Isolation and characterization of rat plasma fibronectin

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Rat plasma fibronectin has been isolated and characterized and monospecific antibodies were prepared to it. Two components of fresh rat plasma (in the presence of proteinase inhibitors) bound to a gelatin–Sephadex affinity column. One protein was eluted with 4.0M-urea and was identified as fibronectin. Another protein was eluted from the gelatin–Sephadex column with 8.0M-urea and was identified as a 70000- M_r collagen-binding molecule. This 70000- M_r fragment was found to be a normal constituent of blood plasma, and its presence did not represent a proteolytic degradation product formed during isolation. The antibodies prepared against rat fibronectin only weakly cross-reacted with plasma fibronectins of chicken, horse and human. These studies shed light on the metabolic interrelationships between fibronectin and other collagen-binding molecules.

Fibronectin is a pericellular glycoprotein involved in extracellular matrix–cell interaction (Hynes, 1976; Vaheri *et al.*, 1978; Yamada & Olden, 1978; Kleinmann *et al.*, 1979; Pearlstein *et al.*, 1980). A structurally related and immunologically cross-reactive protein, plasma fibronectin, occurs in the circulating blood plasma and was previously known as ‘cold-insoluble globulin’ (Mosesson, 1978; Yamada & Kennedy, 1979). During the course of our investigation of collagenous matrix–cell interactions *in vivo* (Reddi, 1976), we described the importance of fibronectin in the early stages of mesenchymal-cell proliferation (Weiss & Reddi, 1980, 1981). We realized that despite the use of the rat in numerous experimental studies on matrix–cell interactions, there was a paucity of information on the chemical characterization of rat fibronectin. In view of this we have isolated and characterized the rat plasma fibronectin.

Materials and methods

Isolation of plasma fibronectin

Gelatin–Sephadex chromatography. Pig skin gelatin (Sigma) was coupled to Sephadex 4B

Abbreviations used: SDS, sodium dodecyl sulphate; ELISA, enzyme-linked immunoabsorbent assay; IgG, immunoglobulin G.

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(Pharmacia) at 56°C in 1.0M-NaCl/0.5M- H_3BO_3 , pH9.0 to prepare the gelatin–Sephadex affinity column (as described by Engvall & Ruoslahti, 1972). The pig skin gelatin was dissolved in the above buffer at 56°C (1mg/ml) and then centrifuged to remove undissolved particles. The Sephadex 4B was activated by pretreating with CNBr (Cuatrecasas, 1970). The collagen solution was mixed with the gel slurry for 3h at 56°C. The reaction was stopped with 1.0M-ethanolamine, pH8.0, for 2h. The gelatin–Sephadex was then washed alternately with 0.1M-sodium acetate, pH4, and 0.1M- H_3BO_3 , pH9, for four cycles. The gel was then equilibrated with 0.02M- NaH_2PO_4 /0.15M-NaCl/0.5mm-phenylmethanesulphonyl fluoride before use. Freshly obtained rat plasma, with EDTA as an anticoagulant, and chilled on ice immediately after exsanguination, was applied to the gelatin–Sephadex column (4 × 25 cm; 75 ml of plasma). Phenylmethanesulphonyl fluoride (0.5mm final concn.) was used with EDTA in blood-collection tubes. The eluant was monitored for protein by absorbance at 280nm. The column was washed with 5 vol. of 1.0M-urea/0.05M-Tris/HCl/0.02M- NaH_2PO_4 /0.15M-NaCl/0.5mm-phenylmethanesulphonyl fluoride, pH7.4. Fibronectin was eluted with 2 column vol. of 4.0M-urea/0.02M- NaH_2PO_4 /0.15M-NaCl/0.05M-Tris/HCl/0.5mm-phenylmethanesulphonyl fluoride, pH7.4. All chromatography was performed at 4°C. The column was then washed with 8.0M-urea/0.02M- NaH_2PO_4 /0.15M-NaCl/0.05M-Tris/HCl/0.5mm-

phenylmethanesulphonyl fluoride, pH 7.4, to remove all tightly bound components.

Gel filtration. The peak that was eluted by 4.0M-urea from the gelatin–Sepharose affinity column was dialysed against 20 vol. of water at 4°C for 48 h and was then rechromatographed on Sepharose 4B-CL (0.9 cm × 90 cm column) with an eluting buffer containing 0.02M-NaH₂PO₄/0.15M-NaCl/0.5 mM-phenylmethanesulphonyl fluoride, pH 7.4 at 22°C. The proteins were monitored for A_{280} .

Gel electrophoresis. Samples were subjected to 0.1% SDS/5%-polyacrylamide-gel electrophoresis (Laemmli, 1970), vertical slab gels (1.5 mm thick) being used. Samples were heated in a boiling-water bath for 5 min with 0.1M- β -mercaptoethanol, 0.1% SDS and glycine/Tris electrode buffer, pH 8.8.

Amino acid analysis. Samples were dissolved in constant-boiling 6M-HCl in a N₂ atmosphere and then hydrolysed at 106°C for 24 h.

Preparation of antibodies

Production of the antiserum. The freeze-dried preparation eluted by 4M-urea from the gelatin–Sepharose columns was treated for 5 min in a boiling-water bath in sample buffer (0.1M- β -mercaptoethanol/0.1% SDS/1.0% glycerol/glycine/Tris/HCl electrode buffer, pH 8.5), and then subjected to preparative 0.1% SDS/5%-polyacrylamide-gel electrophoresis. Gel slices containing fibronectin were then homogenized with incomplete Freund's Adjuvant and injected into male New Zealand rabbits. Booster injections were given 2 and 4 weeks later.

Isolation of purified antifibronectin antibodies. The freeze dried preparation eluted by 4.0M-urea, containing the fibronectin, was further purified by preparative electrophoresis. This purified fibronectin, from the gelatin–Sepharose column, was coupled to CNBr-activated Sepharose 4B at 4°C in 0.5M-H₃BO₃/1.0M-NaCl for 15 h with gentle agitation. The antiserum (20 ml) was applied to the fibronectin–Sepharose affinity column (1.5 cm × 12 cm) at 4°C. The column was washed with buffer containing 0.002% NaN₃, 0.05M-Tris/HCl and 0.15M-NaCl, pH 7.5. The eluant was monitored for protein by absorbance at 280 nm. The fibronectin specific antibody was eluted with 3.0M-KSCN, 0.02M-NaH₂PO₄, pH 6.0. The antibody was then immediately dialysed against phosphate-buffered saline (0.02M-NaH₂PO₄/0.15M-NaCl) at 4°C.

Antibody cross-reactivity

The antibody specificity was monitored by the Ouchterlony immunodiffusion technique. The antibody was placed in the centre well whereas soluble rat fibronectin preparations, and rat Types I and II collagens, rat fibrin and rat albumin were placed in the surrounding wells.

The species specificity of the anti-(rat fibronectin) antibody was tested by comparing horse, chicken and human fibronectins. Sera of the animals were commercially obtained (Gibco) and the human serum from the National Institutes of Health blood bank, subjected to gelatin–Sepharose affinity chromatography and the fibronectin isolated as described above. An enzyme-linked immunoabsorbent assay ('ELISA') was used to assess the cross-reactivity (Rennard *et al.*, 1980). The degree of competitive inhibition of the rat specific antibody and the rat fibronectin with non-rat fibronectins were observed.

The ELISA assay used was similar to that described by Rennard *et al.* (1980) and Vuento & Vaheri (1978). Immulon micro-ELISA plates (Dynatech) were coated with 100–200 ng of purified rat plasma fibronectin per well. The species-specific fibronectin was incubated with the purified rabbit antibody to rat plasma fibronectin for 60 min in phosphate-buffered saline with 0.05% Tween 20. The soluble antigen–antibody mixture was then placed in the micro-ELISA wells and incubated for 60 min. After washing, peroxidase conjugated anti-(rabbit IgG) made in goat (Miles) was placed in the wells for 60 min. After washing, a substrate solution containing H₂O₂ and *o*-phenylenediamine was added. The reaction was stopped with 8.0M-H₂SO₄ and the absorbance read at 480 nm. A standard curve was constructed with purified rat plasma fibronectin and was used to calculate the plasma fibronectin concentration.

Immunofluorescent staining. Tissue pieces from rat skin, spleen, liver and kidney were immediately placed on solid CO₂ (–70°C) and then frozen, unfixed, in O.C.T. embedding medium (Miles). Frozen sections (6 μ m thick) were cut in a cryostat at –20°C within 3 h of autopsy. The protein concentration of the antibody solution was 20 μ g/ml as determined by the Lowry *et al.* (1951) procedure. After incubation, the sections were washed three times for 5 min each in phosphate-buffered saline, then air-dried. The sections were incubated for 30 min at 22°C with fluorescein isothiocyanate-conjugated goat antibodies to rabbit IgG (diluted 1:20 with phosphate-buffered saline; Miles). A control was observed for each tissue by using preimmune rabbit sera as the first incubating solution. Another control, which consisted of pre-absorbing the antibody with purified antigen, yielded no fluorescence.

Results

The elution profile from the gelatin–Sepharose affinity column is shown in Fig. 1. The peaks were analysed by gel electrophoresis under reducing conditions (Fig. 2), and apparent molecular weights were determined. The material eluted by 4.0M-urea

Fig. 1. Gelatin-Sepharose affinity chromatography of fibronectin from rat plasma

Fresh rat plasma was passed through a gelatin-Sepharose affinity column and the eluant was monitored for protein absorbance at 280 nm (ordinate). The column was then washed with 4.0M-urea and 8.0M-urea (arrows) (see the Materials and methods section for the complete procedure).

Fig. 2. SDS/5%-polyacrylamide-gel electrophoresis of proteins eluted from gelatin-Sepharose affinity column

Proteins were stained with Coomassie Blue. (a) Fraction eluted from gelatin-Sepharose column by 1.0M-urea; (b) fraction eluted from gelatin-Sepharose column by 4.0M-urea; (c) fraction eluted from gelatin-Sepharose column by 8.0M-urea; (d) molecular-weight standards: myosin (200 000), β -galactosidase (130 000), phosphorylase (94 000), bovine serum albumin (69 000), and ovalbumin (43 000).

contained primarily fibronectin (220 000 M_r) and another protein (70 000 M_r). The material eluted by 8.0M-urea contained primarily the 70 000 M_r protein with a small amount of the fibronectin. Amino acid analysis of the material eluted by 4.0M-urea was identical with that given in previous reports for

fibronectin (Table 1). Amino acid composition of the protein eluted by 8.0M-urea was similar to the 70 000- M_r collagen-binding fragment reported by Balian *et al.* (1979).

The material eluted by 4.0M-urea from the gelatin-Sepharose affinity column was chromatographed on a Sepharose 4B CL column, 0.02M- NaH_2PO_4 /0.15M- NaCl , pH 7.5, being used as the eluting buffer (Fig. 3). Four peaks were separated and the amino acid compositions of these fractions are given in Table 2.

The material eluted by 4.0M-urea from the gelatin-Sepharose affinity column was electrophoresed (5%-polyacrylamide slab gels containing 0.1% SDS) under reducing conditions. The fibronectin band was sliced and used for production of antiserum as described in the Materials and methods section. The two bands in the material eluted by 4M-urea were separated by preparative electrophoresis. The purified fibronectin was coupled to CNBr-activated Sepharose 4B and the anti-fibronectin antiserum was passed through the column. The monospecific antibody was eluted with 3.0M-KSCN (see the chromatogram in Fig. 4).

The antibody did not cross-react with Types I or II collagens, albumin or fibrin, as determined by immunodiffusion or the ELISA technique. Fibronectins from chicken, horse and human only weakly cross-reacted with the antibody made against rat plasma fibronectin (Fig. 5). The fraction eluted by 8.0M-urea from the gelatin-Sepharose affinity column (the 70 000- M_r protein) weakly cross-reacted with the antibody (Fig. 5).

In order to further assess the specificity of the antibody, it was used to localize fibronectin in various rat tissues. Fibronectin was absent from the epidermis, but present in the dermis (Plate 1a). The antigen was also present in the glomerulus of the kidney (Plate 1b), and the connective tissue of the spleen (Plate 1c). Fibronectin could be seen

Table 1. Amino acid composition of fractions from gelatin–Sephacrose affinity chromatography

Composition (residues/1000 residues)				
	Human fibronectin (Balian <i>et al.</i> , 1979)	Fraction eluted from gelatin–Sephacrose column by 4 M-urea	Collagen-binding 70 000- <i>M_r</i> fragment from human fibro- nectin (Balian <i>et al.</i> , 1979)	Fraction eluted from gelatin–Sephacrose column by 8 M-urea
Asp	97	96	112	72
Ser	78	78	52	70
Thr	107	112	87	101
Glu	118	134	136	134
Pro	80	83	46	56
Gly	91	88	116	114
Ala	45	48	32	31
Val	78	79	51	65
Met	10	11	25	26
Ile	44	42	30	45
Leu	55	61	31	53
Tyr	41	43	56	41
Phe	25	24	30	26
His	24	26	31	26
Lys	36	39	36	40
Arg	54	56	50	57

Fig. 3. Sephacrose 4B-CL chromatography of the fraction eluted by 4.0 M-urea from the gelatin–Sephacrose affinity column. Eluted protein was monitored for absorbance at 280 nm.

Fig. 4. Fibronectin–Sephacrose affinity chromatography of anti-fibronectin serum. Rabbit anti-(rat fibronectin) antiserum was eluted with 3.0 M-KSCN. Protein was monitored by absorbance at 280 nm.

EXPLANATION OF PLATE 1

Immunofluorescence localization of fibronectin in fresh frozen sections of rat liver tissues (magnification $\times 250$)
 (a) Skin: fibronectin is absent from the epidermis (E), but present in the dermis, associated with blood vessels (arrow);
 (b) kidney: fibronectin is absent from the tubule cells (T) but present in the glomerulus (G) and connective tissues;
 (c) spleen: fibronectin is associated with the mesenchymal connective tissues; a blood vessel (arrow) is reactive towards the anti-fibronectin antibody;
 (d) liver: fibronectin is not associated with hepatocytes, but present in the connective tissue, possibly associated with Kupfer cells or endothelial cells.

Fig. 5. Species specificity of anti-(rat fibronectin) antibody
Displacement curves of fibronectin from chicken, horse, human and the fraction eluted from the gelatin–Sephacrose column by 8.0M-urea were determined by using the ELISA technique.

Table 2. Amino acid composition of fractions from
Sephacrose 4B-CL gel filtration
Composition (residues/1000 residues)

		Fractions			
		1	2	3	4
Rat fibronectin					
Asp	97	70	88	67	107
Ser	78	88	70	77	92
Thr	107	45	108	73	57
Glu	118	124	122	128	133
Pro	80	69	85	58	34
Gly	91	154	128	137	161
Ala	45	62	56	78	97
Val	78	44	85	76	60
Met	10	12	10	31	07
Ile	44	38	48	38	39
Leu	55	58	66	73	64
Tyr	41	17	40	14	14
Phe	25	31	25	36	27
His	24	87	20	21	27
Lys	36	64	36	60	32
Arg	54	36	57	46	41

associated with blood vessels in the liver (Plate 1d). The localization of antibody in the specific tissues and its association with specific cell types supports the specificity of the antibody. Similar distributions of fibronectin were observed in these

tissues by Linder *et al.* (1978) and Oberly *et al.* (1979).

Discussion

We have isolated and characterized rat plasma fibronectin and prepared specific antibodies to it. The gelatin–Sephacrose affinity chromatography of rat plasma indicated that fibronectin was eluted by 4.0M-urea, along with a small amount of a 70 000-*M_r* protein. The 70 000-*M_r* protein had a higher affinity for gelatin, since it was eluted from the column with 8.0M-urea. Amino acid compositions suggest that this 70 000-*M_r* protein may be similar to the 70 000-*M_r* collagen-binding fragment of the human fibronectin molecule, in that it also binds to collagen and is eluted by 8.0M-urea. Whereas the collagen-binding portion of the fibronectin molecule was shown to contain the disulphide linkage, it is also true that the 70 000-*M_r* protein described here is enriched in methionine. There are other similarities in amino acid composition between the 70 000-*M_r* protein and the collagen-binding fragment with respect to decreased valine, leucine and proline contents. However, unlike the collagen-binding fragment, the 70 000-*M_r* protein had a significantly decreased aspartic acid content. The fact that this 70 000-*M_r* protein was only weakly cross-reactive with fibronectin anti-

body agrees with the previous reports of the nature of the collagen-binding fragment, but also suggests that the 70000- M_r protein may not be derived from fibronectin (i.e. if the antigenic site is associated with the collagen-binding fragment). The possibility that the 70000- M_r protein may arise as a proteolytic artefact was next examined. In an effort to inhibit proteinase activity, phenylmethanesulphonyl fluoride was used in all eluting buffers and only fresh plasma was used and processed the same day. Despite the use of EDTA and phenylmethanesulphonyl fluoride together in the blood collection tubes, the 70000- M_r fragment was still present. In numerous tissues there may be more than one fibronectin-related or fibronectin-unrelated molecules that have the ability to bind to collagen. In fact the differences seen in amino acid composition between the 70000- M_r collagen-binding fragment of human fibronectin and the 70000- M_r protein presently described suggest that this molecule is a distinct collagen-binding protein unrelated to fibronectin. Whatever the source of this protein, these data indicate that the 70000- M_r protein has a higher affinity for gelatin than fibronectin and may be present in the blood plasma as a separate entity from fibronectin.

Other peaks produced by Sepharose 4B-Cl ultrafiltration may represent other gelatin-binding components. Future studies may shed more light on the metabolic interrelationships between fibronectin and other fragments of fibronectin in a variety of biological functions (Gold & Pearlstein, 1980; Yamada *et al.*, 1980).

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References

- Balian, G., Glick, E. M., Crouch, E., Davidson, J. M. & Bornstein P. (1979) *J. Biol. Chem.* **254**, 1429–1432
- Cuatrecasas, P., (1970) *J. Biol. Chem.* **245**, 3059–3065
- Engvall, E. & Ruoslahti, E., (1972) *Int. J. Cancer* **20**, 1–15
- Gold, L. I. & Pearlstein, E. (1980) *Biochem. J.* **186**, 551–559
- Hynes, R. O. (1976) *Biochim. Biophys. Acta* **459**, 73–107
- Kleinman, H. K., Hewitt, A. T., Murray, J. C., Liotta, L. A., Rennard, S. I., Pennypacker, J. P., McGoodwin, E. B., Martin, G. R. & Fisherman, P. H. (1979) *J. Supramol. Struct.* **11**, 69–78
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Linder, E., Lehto, V. P. & Vaheri, A. (1978) *Ann. N.Y. Acad. Sci.* **312**, 151–159
- Lowry, O. H., Rosebrough, N. J., Farr, A. H. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Mosesson, M. W. (1978) *Ann. N.Y. Acad. Sci.* **312**, 11–30
- Oberly, T. D., Mosher, D. F. & Mills, M. D. (1979) *Am. J. Pathol.* **96**, 651–662
- Pearlstein, E., Hold, L. & Garcia-Pardo, A. (1980) *Mol. Cell Biochem.* **29**, 103–123
- Reddi, A. H. (1976) in *Biochemistry of Collagen* (Ramachandran, G. N. & Reddi, A. H., eds.), pp. 449–478, Plenum Press, New York
- Rennard, S. I., Berg, R., Martin, C. R., Foidart, J. M. & Gehron-Robey, P. (1980) *Anal. Biochem.* **104**, 205–214
- Vaheri, A., Ruoslahti, E. & Mosher, D. F. (1978) *Ann. N.Y. Acad. Sci.* **312**, 1–456
- Vuoto, M. & Vaheri, A. (1978) *Biochem. J.* **175**, 333–336
- Yamada, K. M. & Kennedy, D. W. (1979) *J. Cell Biol.* **80**, 492–498
- Yamada, K. M. & Olden, K. (1978) *Nature (London)* **275**, 179–184
- Yamada, K. M., Kennedy, D. W., Kimata, K. & Pratt, R. M. (1980) *J. Biol. Chem.* **255**, 6055–6063
- Weiss, R. E. & Reddi, A. H. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 2074–2078
- Weiss, R. E. & Reddi, A. H. (1981) *J. Cell. Biol.* **88**, 630–636