Polypeptide heterogeneity of hamster and calf fibronectins

Sergio D. J. PENA,* Gary MILLS, R. Colin HUGHES and John D. APLIN
National Institute for Medical Research, Mill Hill, London NW7 1AA, U.K.

(Received 26 November 1979)

The adhesive glycoprotein fibronectin has been isolated from fresh hamster plasma by affinity chromatography on gelatin coupled to Sepharose beads by the method of Engvall & Ruoslahti [*Int. J. Cancer* (1979) 20, 1–5]. Polyacrylamide-gel electrophoresis of material heated in sodium dodecyl sulphate and 2-mercaptoethanol shows two prominent polypeptide subunits of approx. mol.wts. 215 000 and 200 000, with variable amounts of lower-molecular-weight fragments. The unexpected polypeptide heterogeneity of different preparations of hamster fibronectins and bovine serum fibronectin is shown to be partly an artefact and is generated during isolation and storage of purified fibronectin. Treatment of each hamster fibronectin subunit or a smaller fragment of approx. mol.wt. 140 000 with thermolysin or trypsin after radioiodination produces similar patterns of tyrosine-containing peptides, indicating similar primary amino-acid sequences. Antibodies raised against the major subunits of hamster plasma fibronectin were coupled to Sepharose beads and used in conjunction with gelatin affinity chromatography to isolate fibronectins extracted with urea from baby-hamster kidney (BHK) cells and present in the long-term culture medium of these cells. The cell and medium fibronectins are similar to hamster plasma fibronectin in amino-acid and carbohydrate composition and also produce very similar peptide 'maps'. We conclude that the various forms of hamster fibronectins are structurally analogous in agreement with indistinguishable biological properties in mediating the substrate adhesion of BHK cells [Pena & Hughes (1978) *Cell Biol. Int. Rep.* 3, 339–344].

Fibronectin, also called large external transformation-sensitive ('LETS') glycoprotein, fibroblast surface antigen ('SFA') or cell-surface protein ('CSP'), is a glycoprotein of high molecular weight synthesized by fibroblastic cell lines in culture as well as by some epithelial and endothelial cells, amniotic cells, myoblasts and astroglial cells (Hynes, 1976; Vaheri & Mosher, 1978; Yamada & Olden, 1978). Although the bulk of fibronectin synthesized by fibroblasts is recovered from the culture medium (Mosher *et al.*, 1977), a significant part of the glycoprotein is associated with the cell surface, apparently in a lipid-poor 'glycocalyx' fraction (Graham *et al.*, 1975). Fibronectin is also present in normal plasma in a form known as cold-insoluble globulin ('Cig') and most likely produced by endothelial cells (Jaffe & Mosher, 1978).

The activities of the various forms of fibronectin

isolated from serum or from fibroblasts in mediating the adhesion of cells to substratum (Pearlstein, 1976; Pena & Hughes, 1978a; Grinnell & Minter, 1978; Murray *et al.*, 1979), appear to be very similar (Pena & Hughes, 1978b; Yamada & Olden, 1978). However, clear differences between these various preparations have been demonstrated. The cell-associated form of fibronectin is a haemagglutinin, whereas the plasma form is much less active (Yamada & Olden, 1978; Yamada *et al.*, 1978). The plasma form is a soluble circulating glycoprotein, whereas cell-associated fibronectin can be extracted from cells only under dissociating conditions using urea or guanidine hydrochloride (Nairn & Hughes, 1976; Hughes & Nairn, 1978; Hynes *et al.*, 1976). Treatment of normal fibroblasts with non-ionic detergents such as Triton X-100 leaves an insoluble residue consisting substantially of fibronectin and protein subunits of the cytoplasmic contractile elements (Culp & Buniel, 1976; Chen *et al.*, 1978). Furthermore, addition of cellular fibronectin to transformed cells induces a marked shape change (Yamada *et al.*, 1976; Ali *et al.*, 1977) and

Abbreviation used: SDS, sodium dodecyl sulphate.

* Present address: Montreal Neurological Institute, 3801 University Street, Montreal, Quebec H3A 2B4, Canada.

re-alignment of actin microfilaments in submembranous sheaths and in bundles traversing the cell (Mautner & Hynes, 1977), whereas the plasma form is less active. Electron-microscopic observations have strongly suggested some form of association of cell-surface fibronectin with cytoplasmic actin filaments (Singer, 1979), perhaps through an intermediate (glyco)protein component integrated into and spanning the surface-membrane bilayer (Hegness *et al.*, 1978; Hughes *et al.*, 1979a).

In order to examine the biological properties of the various forms of fibronectins and their interactions with cells, we began to isolate fibronectin from baby-hamster kidney fibroblasts (BHK cells), from the spent culture medium of such cells and from fresh hamster plasma. Some properties of these three forms of hamster fibronectin are described. An unexpected heterogeneity was found for different preparations of each of the fibronectins. Other studies have also indicated considerable heterogeneity in preparations of human plasma fibronectin and the fibronectin in amniotic fluid (Mosesson *et al.*, 1975; Chen *et al.*, 1976, 1977; Jilek & Hormann, 1977). The possible sources of such heterogeneity are discussed.

Materials and methods

Chemicals and media

SDS, urea (AnalaR grade), acrylamide, *NN'*-methylenebisacrylamide and ammonium persulphate were from BDH Ltd. Trypsin, thermolysin, 2-mercaptoethanol, Tris, Caps [3-(cyclohexylamino)propanesulphonic acid] and phenylmethanesulphonyl fluoride were from Sigma Chemical Corp. Eagle's medium and foetal-calf serum were from Flow Laboratories, Irvine, Ayrshire, Scotland, U.K. Na¹²⁵I was from The Radiochemical Centre, Amersham, Bucks., U.K.

Cell cultures

BHK cells were grown routinely at 36°C in Eagle's medium supplemented with tryptose phosphate and 10% (v/v) foetal-calf serum as described previously (Meager *et al.*, 1976). Trypsin treatment of cell monolayers was carried out in trypsin/EDTA solution also as described by Meager *et al.* (1976).

In order to collect culture medium for preparation of secreted fibronectins, near confluent BHK-cell monolayers were rinsed in serum-free Waymouth medium MAB 87/3 and incubated at 36°C in this medium (125 ml per 120 cm²-area tissue-culture flask) for 24–48 h (Sear *et al.*, 1977). In some experiments, BHK cells were grown to confluency at 36°C in Eagle's medium supplemented with serum depleted of fibronectin by passage twice through a gelatin–Sephadex affinity column (Engvall & Ruoslahti, 1977; Dessau *et al.*,

1978) as described below. These culture fluids were then used to isolate hamster fibronectin.

Extraction of cells

The general procedure of Brown *et al.* (1976) was used. In a typical experiment, confluent monolayers of BHK cells (5×10^8) were suspended by scraping into phosphate-buffered saline (50 ml) (Meager *et al.*, 1976) and washed twice with phosphate-buffered saline (50 ml) by centrifugation at 4°C before extraction in 0.5% Nonidet P-40, 2 mM-phenylmethanesulphonyl fluoride, 10 mM-Tris/HCl buffer, pH 8.2 (20 ml) at 4°C for 30 min. The insoluble residue was recovered by centrifugation and mixed with 8 M-urea in 50 mM-Tris/HCl (pH 7.5)/2 mM-phenylmethanesulphonyl fluoride. After incubation at 4°C for 20 min, the solution was diluted with 50 mM-Tris/HCl (pH 7.5)/2 mM-phenylmethanesulphonyl fluoride to give 1.5 M-urea final concentration. The clear supernatant obtained after centrifugation at 30000 g for 50 min was used for fibronectin purification.

Hamster plasma fibronectin

Adult Syrian hamsters were bled by heart puncture into citrated syringes (0.1 ml; 100 units/tube). The plasma was separated by centrifugation at 1000 g for 10 min, followed by centrifugation at 30000 g for 30 min, and applied to a gelatin–Sephadex 4B affinity column as described by Engvall & Ruoslahti (1977). Pig skin gelatin (500 mg; Sigma) was coupled to CNBr-activated Sephadex 4B (5 g) as recommended by the manufacturers (Pharmacia), packed into columns (11–12 ml) and washed alternatively with column buffer (phosphate-buffered saline/0.1 M-sodium citrate) and elution buffer (8 M-urea in 0.05 M-Tris/HCl buffer, pH 7.5). Hamster plasma (20 ml portions) was added to the column at room temperature in column buffer at approx. 0.4 ml/min. After washing with column buffer until the A_{280} decreased to background value, fibronectin was removed with elution buffer. In later experiments the plasma (20 ml) was stirred for 1–2 h with Sephadex 4B (5 g) and the supernatant, recovered by centrifugation, was applied to the gelatin affinity column. Fibronectin eluted by 8 M-urea from the gelatin–Sephadex column was detected by A_{280} and either diluted to 1.5 M-urea with 0.15 M-NaCl/0.1 M-CaCl₂/10 mM-Caps adjusted with 1 M-NaOH to pH 11 (Yamada *et al.*, 1977) or dialysed overnight at 2°C against this buffer. The diluted solutions were then neutralized by addition of 0.1 M-NaH₂PO₄ and stored at –20°C in small portions (approx. 0.4 mg/ml). The yield from a typical run was 4–5 mg of protein as determined by the Lowry *et al.* (1951) method or by absorbance measurements ($A_{280}^{1\%}$ 12.4).

When the material eluted unretarded from the

gelatin–Sephacrose 4B column was rechromatographed on the affinity column, no protein material was detected by A_{280} in the fractions eluted with 8M-urea, showing that essentially all of the fibronectin was removed from plasma by a single adsorption to gelatin–Sephacrose 4B.

Polyacrylamide-gel electrophoresis

Protein samples (usually 50–400 μ g) were mixed with 2 vol. of cold (-20°C) ethanol and the precipitate was collected after 16 h at -20°C by centrifugation at 30000 g for 1 h. The precipitates were heated in 1% (w/v) SDS with or without 1% (v/v) 2-mercaptoethanol at 90° for 3–5 min and portions applied to the polyacrylamide slab gel for electrophoresis as described previously (Pena *et al.*, 1979).

In some experiments, protein samples (50–400 μ g) were dialysed against distilled water at 2°C overnight, freeze-dried and dissolved in hot SDS (plus or minus 2-mercaptoethanol) for electrophoresis.

Preparation of antisera

Hamster plasma fibronectin (0.45 mg) was disrupted by heating in 1% SDS plus 1% 2-mercaptoethanol (1 ml) and applied to a 7.5% (w/v) polyacrylamide slab gel for electrophoresis. The gel was stained with Coomassie Blue and destained by routine procedures (Pena *et al.*, 1979). The region of the gel containing the two most intensely stained bands (see Fig. 1, arrows 1 and 2) was cut into small pieces and passed through a gauge-21 needle and further disrupted in a Dounce homogenizer containing 2 ml of complete Freund's adjuvant. The homogenate was then injected subcutaneously into the back of two female New Zealand White rabbits at multiple sites. In some experiments the gel pieces were extracted overnight with 0.1% SDS, followed by homogenization by 12 strokes in a Dounce homogenizer and the clear filtrate obtained by passage through glass wool made 0.1 M with respect to KCl. The precipitate forming after 24 h at 2°C was recovered by centrifugation, emulsified with complete Freund's adjuvant (2 ml) and injected into rabbits. After booster injections at 2 and at 4 weeks after the initial injection, the rabbits were bled by heart puncture after a further 3–4 weeks. Portions of the immune serum (total volume 180 ml) was mixed with 4 vol. of foetal-calf serum to remove antibodies cross-reacting with calf serum fibronectin. The precipitate formed at 2°C after 2–3 days was removed by centrifugation, and the supernatant was treated with Na_2SO_4 to prepare the immunoglobulin fraction (Deutsch, 1967). This was dissolved in phosphate-buffered saline to give the original volume of serum, filtered through Millipore filters (0.22 μm pore size) and stored at 2°C .

Immunoabsorbent column chromatography

The immunoglobulin fraction (30 ml; approx. 450 mg of protein) was dialysed against 0.1M- NaHCO_3 at 4°C overnight and added to CNBr-activated Sepharose (7 g). After gentle mixing overnight at 4°C , approx. 90% of the immunoglobulins was coupled to the gel, as measured by the A_{280} of the supernatant remaining after centrifugation. The gel beads were washed with 1M-Tris/HCl, pH 9, and then alternately with 0.1M- NaHCO_3 /0.5M-NaCl, pH 8.3, followed by 0.1M-acetic acid/0.5M-NaCl, pH 4. Finally, the beads were washed with phosphate-buffered saline and packed into a column (10 ml). In a typical affinity-chromatography experiment, hamster plasma (8 ml) was added (at 0.5 ml/min) to the immunoabsorbent at 4°C and eluted with phosphate-buffered saline (45 ml) followed by 8M-urea in 0.05M-Tris buffer, pH 7.5 (15 ml). The fractions (2 ml) were assayed for protein by measuring A_{280} , and peak fractions containing fibronectin were pooled and dialysed to remove urea as before.

Immunofluorescence experiments

BHK cells were grown in Eagle's medium/fibronectin-depleted foetal-calf serum (Pena & Hughes, 1978a,b) to near confluence in 60 mm-diameter tissue-culture dishes containing glass coverslips. The cell monolayers were rinsed in phosphate-buffered saline and fixed in 4% (v/v) formaldehyde in buffered saline at room temperature for 10–15 min. The coverslips were then incubated in 35 mm-diameter dishes containing anti-fibronectin immunoglobulin (1 ml; diluted 1:4 with phosphate-buffered saline) for 30 min, rinsed, and stained by incubation in fluorescein-conjugated goat anti-(rabbit immunoglobulin) serum (Miles Laboratories, Stoke Poges, Slough, Berks., U.K.) for 30 min. The cells were rinsed in phosphate-buffered saline followed by water and were examined for immunofluorescence staining in a Leitz Orthoplan microscope. Photographs were taken with Kodak Ektachrome film and exposure times of 5–20 s.

Immunodiffusion

The method of Ouchterlony (1958) was used.

Iodination and peptide 'mapping'

Fibronectin preparations (0.2–0.25 mg) were precipitated by addition of 2 vol. of ethanol at -20°C and dissolved in 1% SDS/0.25M-sodium phosphate buffer, pH 7.4 (0.25 ml), by heating for 5 min at 90°C . Na^{125}I (100 μCi) in 0.05M-sodium phosphate buffer, pH 7.4 (0.1 ml), was added to each fibronectin solution at room temperature, followed by chloramine-T (0.025 ml; 2.5 mg/ml) in 0.05M-sodium phosphate buffer, pH 7.4. After incubation for

10–15 s the reaction was stopped by addition of sodium metabisulphite (0.05 ml; 2.5 mg/ml). The iodinated samples were dialysed for 2 days at 2°C against 1% SDS in 0.05 M-Tris/HCl buffer, pH 7.5. The dialysed fractions were heated in 1% SDS/1% mercaptoethanol and then applied to 7.5% (w/v) polyacrylamide slab gels for electrophoresis. After drying and radioautography the major labelled bands were eluted from the acrylamide gels by Dounce homogenization in 0.1% SDS/Tris/HCl buffer, pH 7.5. The clear supernatants, collected by centrifugation at 10000 g for 30 min and containing 70–80% of the total ^{125}I radioactivity present in the region of the polyacrylamide gel taken for extraction, were dialysed against distilled water and freeze-dried. The freeze-dried material was dissolved in 0.2 M- NH_4HCO_3 and digested with either 100 μg of trypsin or 100 μg of thermolysin for 16 h at 37°C. After digestion the solutions were centrifuged at 1000 g for 5 min. The supernatants containing at least 90% of total radioactivity were freeze-dried and applied in a pyridine/acetic acid/water mixture (1:10:989, by vol.; pH 3.5; 0.025–0.20 ml) to full sheets of Whatman no. 3MM chromatography paper (45 cm $\times$ 57 cm). Usually at least 10^5 c.p.m. were present. A portion (10 μl) of the dye markers Methyl Green and ϵ -2,4-dinitrophenyl-lysine [1% (w/v) solution] was added to each paper sheet and electrophoresis was at 3 kV and 50 mA for 65 min. The dried papers were then chromatographed for 16–20 h with butan-1-ol/acetic acid/water (4:1:5, upper phase) in a direction at 90° to that used for electrophoresis. Finally the papers were dried again and exposed to Kodak X-ray film (Kodirex Auto-process AP54; Kodak Ltd.) for several weeks to locate radioactive peptides.

Amino-acid and carbohydrate analysis

Fibronectin preparations (0.9–1.7 mg) were hydrolysed in 6 M-HCl at 110°C for 24 h. Amino acids were identified and quantified on a Beckman model 121 MB amino-acid analyser. Carbohydrate analysis was performed essentially as described by Clamp *et al.* (1971). Samples were hydrolysed at 85°C for 6 h to methyl glycosides in methanolic HCl and quantified on a Hewlett–Packard gas chromatograph using helium gas, starting at 120°C, maintained for 20 min, then 10°C $\cdot$ min $^{-1}$ to 210°C, maintained until sialic acid was eluted.

Radioiodinated-lectin staining of gels

Concanavalin A was iodinated with ^{125}I and purified by affinity chromatography as described previously (Butters & Hughes, 1978). Stained fixed gels were rinsed in 0.5 M-sodium phosphate buffer, pH 6.8, for 3 h at room temperature, washed overnight with 0.05 M-phosphate buffer, pH 6.8, containing 0.5 M-NaCl and then exposed to radio-

active lectin (100 μg ; approx. 1×10^8 – 2×10^8 c.p.m. in 25 ml of 0.05 M-phosphates buffer/0.5 M-NaCl, pH 6.8) at room temperature for 30 min–2 h. The gels were then washed extensively in 0.05 M-phosphate/0.5 M-NaCl, pH 6.8, dried and radioautographed by using Kodak X-ray No-Screen film exposed for 1–2 days.

Results

Hamster plasma fibronectin

The basic method of Engvall & Ruoslahti (1977) was used successfully to prepare hamster plasma fibronectin in good yield and in a single step by

Fig. 1. SDS/polyacrylamide-gel electrophoresis of hamster plasma fibronectin purified by gelatin–Sepharose affinity chromatography

Electrophoresis on 7.5% (w/v) acrylamide of fibronectin (approx. 50 μg) disrupted in 1% SDS/1% 2-mercaptoethanol, showing minor bands of mol.wts. 185 000 (3) and 170 000 (4) migrating close to the prominent doublet of 215 000 (1) and 200 000 (2) subunits. The gel was stained with Coomassie Brilliant Blue. Migration was from top to bottom.

affinity chromatography on gelatin–Sephacrose. The amino-acid and carbohydrate compositions of such preparations were closely similar to other reported values (Table 1).

SDS/polyacrylamide-gel electrophoresis of a typical product after reduction shows (Fig. 1) a closely spaced doublet of polypeptides with approx. mol.wts. 215 000 and 200 000. In addition, a number of relatively minor stained bands were present (Fig. 1), in particular two bands of apparent mol.wts. 185 000 and 170 000. In addition to these major subunits and high-molecular-weight fragments, some preparations of hamster plasma fibronectin with amino-acid and carbohydrate compositions closely similar to those shown in Table 1, contained components of relatively lower molecular weights (Fig. 2). The number and relative proportions of these components varied between preparations obtained by using apparently identical conditions of gelatin–Sephacrose chromatography. The predominant additional polypeptide subunits were of approx. mol.wts. 140 000 and 75 000 (Fig. 2). The 140 000-mol.wt. fragment contained much of the carbohydrate, as shown by the significant binding of concanavalin A to this region of a resolving gel (Fig. 2). The 75 000-mol.wt. fragment, by contrast, was only lightly stained. Some preparations of hamster plasma fibronectin contain very small amounts of the 215 000-mol.wt. polypeptide species (Fig. 2). All of these polypeptides originated from a higher-molecular-weight (nominally 360 000) polypeptide

Fig. 2. SDS/polyacrylamide-gel electrophoresis of non-reduced (NR) and reduced (R) hamster plasma fibronectin

Another preparation of fibronectin isolated as indicated in Fig. 1 was disrupted with 1% SDS in absence or presence of 1% 2-mercaptoethanol. The final concentration of acrylamide was 5% and the bands were located with Coomassie Brilliant Blue (Coomassie) or by staining with ¹²⁵I-labelled concanavalin A (¹²⁵I-con A) followed by radioautography. The molecular weights of major bands were estimated from their migration relative to standard proteins of known molecular weight. The small arrow indicates the position of native non-reduced fibronectin in the lectin-stained gel.

Table 1. Amino-acid analysis of fibronectins

References: *present results; †Carter *et al.* (1978); Fukuda & Hakomori (1979); ‡Yamada *et al.* (1978); §Mosesson (1978). Abbreviation used: ND, not determined.

Source ...	Content (mol/100 mol)						
	Hamster*			Hamster†		Chick cell‡	Human plasma§
	Plasma	Cell	Medium	Cell	Medium		
Amino acid							
Lys	3.9	2.4	2.7	3.1	3.2	3.4	3.7
His	2.4	1.8	1.8	2.2	1.6	1.6	2.1
Arg	5.3	4.9	5.7	4.6	4.5	5.3	5.3
Asp	9.3	8.9	7.8	9.5	10.3	9.4	9.5
Thr	10.4	11.2	8.2	10.4	10.0	10.0	9.9
Ser	8.3	7.2	6.8	9.6	7.3	7.4	7.0
Glu	12.1	15.6	11.4	12.4	11.6	11.7	11.9
Pro	7.4	6.4	7.5	7.5	8.3	7.7	7.8
Gly	9.3	9.0	8.7	10.2	10.5	10.6	8.2
Ala	9.6	8.9	11.2	7.1	5.2	4.8	4.4
‡Cys	1.6	1.4	1.7	ND	2.8	1.8	2.7
Val	7.8	9.2	7.2	7.0	7.7	6.6	8.3
Met	0.2	0.6	0.4	ND	1.1	1.3	1.2
Ile	4.7	5.0	3.6	4.7	3.4	3.8	4.5
Leu	5.6	5.0	3.9	5.8	6.7	5.5	5.9
Tyr	3.6	3.1	2.6	3.8	3.7	3.7	4.7
Phe	2.6	2.2	2.0	2.4	2.1	1.9	2.8
Trp	ND	ND	ND	ND	ND	3.6	ND

Table 2. Carbohydrate analysis of fibronectins
Footnote symbols refer to references given in Table 1. Abbreviation used: ND, not determined.

	Molar ratios						
	Hamster*			Hamster†		Chick cell‡	Human Plasma§
	Plasma	Cell	Medium	Cell	Medium		
Man	1.0	1.0	1.0	1.0	1.0	1.0	ND
Gal	0.78	0.69	0.74	0.67	0.72	0.62	ND
GlcNAc	1.04	1.14	1.08	1.26	1.38	1.0	ND
GalNAc	0.21	0.22	0.17	0.31	0.13	0	ND
Glc	0.74	1.01	0.83	ND	ND	0.88	ND
Fuc	0.21	ND	ND	0.34	0.29	ND	ND
NeuNAc	0.18	0.21	0.13	0.12	0.13	0.30	ND

Fig. 3. Peptide 'mapping' of hamster fibronectin subunits
Hamster plasma fibronectin subunits of mol.wts. 215 000 (a and d), 200 000 (b and e) and 140 000 (c and f) were eluted from a 7.5% (w/v) polyacrylamide gel after electrophoresis of ¹²⁵I-radioiodinated material. Each subunit was treated with trypsin (d-f) or thermolysin (a-c) and the mixtures of ¹²⁵I-labelled-tyrosine-containing peptides were analysed by electrophoresis at pH 3.5 (horizontal direction) followed by chromatography in butan-1-ol/acetic acid/water solvent (vertical direction). The radioactive peptides were located by autoradiography and major common features are indicated by arrows.

species detected by SDS/polyacrylamide-gel electrophoresis of the non-reduced fibronectin (Fig. 2).

Evidence for the structural interrelationship of the different molecular-weight species of polypeptide subunits is shown in Fig. 3. Plasma fibronectin (175 μ g) was labelled by chloramine-T-catalysed radioiodination and subjected to electrophoresis on a 7.5%-polyacrylamide slab gel after disruption in SDS and 2-mercaptoethanol. After radioautographic detection, appropriate regions of the gel corresponding to each of the different molecular-weight species were eluted and subjected to proteolysis. Similar mixtures of radiolabelled peptides were produced by proteolysis of the 215 000-, 200 000- and 140 000-mol.wt. species with trypsin (Figs. 3d, 3e and 3f) or thermolysin (Figs. 3a, 3b and 3c). Furthermore, a spectrum of peptides similar to those shown in Figs. 3d, 3e and 3f were produced from the bands corresponding to 185 000 and 170 000 mol.wt. and separated by prolonged electrophoresis and subjected to trypsin treatment (results not shown).

Further evidence that the heterogeneity observed in some hamster-plasma-fibronectin preparations is probably not due to contamination by extraneous proteins was obtained. An immunoabsorbent column was prepared containing antibodies raised against the 215 000–200 000 mol.wt. species of the hamster plasma fibronectin sample shown in Fig. 1. This antiserum reacts with hamster plasma but not bovine fibronectin (Fig. 4a) after adsorption with calf serum and stains BHK cells for fibronectin, associated in an extensive fibrillar array over the cell surface (Fig. 4b). When hamster plasma was passed through an affinity column of this antibody immobilized on Sepharose 4B, protein was eluted from the column with 8 M-urea (Fig. 5a) that adsorbed up to 80% [as estimated with the Lowry *et al.* (1951) method] to a gelatin–Sepharose affinity column (Fig. 5b) and was eluted from such a column by 8 M-urea. SDS/polyacrylamide-gel electrophoresis of some batches of material prepared in this way showed a polypeptide heterogeneity similar to some fibronectin samples prepared from hamster plasma by gelatin–Sepharose alone (see Fig. 2).

Bovine serum fibronectin

A heterogeneity similar to that described for hamster plasma fibronectin was obtained during preparation of fibronectin from different batches of calf serum. Calf serum fibronectin, isolated by gelatin–Sepharose affinity chromatography under the standard conditions described in the Materials and methods section, usually gave a major band on polyacrylamide-gel electrophoresis with nominal mol.wt. 400 000 and after reduction, major bands of nominal mol.wts. 220 000 and 212 000 (Fig. 6a), exactly as described by Iwanaga *et al.* (1978).

Fig. 4. *Immunodiffusion and immunofluorescence* (a) Immunodiffusion in agarose of rabbit anti-(hamster plasma fibronectin) (centre well) against hamster plasma (1), bovine serum (2), or human plasma (3); (b) and (c) immunofluorescence of BHK cells stained with anti-fibronectin rabbit serum (b) or pre-immune rabbit serum (c) followed by fluorescein-conjugated anti-(rabbit immunoglobulin) goat serum. Magnification $\times 780$.

However, other samples of bovine serum fibronectins often contained considerable amounts of lower-molecular-weight species after reduction. These fibronectin samples produced the major

Fig. 5. Affinity chromatography of hamster plasma fibronectin

Hamster plasma was passed (a) through a column of Sepharose conjugated with the immunoglobulin fraction of rabbit anti-(hamster fibronectin) serum as described in the Materials and methods section. The second peak eluted by 8M-urea was dialysed and passed through a gelatin-Sepharose column (b) and the final fraction containing fibronectin was again dialysed to remove urea.

subunits of mol.wts. 220 000, 212 000, together with polypeptides of mol.wts. 195 000, 164 000, 112 000, 83 000, 66 000, 54 000 and 29 000. The possibility that serum proteinases are adsorbed to affinity-column supports and cause degradation of fibronectin during the several hours at room temperature required for chromatography was examined as follows. Bovine serum (50 ml) was passed through a gelatin-Sepharose column and non-adsorbed material eluted in phosphate-buffered saline under the standard conditions described in the Materials and methods section. Then the column was washed with 1M-arginine (Vuento & Vaheri, 1979) to elute fibronectin under non-denaturing conditions. When samples of fibronectin prepared in this way were left at room temperature in phosphate-buffered saline, pH 7.2, containing 0.2% NaN₃, a gradual increase was observed in the polypeptide heterogeneity of the reduced products, and eventually the 220 000–212 000-mol.wt. subunits were almost completely converted into lower-molecular-weight products (Fig. 6).

Fig. 6. SDS/polyacrylamide-gel electrophoresis of bovine serum fibronectin eluted from gelatin-Sepharose with 1M-agarose and kept at room temperature for various times before storage at -70°C (a, 0 days; b, 10 days; c, 40 days)

Samples containing equivalent protein (approx. 50 μg) were thawed, immediately heated in 1% SDS/1% β -mercaptoethanol and analysed by 7.5% polyacrylamide-gel electrophoresis followed by Coomassie Brilliant Blue staining. The arrows indicate mol.wts. of 195 000 (1), 164 000 (2), 112 000 (3), 83 000 (4), 66 000 (5), 54 000 (6) and 29 000 (7). Abbreviation used: FN, major polypeptide subunits of fibronectin.

Modifications of the isolation procedures

Attempts were made to limit the extent of degradation of fibronectin during storage of plasma or sera and subsequent purification steps. The broad-spectrum proteinase inhibitor phenylmethanesulphonyl fluoride was added to fresh plasma or serum and included in buffers throughout the isolation procedures. Similar experiments were

carried out with other inhibitors such as di-isopropyl phosphofluoridate 1-chloro-4-phenyl-3-L-tosylamidobutan-2-one ('TPCK'), 7-amino-1-chloro-3-L-tosylamidoheptan-2-one ('TLCK') and *p*-tosyl-L-arginine methyl ester ('TAME') hydrochloride, either alone or in mixture. No substantial improvement in the reproducibility of preparing fibronectins with a simple polypeptide composition was achieved. Chromatography on Sepharose-gelatin using faster elution rates gave no improvement and neither did a batch procedure in which serum was stirred gently with the derivatized Sepharose beads for 4–16 h at 2°C, followed by packing the beads into a column and elution of fibronectin with 8M-urea. Various modifications of the basic method (Engvall & Ruoslahti, 1977) of column chromatography were examined, including the stepwise elution of the column with column buffer, 1M-urea or 0.5% Triton X-100 in phosphate-buffered saline (10 ml), followed by extensive washing with column buffer until the A_{280} decreased to background values, before elution of the bound fibronectin with 8M-urea. Again, these modified procedures often gave products showing extensive polypeptide heterogeneity and appeared to offer no advantages over the standard method. However, phenylmethanesulphonyl fluoride was added as a routine to all samples of plasma sera or cell extracts used for fibronectin purification.

Hamster fibroblast fibronectins

The products synthesized and secreted by confluent BHK cells were collected by incubation of cells at 36°C in serum-free Waymouths' medium over 2–3 days. During this time the cells remained firmly attached to the tissue-culture flask and were impermeable to Trypan Blue. The medium after centrifugation at 3000 *g* for 1 h at 4°C was adjusted to 70% saturation with $(\text{NH}_4)_2\text{SO}_4$ (56.6 g) and the precipitate formed after 16 h at 4°C was collected by centrifugation. The precipitated material was dissolved in phosphate-buffered saline (15 ml), treated with Sepharose 4B (5 g) and then fractionated by affinity chromatography on an anti-(hamster plasma fibronectin)-Sepharose 4B column (see Fig. 5a). The material eluted with 8M-urea was dialysed for 16 h at 4°C against phosphate-buffered saline and then passed through a gelatin-Sepharose affinity column (see Fig. 5b). Approx. 1.1 mg of protein/ 10^8 cells was eluted from the column with 8M-urea.

Purification of BHK-cell fibronectin was carried out in essentially the same way by using the 8M-urea extracts of confluent cells, previously treated with a non-ionic detergent to remove the bulk of cellular proteins (Brown *et al.*, 1976). A yield of approx. 1 mg of fibronectin/ 10^8 cells was obtained.

Analysis of the polypeptide compositions of different preparations of BHK-cell fibronectins

Fig. 7. Peptide 'mapping' of BHK-cell fibronectin isolated by affinity chromatography

The preparation was radiolabelled as described in Fig. 3 and digested with thermolysin (a) or trypsin (b) before electrophoresis at pH 3.5 (horizontal direction) followed by chromatography. Radioactive spots were located by radioautography.

treated with reducing agent again showed prominent subunits of 215 000 and 200 000 mol.wt. and, in addition, components migrating faster in the polyacrylamide gel. Again, the contribution of these latter polypeptide species to the total polypeptide constitution of the purified fibronectins varied from batch to batch. The BHK cellular fibronectins have amino-acid and carbohydrate compositions very similar to those of hamster plasma fibronectin (Table 1), and the peptide 'maps' produced from cell (Fig. 7) or medium (results not shown) fibronectins are similar to those of hamster plasma fibronectin subunits (Fig. 3). The analyses of BHK-cell fibronectins are also closely similar to data published for primary embryo fibroblasts (Carter *et al.*, 1978; Fukuda & Hakomori, 1979).

Discussion

The reason for the variation in polypeptide constitution of hamster plasma fibronectin is unknown. Since a number of animals were used to

provide sufficient plasma for a single preparative run, it seems unlikely that the results are due to individual variation in fibronectin structure. More probably, the small differences may relate to the time taken to collect plasma, allowing different degrees of degradation or fragmentation of the native fibronectin to occur by serum enzymes or by enzymes released from damaged cells, for example, platelets. Some support for this notion comes from the appearance of polypeptide heterogeneity in bovine serum fibronectin eluted from gelatin-Sepharose under non-denaturing conditions with 1 M-arginine and stored at room temperature (see Fig. 6). Furthermore, Iwanaga *et al.* (1978) and Mosesson (1978) have shown that the proportion of lower-molecular-weight fragments in highly purified bovine- and human-plasma-fibronectin preparations respectively are increased by relatively brief treatment with plasmin or kallikrein. However, incorporation of general serine proteinase inhibitors such as phenylmethanesulphonyl fluoride into the collecting tube did not significantly improve the polypeptide homogeneity of some batches of isolated bovine or hamster plasma fibronectin; neither does this inhibitor prevent degradation of fibronectins prepared from BHK cells or spent culture fluids of confluent monolayers of these cells, presumably by proteinases derived from the cells themselves or from serum incorporated into the culture medium. Proteinase inhibitors such as *N*-ethylmaleimide were not used, since fibronectin contains free thiol groups that may be required for biological activity (Wagner & Hynes, 1979). Therefore thiol-containing proteinases may be responsible for the degradation observed in some fibronectin preparations.

It is noteworthy that the fragmentation of hamster plasma fibronectin is rather characteristic and is different to that of bovine serum fibronectin. Evidence suggests that the C-terminal domain of bovine (Iwanaga *et al.*, 1978) and primary-hamster-embryo fibroblast fibronectins (Carter *et al.*, 1978; Fukuda & Hakomori, 1979) are relatively poor in carbohydrate. Therefore the relatively poor staining (see Fig. 2) of the 75 000-mol.wt. fragment of hamster-plasma-fibronectin preparations, with concanavalin A, a lectin of broad specificity for many carbohydrate chains of glycoproteins, is consistent with these fragments originating from the C-terminal region of the whole molecule. Since major fragments of 140 000 and 75 000 mol.wt. were often present in equivalent amounts (Fig. 2), it is tempting to suggest that these arise because of a single hydrolytic split of a 215 000-mol.wt. subunit. Indeed, the appearance of the 140 000 and 75 000 mol.wt. species seems to account quantitatively for loss of the 215 000-mol.wt. subunit in some preparations of hamster plasma

fibronectin, whereas the 200 000-mol.wt. subunit appears to be relatively stable (Fig. 2) and persists as a major component. Since all of the bands derive from a native fibronectin of nominal mol.wt. 360 000, presumably intra-chain cleavage of the polypeptide subunits must occur within disulphide-bonded loops. Only after reduction does the native structure show a marked polypeptide heterogeneity. In order to explain the apparent stability of the 200 000-mol.wt. fragment, we suggest that hydrolysis at a terminal site decreases the susceptibility of the 215 000-mol.wt. polypeptide subunit to proteolytic cleavage to form the 140 000- and 75 000-mol.wt. species.

The biological significance of the multiple forms of hamster fibronectins indicated by the present study remains to be established. Experience gained over the last 2 years strongly suggests that the biological activities of various hamster fibronectin preparations in mediating the attachment and spreading of trypsin-treated hamster fibroblasts to plastic surfaces or to collagen (Pena & Hughes, 1978*a,b*; Hughes *et al.*, 1979*a,b*) are very similar. Thus a limited degradation apparently has little effect on these functions of fibronectin. It is possible, however, that other properties (Yamada & Olden, 1978) may be modified by fragmentation, and, if so, this process may have physiological significance.

We have shown elsewhere that the various forms of hamster fibronectin isolated from plasma or fibroblasts have similar activities in mediating BHK-cell adhesion (Pena & Hughes, 1978*a*; Hughes *et al.*, 1979*b*), and these conclusions are confirmed by the very similar chemical compositions of the different fibronectins and their similar peptide 'maps', indicating closely similar primary amino-acid sequences. Furthermore, although not homologous, as shown by peptide 'mapping' (Nairn & Hughes, 1976; Hughes & Nairn, 1978), fibronectins from species as widely separated phylogenetically as the hamster, human and chick show very similar gross chemical compositions (Table 1), in addition to being analogous dimeric molecules of similar size (Yamada & Olden, 1978). If, as seems likely, fibronectin has been conserved to perform similar functions in different species, then such similarities in overall structure are to be expected and are consistent with the ability of fibronectins of various species to mediate equally well the adhesion of hamster cells to substrata as described previously (Pena & Hughes, 1978*a*).

We thank the Medical Research Council of Canada for a Fellowship to S. D. J. P. and the Medical Research Council (U.K.) for a Training Fellowship awarded to J. D. A. We thank Mr. T. D. Butters for help and advice in performing the lectin staining of gels.

References

- Ali, I. U., Mautner, V., Lanza, R. & Hynes, R. O. (1977) *Cell* **11**, 115–126
- Butters, T. D. & Hughes, R. C. (1978) *Carbohydr. Res.* **61**, 159–168
- Brown, S., Lovinson, W. & Spudich, J. A. (1976) *J. Supramol. Struct.* **5**, 119–130
- Carter, W. G., Fukuda, M., Lingwood, C. & Hakomori, S. (1978) *Ann. N.Y. Acad. Sci.* **312**, 160–177
- Chen, A. B., Mosesson, M. W. & Solish, G. I. (1976) *Am. J. Obstet. Gynecol.* **125**, 958–961
- Chen, A. B., Amrani, D. L. & Mosesson, M. W. (1977) *Biochim. Biophys. Acta* **493**, 310–322
- Chen, L. B., Murray, A., Segal, R. A., Bushnell, A. & Walsh, M. L. (1978) *Cell* **14**, 377–391
- Clamp, J. R., Bhatti, T. & Chambers, R. E. (1971) *Methods Biochem. Anal.* **19**, 229–262
- Culp, L. A. & Buniel, J. F. (1976) *J. Cell. Physiol.* **88**, 89–106
- Dessau, W., Jilek, F., Adelmann, B. C. & Hormann, H. (1978) *Biochim. Biophys. Acta* **533**, 227–237
- Deutsch, H. F. (1976) *Methods Immunol. Immunochem.* **1**, 319–320
- Engvall, E. & Ruoslahti, E. (1977) *Int. J. Cancer* **20**, 1–5
- Fukuda, M. & Hakomori, S. (1979) *J. Biol. Chem.* **254**, 5442–5450
- Graham, J. M., Hynes, R. O., Davidson, E. A. & Bainton, D. F. (1975) *Cell* **4**, 353–365
- Grinnell, F. & Minter, D. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 4408–4412
- Hegeness, M. H., Ash, J. F. & Singer, S. J. (1978) *Ann. N.Y. Acad. Sci.* **312**, 414–417
- Hughes, R. C. & Nairn, R. (1978) *Ann. N.Y. Acad. Sci.* **312**, 192–206
- Hughes, R. C., Pena, S. D. J. & Vischer, P. (1979a) in *Cell Adhesion and Motility* (Curtis, A. & Pitts, J. D., eds.), pp. 329–356, Cambridge University Press, Cambridge
- Hughes, R. C., Pena, S. D. J., Clark, J. & Dourmashkin, R. R. (1979b) *Exp. Cell Res.* **121**, 307–314
- Hynes, R. O. (1976) *Biochim. Biophys. Acta* **458**, 73–107
- Hynes, R. O., Destree, A. T. & Mautner, V. (1976) *J. Supramol. Struct.* **2** (Suppl. 1), 189–201
- Iwanaga, S., Suzuki, K. & Hashimoto, S. (1978) *Ann. N.Y. Acad. Sci.* **312**, 56–73
- Jaffe, E. A. & Mosher, D. F. (1978) *Ann. N.Y. Acad. Sci.* **312**, 122–131
- Jilek, F. & Hormann, H. (1977) *Hoppe-Seyler's Z. Physiol. Chem.* **358**, 133–136
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Mautner, V. & Hynes, R. O. (1977) *J. Cell Biol.* **75**, 743–768
- Meager, A., Unkitchanukit, A. & Hughes, R. C. (1976) *Biochem. J.* **154**, 113–124
- Mosesson, M. W. (1978) *Ann. N.Y. Acad. Sci.* **312**, 11–30
- Mosesson, M. W., Chen, A. B. & Huseby, P. M. (1975) *Biochim. Biophys. Acta* **386**, 509–524
- Mosher, D. F., Saksela, O., Keski-Oja, J. & Vaheri, A. (1977) *J. Supramol. Struct.* **6**, 551–557
- Murray, J. C., Stingl, G., Kleinman, H. K., Martin, G. R. & Katz, S. I. (1979) *J. Cell Biol.* **80**, 197–202
- Nairn, R. & Hughes, R. C. (1976) *Biochem. Soc. Trans.* **4**, 165–167
- Ouchterlony, O. (1958) *Prog. Allergy* **5**, 1–78
- Pearlstein, E. (1976) *Nature (London)* **262**, 497–500
- Pena, S. D. J. & Hughes, R. C. (1978a) *Nature (London)* **276**, 80–83
- Pena, S. D. J. & Hughes, R. C. (1978b) *Cell Biol. Int. Rep.* **2**, 339–342
- Pena, S. D. J., Mills, G. & Hughes, R. C. (1979) *Biochim. Biophys. Acta* **550**, 100–109
- Sear, C., Grant, M. E. & Jackson, D. S. (1977) *Biochem. J.* **168**, 91–103
- Singer, I. I. (1979) *Cell* **16**, 675–685
- Vaheri, A. & Mosher, D. F. (1978) *Biochim. Biophys. Acta* **516**, 1–25
- Vuento, M. & Vaheri, A. (1979) *Biochem. J.* **183**, 331–337
- Wagner, W. P. & Hynes, R. O. (1979) *J. Biol. Chem.* **254**, 6746–6755
- Yamada, K. M. & Olden, K. (1978) *Nature (London)* **275**, 179–184
- Yamada, K. M., Yamada, S. S. & Pastan, I. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 1217–1221
- Yamada, K. M., Schlesinger, D. H., Kennedy, D. W. & Pastan, I. (1977) *Biochemistry* **16**, 5552–5559
- Yamada, K. M., Olden, K. & Pastan, I. (1978) *Ann. N.Y. Acad. Sci.* **312**, 256–277