinations showed that about 1.5–2.5 mg of protein were eluted from the columns of carboxy-group-modified fibronectin-Sepharose, immunoglobulin and albumin-Sepharose, whereas only 0.2 mg of protein was eluted from columns of the native proteins coupled to Sepharose. Fibronectin-Sepharose was also tested by affinity chromatography after succinylation, reduction and alkylation or heat-denaturation. In these cases the amount of protein bound to, and eluted from, the columns was approx. 5–10% of the amount eluted from carboxy-group-modified fibronectin.

An electrophoretic analysis of the material eluted from the columns of carboxy-group-modified immobilized fibronectin, immunoglobulin G and albumin is shown in Fig. 3. The analysis revealed a

major polypeptide band having a migration rate identical with that of fibronectin. This band was missing or strongly reduced when serum was passed through a column of gelatin-Sepharose before affinity chromatography on columns of carboxy-group-modified proteins (Fig. 3). It was concluded that most or all of the material with M_r 220 000 was fibronectin. In addition to this polypeptide band, a group of prominent bands having apparent M_r in the range of 60 000–90 000 was consistently present. In the material eluted from carboxy-group-modified albumin-Sepharose, these bands (M_r 60 000–90 000) were relatively stronger than the fibronectin band (Fig. 3).

Effect of pH on the binding reaction between fibronectin and carboxy-group-modified proteins

With the object of obtaining information about the nature of molecular interactions involved in the binding reactions, we characterized the effects of various experimental conditions on the binding. These experiments were performed by using the solid-phase radioassay. The effect of pH was studied by using a series of buffer systems having buffering capacity in the pH range 2–11. All experiments were carried out at room temperature. Fig. 4 shows the results obtained with carboxy-group-modified fibronectin-Sepharose. It was found that the binding had a distinct pH optimum of 6–8, with a rapid decrease of binding on both sides of the optimal range. There was a slight increase of binding at pH 3.0, but binding to control Sepharose gel was also increased at this pH value. There were differences between the various buffer systems. Thus a higher binding was obtained at pH 5.5 in phosphate buffer than in citrate buffer. Similar results were obtained with carboxy-group-modified immunoglobulin G-Sepharose. Carboxy-group-modified albumin-Sepharose showed a slightly narrower pH optimum of 6.0–7.5 (results not shown).

Effect of ionic strength

The effect of ionic strength was studied by increasing the concentration of NaCl in the incubation mixture. The binding of ^{125}I -labelled fibronectin to the carboxy-group-modified proteins was inhibited by NaCl. However, there were differences between the various proteins. NaCl at 100 mM inhibited the binding of labelled fibronectin to modified fibronectin-Sepharose 5%, 250 mM-NaCl inhibited 29%, 500 mM-NaCl inhibited 53% and 1 M-NaCl inhibited 57%. In contrast with this finding, binding of labelled fibronectin to modified immunoglobulin G and albumin was more sensitive to increase in ionic strength, being almost completely inhibited by 0.5 M-NaCl and more than 70% inhibited by 0.25 M-NaCl.

Table 4. Inhibition of binding of ¹²⁵I-labelled fibronectin to immobilized carboxy-group-modified fibronectin, immunoglobulin G and albumin by some human proteins

The experiments were carried out by using the solid-phase radioassay method described in the Materials and methods section. Diluent buffer was 10 mM-Tris/HCl (pH 7.5)/0.9% NaCl/0.02% NaN₃, containing 1 mg of bovine serum albumin/ml, to which buffer the various proteins tested were added. The concentrations of the proteins tested (μg/ml) giving a 50% inhibition of binding are given. The calculation of inhibition of binding is defined in the Materials and methods section. The concentration of radiolabelled fibronectin was 5 ng/ml. Abbreviation used: n.d., not determined.

Inhibitor	Concentration (μg/ml) giving 50% inhibition of binding of ¹²⁵ I-labelled fibronectin to the following carboxy-group-modified immobilized proteins:		
	Fibronectin	Immunoglobulin G	Albumin
Fibronectin	10	10	60
Albumin			
Untreated	2300	790	2300
Heat-denatured	290	70	240
Carboxy-group-modified	290	320	n.d.
Immunoglobulin G			
Untreated	>30 000	29 000	20 000
Carboxy-group-modified	50	50	n.d.

Fig. 2. Ouchterlony immunodiffusion analysis The centre well contains anti-fibronectin antiserum (20 μl); 1, human serum (20 μl); 2, protein eluted from carboxy-group-modified fibronectin-Sepharose with urea (0.8 mg/ml, 20 μl); 3, serum passed through a column of carboxy-group-modified fibronectin-Sepharose and concentrated to the original volume (20 μl).

Fig. 3. Dodecyl sulphate/polyacrylamide-gel electrophoresis

1, Human serum (0.1 μl); 2, proteins eluted from carboxy-group-modified albumin-Sepharose (20 μg); 3, proteins eluted from carboxy-group-modified immunoglobulin G-Sepharose (20 μg); 4, proteins eluted from carboxy-group-modified fibronectin-Sepharose (20 μg); 5, as 4, but serum depleted of fibronectin before affinity chromatography on carboxy-group-modified fibronectin-Sepharose. Approx. 11 μg of protein were applied to gel. Migration was from top to bottom. The Figure combines results from different electrophoresis runs. The migration positions of M_r markers are shown by arrows on the right. They apply closely to lanes 4–5, but only approximately to lanes 1–3.

Effect of bivalent cations

The effect of bivalent cations was studied by adding CaCl₂, MgCl₂ or EDTA to the assay mixture to a final concentration of 1 mM. These salts had no effect on the binding of labelled fibronectin to

Fig. 4. Effect of pH on the binding of ^{125}I -labelled fibronectin to carboxy-group-modified fibronectin-Sephadex

Portions of carboxy-group-modified fibronectin (20 μl) were equilibrated with ^{125}I -labelled fibronectin for 16 h at room temperature in various buffers (0.98 ml). The concentration of the buffers was 50 mM. All buffers contained 1 mg of bovine serum albumin/ml. The buffer systems used were glycine/HCl, pH 2.0–3.0 (○), sodium citrate, pH 3.0–5.5 (●), potassium phosphate, pH 5.5–8.0 (△), Tris/HCl, pH 7.0–9.5 (▲) and glycine/NaOH, pH 8.5–11.0 (■); Sephadex 4B gel was used as a control (□).

carboxy-group-modified fibronectin, immunoglobulin G or albumin.

Effect of urea and detergents

We also tested the effect of urea and detergents on the binding reactions. Binding was completely inhibited by 4 M-urea and significantly inhibited by 1–2 M-urea. The non-ionic detergents Triton X-100, Lubrol WX, Tween 20 and Tween 80 did not inhibit the binding reactions at concentrations of up to 5%. However, they did interfere with the binding in some experiments with carboxy-group-modified albumin-Sephadex, since the binding of labelled fibronectin to this protein was enhanced 2-fold by Lubrol WX, Tween 20 and by Tween 80. The anionic detergents sodium dodecyl sulphate and sodium deoxycholate were strongly inhibitory, even at concentrations as low as 1–10 mM.

Gel chromatography

Attempts to determine the size of the complexes between ^{125}I -labelled fibronectin and carboxy-group-modified proteins by gel chromatography were unsuccessful. Labelled fibronectin alone eluted from the column of Sephadex 4 B as a single symmetrical peak in a volume of $1.8 \times V_0$. When fibronectin was incubated with the carboxy-group-modified proteins before chromatography, only 20% of the radioactivity eluted in control experiments was

recovered from the column, and this eluted later than control fibronectin (results not shown). These results suggested adsorption of the complexes to the column.

Discussion

We have shown that fibronectin binds *in vitro* to carboxy-group-modified fibronectin, immunoglobulin G and albumin. This phenomenon was not recognized previously, although some studies dealing with chemical modification of fibronectin have been published (Grinnell & Minter, 1979; Gold & Pearlstein, 1979; Vuento & Salonen, 1981; Vuento *et al.*, 1982). We also demonstrated a direct association of fibronectin with itself. This self-association is in line with the previously observed polymerization tendency of fibronectin (Vuento *et al.*, 1980a).

More than half of the proteins tested could be induced to bind to fibronectin by carboxy-group modification. Fibronectin, immunoglobulin G and albumin were selected for the present study because they represent major plasma and serum proteins. On the other hand, some of the proteins could not be induced to bind fibronectin by carboxy-group modification, e.g. soya-bean trypsin inhibitor and α_1 -acid glycoprotein (Table 1).

The conditions of the modification reaction with carbodi-imide, that is, pH 4.75 and the presence of 1 M-glycine ethyl ester, are known to give rise to a specific modification of carboxy groups (Carraway & Koshland, 1972). A modification of tyrosine residues is possible, but this is reversible upon incubation with hydroxylamine (Carraway & Koshland, 1972). The failure of hydroxylamine to reverse the binding (Tables 1–3) suggests that a modification of tyrosine residues has not contributed to the observed binding reactions. Modification of thiol groups by carbodi-imide is probably not either an important factor. Fibronectin contains less than 0.5 free thiol groups per molecule (Chen *et al.*, 1977). Although albumin and immunoglobulin G may contain free thiol groups, these would be blocked during reduction and alkylation. This modification did not give rise to binding of fibronectin to immunoglobulin G or albumin (Tables 2 and 3).

Our attempts to clarify the mechanism by which modified proteins bind fibronectin did not give conclusive results. However, it is evident that: (1) the binding takes place at physiological pH and ionic strength; and (2) that different proteins vary in their binding. The binding can be inhibited by high and low pH and by high concentrations of salt, but also by albumin, urea and detergents, which leaves the binding mechanism open to speculation.

It is not evident how the described binding relates to the previously described binding of fibronectin to

collagen, fibrin, cell surfaces, glycosaminoglycans etc. (Ruoslahti *et al.*, 1981). However, some of these binding reactions have been reported to proceed more readily when the ligand is in a denatured or modified state. Thus denatured collagen binds more strongly to fibronectin than does native collagen (Engvall & Ruoslahti, 1977; Jilek & Hörmann, 1978). The same is true for actin (Koteliansky *et al.*, 1981). Binding of fibronectin to fibrin monomers, which are proteolytic derivatives of fibrinogen, is also more effective than binding to fibrinogen (Stemberger & Hörmann, 1976). Conformational factors may also be important in the binding of fibronectin to C1q (Menzel *et al.*, 1981). These findings, together with the results reported here, suggest that fibronectin has the ability to recognize denatured or otherwise modified proteins. This property should be viewed against the well-documented function of plasma fibronectin as an opsonin that mediates removal of debris from circulation. For this function the ability to selectively recognize and bind denatured proteins would be of prime importance.

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