The EMBO Journal vol.5 no.4 pp.665–670, 1986

Adhesion and cytoskeletal organisation of fibroblasts in response to fibronectin fragments

A.Woods[1,4], J.R.Couchman[1,4], S.Johansson[2] and M.Höök[3]

[1]Biosciences Division, Unilever Research, Colworth Laboratory, Sharnbrook, Beds., UK, [2]Department of Medical and Physiological Chemistry, University of Uppsala, The Biomedical Center, S-751 23 Uppsala, Sweden, and [3]Connective Tissue Laboratory, Diabetes Research and Training Center, University of Alabama at Birmingham, Birmingham, AL 35294, USA

[4]Present address: Connective Tissue Laboratory, Diabetes Research and Training Center, University of Alabama at Birmingham, Birmingham, AL 35294, USA

Communicated by A.Vaheri

Fibronectin has been shown previously to promote complete cell adhesion in the absence of other serum components or *de novo* protein synthesis. Recently a sequence of four amino acids from the cell-binding domain of fibronectin has been termed the 'cell recognition site' of this multidomain molecule since it mediates cell attachment and inhibits cell adhesion to intact fibronectin. We show here, however, that substrata coated with an isolated cell-binding domain of fibronectin are not sufficient for complete cell adhesion; cells attach and spread but, unlike those adhering to intact fibronectin, they do not form stress fibres terminating in focal adhesions. An additional external stimulus is needed for this cytoskeletal reorganisation and may be provided by one of two heparin-binding fragments of fibronectin. The two 'signals' required for complete adhesion need not be provided simultaneously since focal adhesion formation can be promoted by stimulating cells pre-spread on a cell-binding fragment of fibronectin with a soluble heparin-binding fragment. This second stimulation may involve cell membrane heparan sulphate proteoglycans.

Key words: adhesion/cytoskeleton/fibronectin/proteoglycans

Introduction

Fibroblast adhesion to a substrate is a multistage process where the initial attachment of cells to the substrate is followed by cell spreading which involves a rearrangement of cytoskeletal and membrane components (Thom *et al.*, 1979; Badley *et al.*, 1980; Grinnell, 1978; Woods *et al.*, 1983). Fully spread normal fibroblasts eventually develop concave cell edges and stress fibres terminating in focal adhesions (Heath and Dunn, 1978), the formation of which requires a cellular contraction (Woods *et al.*, 1983). This 'contracted' state seems to be a prerequisite for growth (Thom *et al.*, 1979; Couchman *et al.*, 1982). Fibroblasts specialised for locomotion persist more in a 'protrusive' adhesion state characterised by a lack of stress fibres or focal adhesions and only progress to the 'contracted' state as motility decreases and growth begins (Couchman and Rees, 1979; Couchman *et al.*, 1982).

Fibronectin substrata have been shown to be sufficient to promote complete 'contracted' fibroblast adhesion even in the absence of cellular protein synthesis (Grinnell, 1978; Couchman *et al.*, 1983) or other serum components (Grinnell and Feld, 1979). The multidomain molecule of fibronectin has been well characterised and domains responsible for specific biological activities such as cell attachment and binding to collagen or heparin have been isolated (see reviews by Hynes and Yamada, 1982; Yamada, 1983). Recently, a four amino acid sequence (R-G-D-S) present in the interior part of the molecule was identified as a cell-binding site, and synthetic peptides containing this sequence have been shown to promote cell attachment and to inhibit attachment of fibroblasts to substrata coated with intact fibronectin (Pierschbacher and Ruoslahti, 1984a). A similar sequence located towards the carboxy terminus of some forms of rat fibronectin can also promote cell attachment (Pierschbacher and Ruoslahti, 1984b) indicating the possibility of multiple 'cell-binding' sites in one molecule of fibronectin.

We have isolated several fragments of human plasma fibronectin after proteolytic cleavage and compared these with intact fibronectin as adhesive substrata for human embryo fibroblasts, to investigate their roles in the adhesion process. The fragments used contain either the cell-binding region without heparin-binding activity [85 kd (Johansson, 1985) and 105 kd], or heparin- but not cell-binding activity [29-kd amino-terminal domain (Fröman *et al.*, 1984) and 31-kd fragment from near the carboxy terminus (Hynes and Yamada, 1982)], or are large enough to encompass both cell- and heparin-binding domains although occurring in monomer form (165 kd, 135 kd). We show here that the cell-binding domain of fibronectin is not sufficient to promote 'contracted' cell adhesion in the absence of cellular protein synthesis; cells attach and spread but have a 'protrusive' morphology with no focal adhesions. To achieve complete adhesion an additional stimulus is required. This may be provided by a heparin-binding domain of fibronectin supplied either as part of the substrate or in soluble form to cells pre-spread on the cell-binding domain. A possible role of cell surface heparan sulphate proteoglycan in the response of cells to fibronectin to generate 'contracted' adhesion is discussed.

Results

The fragments of human plasma fibronectin used in this study are shown in Figure 1 which also indicates their position within the fibronectin monomer and their binding activities. In all studies reported below, endogenous fibronectin synthesis was prevented by the continuous presence of cycloheximide (25 μg/ml).

Attachment to fibronectin and its fragments

Intact fibronectin and the 85-kd cell-binding fragment were equally efficient in promoting cell attachment when equimolar amounts were used as substrates (Figure 2a) as were other fragments containing the cell-binding domain of fibronectin (165, 135 and 105 kd, data not shown). Attachment also occurred, however, to fragments lacking the cell-binding domain but having heparin-binding activity (29 and 31 kd, Figure 2a, b). Attachment on

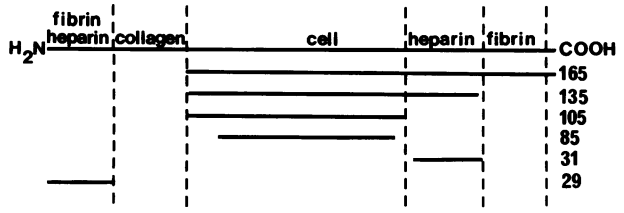

Fig. 1. Diagrammatical representation of the fibronectin monomer and isolated fragments to show the binding activities.

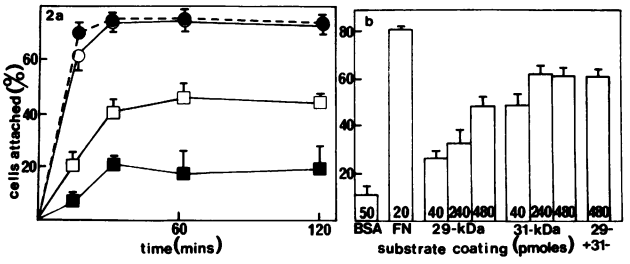

Fig. 2. Attachment of fibroblasts to coated substrates. **(a)** Kinetics of attachment to substrates coated with 20 pmol fibronectin (○ – ○), 40 pmol of isolated 85-kd (● -- ●) or 29-kd (□ – □) fragments or BSA (■ – ■). **(b)** Attachment to increased amounts of 29- or 31-kd fragments, a mixture of both or fibronectin or BSA. The amounts used to coat the substrate are shown on the figure. In **(a,b)** the bars indicate standard errors. Assays were performed in triplicate.

Table I. Attachment of cells to fibronectin or the heparin-binding domains and the effect of antibodies to the heparin-binding fragments

Substrate	Antiserum	Percentage attachment (intact fibronectin = 100%)
Fibronectin	none	100
Fibronectin	αHBF	79
29-kd domain	none	68
29-kd domain	αHBF	28
31-kd domain	none	76
31-kd domain	αHBF	7
31- + 29-kd domains	none	75

Wells were coated with a total of 10 μg protein/well.

heparin-binding fragments increased when increasing amounts of peptide were used to coat the wells (Figure 2b). However, the number of cells attaching never reached that seen with intact fibronectin even when very large amounts of peptide or when a combination of both heparin-binding fragments were used to coat the wells. Very few cells attached to substrates made of bovine serum albumin (BSA).

Attachment of cells to fibronectin substrates was slightly reduced by chicken IgG antibodies raised against the 29-kd fragment (Table I). These antibodies have been shown to recognise both the 29-kd and 31-kd heparin-binding domains, even though they originate from distant parts of the fibronectin molecule (see Materials and methods). This was confirmed in attachment assays where the antibodies specifically reduced human fibroblast attachment to substrates of either of these two domains (Table I). Throughout this report, the antibodies will therefore be termed anti-heparin-binding fragments IgG (αHBF).

Cell spreading on fibronectin and its fragments

Three types of cell morphology were observed depending on whether the cells were allowed to spread on substrates containing both cell- and heparin-binding domains or only one of the two domains.

Cells adhering to intact fibronectin attained a typical 'contracted' (Couchman and Rees, 1979; Woods *et al.*, 1983) fibroblastic morphology with focal adhesions visible by interference reflection microscopy, concave cell edges and prominent stress fibres visible by both phase contrast microscopy and immunofluorescent labelling for actin (Figure 3a – c). The mean area of a fully spread cell on intact fibronectin was 4173 μm^2 (Table II). Fragments of fibronectin containing both cell- and heparin-binding domains (165 and 135 kd) also promoted a 'contracted' cell state with focal adhesion (Table II) and stress fibre (not shown) formation and cells attained mean spread areas not significantly different from those on intact fibronectin. These observations show that the substrate does not have to contain dimeric fibronectin to induce this 'contracted' form of cell spreading.

A second very different type of cell spreading was seen on substrates lacking heparin-binding activity. Although cells on the 85-kd cell-binding fragment spread to cover the same area (Table II), very few cells (10 – 14%) formed focal adhesions, close contacts being predominant (Couchman and Rees, 1979; Woods *et al.*, 1983) (Figure 3d). It is possible that those cells forming focal adhesions were able to escape the cycloheximide block and produce endogenous fibronectin since antibodies specifically recognising heparin-binding domains reduced this figure to 2% (see below, Table III). The phase contrast image of cells spread on 85-kd fragment substrata (Figure 3e) showed that these cells had no prominent stress fibres and furthermore much ruffling membrane was observed, reminiscent of motile fibroblasts (Couchman and Rees, 1979). The actin in these cells was present either diffusely or as peripheral stress fibres (Figure 3f), similar to distributions seen in epithelial cells (Gibson *et al.*, 1983). Similar spread areas, morphology, cytoskeletal organisation (not shown) and lack of focal adhesions (Table II) were seen in cells spread on another isolated fragment containing the cell-binding domain but no heparin-binding activity (105 kd). It therefore appears that the cell-binding domain of fibronectin promotes a 'protrusive' type of spreading, rather than the more static contracted state obtained when a heparin-binding domain is also present.

A third type of cell spreading was obtained on substrates made of isolated heparin-binding fragments, where no cell-binding domain was present. Both the 29-kd amino-terminal and the 31-kd heparin-binding fragments promoted only partial spreading which was reflected in a much reduced spread area when compared with cells spread on fragments containing the cell-binding domain (Table II). Cells did not form focal adhesions (Figure 3g, Table II), ruffling activity was very noticeable (Figure 3h) and stress fibres were absent (Figure 3i).

Cell spreading in response to mixtures of isolated fragments containing cell- and heparin-binding domains

Although neither fragment containing only the cell- or the heparin-binding domain of fibronectin separately promoted the contracted type of spread cell morphology, substrates made from mixtures of these fragments allowed adhesion indistinguishable from that on intact fibronectin. Either of the 29-kd or 31-kd heparin-binding fragments together with the 'cell-binding' domain (85 or 105 kd) promoted the formation of prominent focal adhesions, concave cell edges and stress fibres running centrally through the cell (Figure 4a – d, Table II). Thus fragments containing either of the two binding domains do not need to be

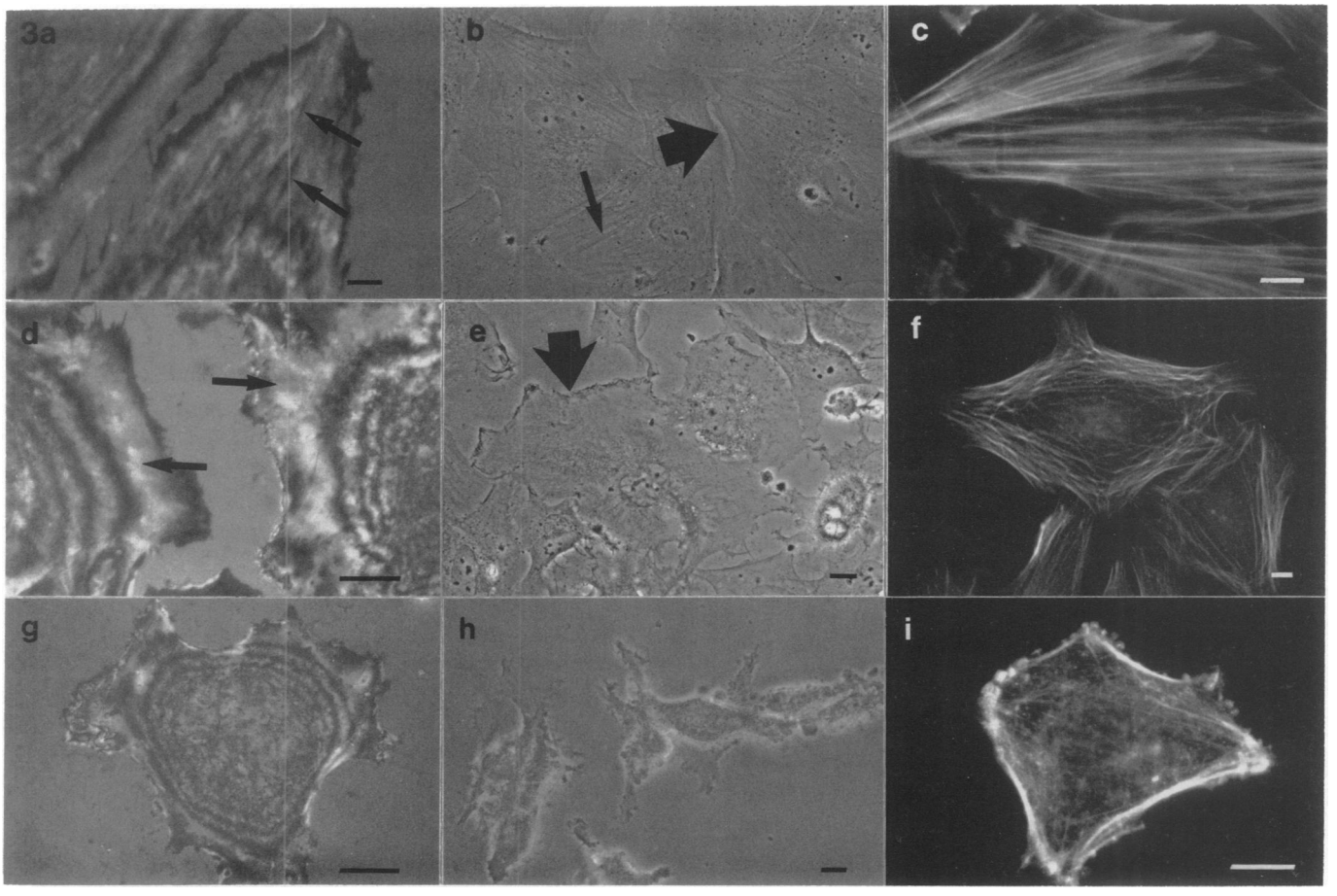

Fig. 3. Cell spreading and organisation on fibronectin or isolated fragments. Interference reflection **(a,d,g)** and phase contrast **(b,e,h)** images and immunofluorescent labelling for actin **(c,f,i)** of cells spread on fibronectin **(a–c)**, 85-kd **(d–f)** or 29-kd **(g–i)** fragments. Arrows indicate focal adhesions in **(a)**, stress fibres in **(b)** and broad lamellae without focal adhesions in **(d)**. Large arrows indicate concave cell edge in **(b)** and ruffling cell edge in **(e)**. Bar = 10 μm.

Table II. Spreading and focal adhesion formation on fibronectin and its fragments

Substratum	Percentage of attached cells spread	Type of spreading	Area of spread cells (± SE) (μm^2)	Percentage[a] of spread cells having focal adhesions
Intact fibronectin	93	contracted	4173 ± 186	79
165-kd	96	contracted	3699 ± 161	85
135-kd	94	contracted	4406 ± 231	75
105-kd	95	protrusive	4904 ± 201	15
85-kd	95	protrusive	4056 ± 299	12
31-kd	89	partial	2285 ± 123	14
29-kd	79	partial	1733 ± 110	13
85- + 29-kd	93	contracted	4191 ± 199	83
85- + 31-kd	95	contracted	4700 ± 195	82
31- + 29-kd	87	partial	1860 ± 105	15
BSA	1	minimal	420 ± 32	0

Coverslips were coated with 5 μg protein/coverslip.
[a]Approximately 10–15% of attached and spread cells showed focal adhesions on any substrate (including uncoated glass). This may represent cells which had escaped the protein synthesis inhibition by cycloheximide.

covalently joined to provide cells with the same stimuli as intact fibronectin. Mixtures of two fragments containing 'cell-binding' domains (not shown) or two fragments containing heparin-binding domains (Figure 4e, f) only promoted partial adhesion, with no focal adhesion or stress fibre formation.

The signal generated by the heparin-binding fragment which converted the protrusive type of adhesion of that of the contracted type did not need to be present during the spreading process. Cells pre-spread on the 85-kd 'cell-binding' fragment for 4 h responded to 0.2 ng/ml of soluble 29-kd fragment by forming

Table III. Focal adhesion formation on intact fibronectin or its fragments: effect of antibodies to the heparin-binding fragments

Substrate	Antiserum	Percentage of spread cells having focal adhesions (intact fibronectin = 100%)
Intact fibronectin	none	100
Intact fibronectin	αHBF	2.5
85-kd domain	none	12
85-kd domain	αHBF	1.5
85- + 29-kd domains	none	94
85- + 29-kd domains	αHBF	1

Coverslips were coated with 5 μg protein of each fragment.

focal adhesions within 1 h, even in the continued presence of cycloheximide (Figure 5a). The percentage of spread cells having focal adhesions rose from 14% on the 'cell-binding' fragment alone to 85 and 67%, respectively after addition of 0.2 ng/ml or 0.02 ng/ml of soluble heparin-binding 29-kd fragment. Morphologically the cells again attained a contracted state of adhesion and, indeed, higher concentrations of heparin-binding fragment (e.g. 2 ng/ml) caused considerable cellular contraction (Figure 5b) resulting in numerous retraction fibrils. The cellular stimulus produced by the 29-kd fragment was dependent on the structure of this domain. Reduced and alkylated 29-kd fragment did not promote focal adhesion formation when added to cells pre-spread on the 85-kd 'cell-binding' fragment (not shown), nor when used as a mixed substrate with the 'cell-binding' fragment (Figure 5c).

Antibodies recognising both the 29-kd and 31-kd heparin-binding fragments (αHBF) reduced focal adhesion formation without affecting spread cell area when cells were seeded on intact fibronectin (Figure 5d) or on a mixed substrate of the 85-kd 'cell-binding' and 29-kd heparin-binding fragments (Table III). The promotion of focal adhesion formation on a mixed substrate of 85- and 31-kd fragments was also prevented by αHBF (not shown). The antibodies also neutralised the effect of the 29-kd fragment in reorganising cells pre-spread on the 'cell-binding' domain (Figure 5e) and prevented the very low level focal adhesion formation normally observed on the 85-kd 'cell-binding' domain.

Discussion

Here we report that in addition to the previously described 'cell-binding region' of fibronectin, there are at least two other domains on the human plasma fibronectin molecule to which cells may attach, both of which are heparin-binding regions. A limited attachment to a similar domain isolated from the amino-terminal of fibronectin has been previously noted (Sekiguchi and Hakomori, 1980) and recently a larger 70-kd amino-terminal fragment has been shown to bind from solution to cells, its binding properties residing mainly in a 27-kd heparin-binding domain (McKeown-Longo and Mosher, 1985). The attachment promoting properties of the 70-kd fragment were, however, minimal in the latter study. In our system, attachment to the heparin-binding domains was lower than that to the 'cell-binding domain', but was dose-dependent and inhibited by antibodies reactive to these heparin-binding domains. Attachment was greater to the 31-kd fragment isolated from the carboxyl side of the 'cell-binding' domain than to the amino-terminal 29-kd domain. This is consistent with their known affinities for heparin (Gold *et al.*, 1983).

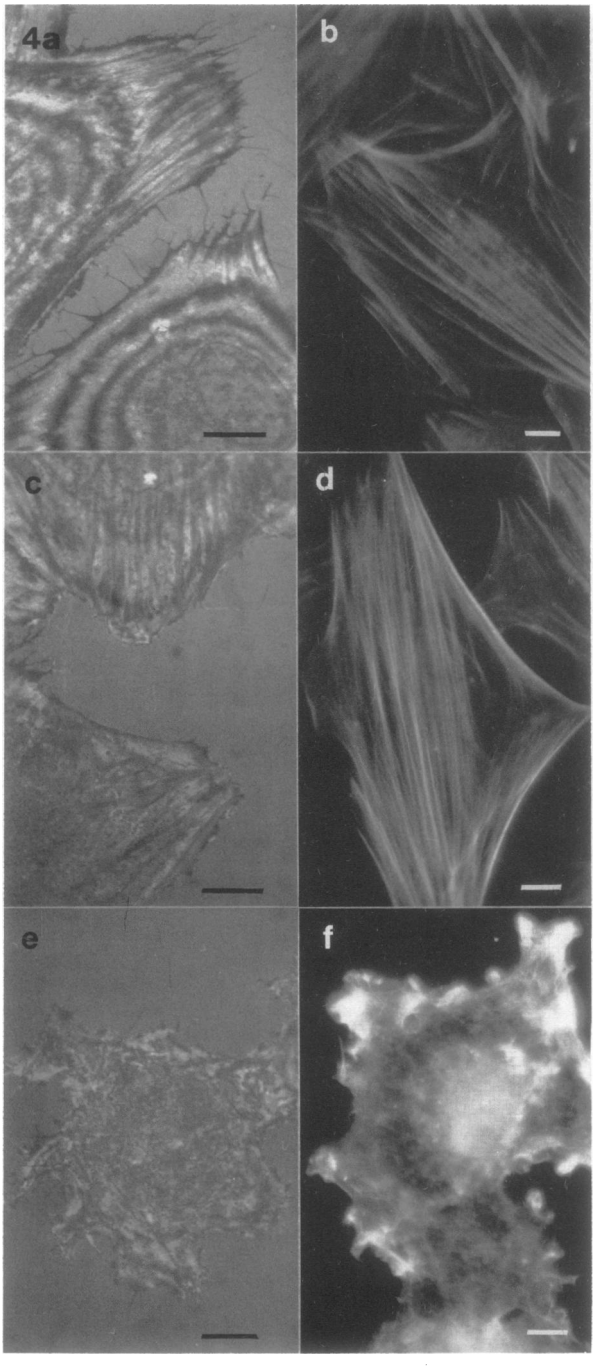

Fig. 4. Cell spreading and organisation on mixtures of fragments. Interference reflection image **(a,c,e)** and immunofluorescent labelling for actin **(b,d,f)** of cells spread on mixed substrates of **(a,b)** 85- + 29-kd fragments; **(c,d)** 85- + 31-kd fragments and **(e,f)** 29- + 31-kd fragments. Bar = 10 μm.

Cell spreading occurred to some extent on all fibronectin fragments used in this study but the type of adhesion state obtained varied. On those fibronectin fragments containing both cell- and heparin-binding activities (165 or 135 kd) spread cell area and morphology resembled that on intact fibronectin. Cells spread on 165- and 135-kd fragments, like those on intact fibronectin, formed focal adhesions and reorganised their cytoskeleton into prominent stress fibres. These cells appeared to be contracted at the cell periphery with concave cell edges characteristic of fibroblasts in a non-motile growth phase (Couchman *et al.*, 1982).

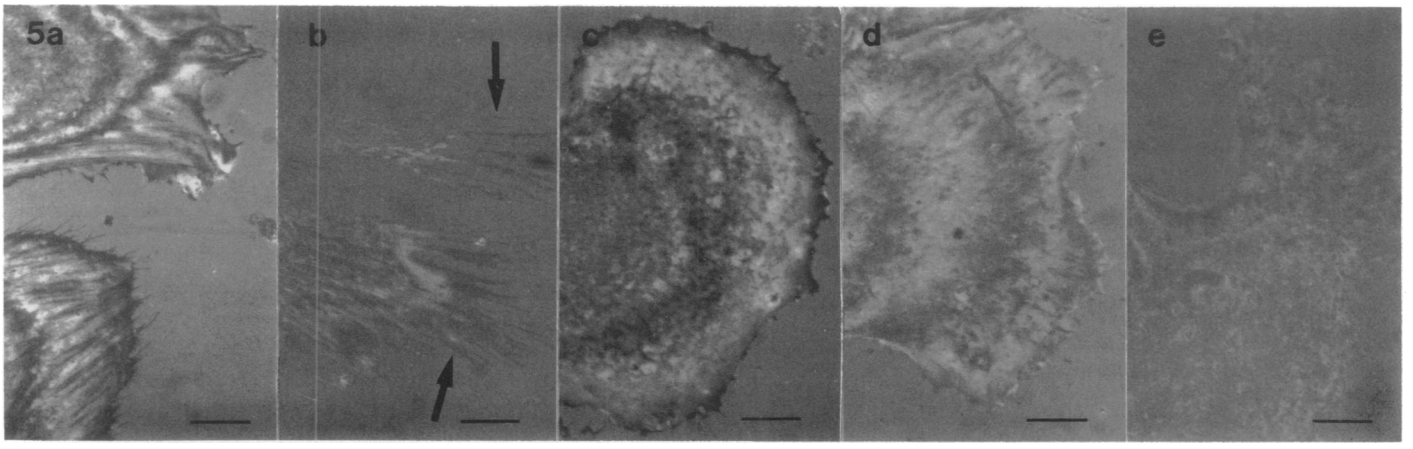

Fig. 5. Stimuli causing focal adhesion formation. All photographs are interference reflection images. **(a,b)** Cells pre-spread on the 85-kd domain 2 h after addition of 0.2 ng/ml **(a)** or 2 ng/ml **(b)** 29-kd fragment. Note focal adhesions in **(a)** and contraction with retraction fibrils (arrows) in **(b)**. **(c)** Cells spread on mixture of 85-kd and reduced and alkylated 29-kd fragments.**(d)** Cells spread on fibronectin in the presence of antibodies to the heparin-binding fragments (αHBF). **(e)** Cells pre-spread on 85-kd substrates treated for 2 h with 0.2 ng/ml 29-kd fragment in the presence of αHBF. Bar = 10 μm.

Cells on the isolated 'cell-binding' domain of fibronectin, however, although attaining a spread area not significantly different from that on intact fibronectin or the 165- or 135-kd fragments, did not form focal adhesions and concomitant stress fibres. They had a morphology consistent in terms of shape and cytoskeletal organisation with a motile protrusive phenotype (Couchman and Rees, 1979; Couchman *et al.*, 1982).

Two conclusions are evident from these results. Firstly, fibronectin need not be in dimeric form (~440 kd) to promote complete cell adhesion with focal adhesions, even in the absence of endogenous protein synthesis, since isolated 165- and 135-kd fragments are sufficient. Secondly, the 'cell-binding' domain alone is not sufficient to promote focal adhesion formation. This seems to require, in addition, a heparin-binding domain present in intact fibronectin and in the 165- and 135-kd fragments. Heparin-binding fragments alone, however, without the 'cell-binding' domain (e.g. fragments of 29 or 31 kd) only promote spreading to a very limited extent. Thus complete adhesion seems to require two stimuli from fibronectin.

The need for a heparin-binding domain was further demonstrated by promotion of focal adhesion formation on substrates composed of a mixture of cell- and either heparin-binding fragments. Indeed, cells pre-spread without focal adhesions on the 'cell-binding' domain could be stimulated to form these and convert from the protrusive to the contracted phenotype by subsequent addition of very small amounts of either heparin-binding domain. It appears unlikely that the added heparin-binding fibronectin fragments combine directly with the substrate-adsorbed 'cell-binding' fragment. The observed effects of these heparin-binding fragments are more likely to be a consequence of an interaction between them and a cell surface component.

Both heparin-binding domains can bind heparan sulphate proteoglycans under physiological conditions (Atherton and Kanwar, 1984). Hence, cell surface heparan sulphate proteoglycans may act as receptors for the heparin-binding fragments. These types of proteoglycans have been shown to be present in several cell types (Kjellén *et al.*, 1981; Rapraeger and Bernfield, 1983; Fransson *et al.*, 1983) and to mediate attachment to platelet factor 4 (Laterra *et al.*, 1983). Recently it has been shown that platelet factor 4, when mixed with the cell-binding domain of fibronectin, promotes a more physiological type of spreading than the cell-binding domain alone (Beyth and Culp, 1984). We find that platelet factor 4 in fact promotes focal adhesion formation in cells pre-spread on the 85-kd 'cell-binding' domain (Woods *et al.*, unpublished) in a similar way to that seen using either heparin-binding fragment. Previous immunohistochemical studies have shown that cell surface distributions of heparan sulphate proteoglycans correspond with concentrations of the actin component of the underlying cytoskeleton during spreading and that, in fully spread cells, these proteoglycans are concentrated over stress fibres and in focal adhesions (Woods *et al.*, 1984). Another extracellular matrix glycoprotein, laminin can also promote fibroblast spreading and focal adhesion formation (Couchman *et al.*, 1983) and this protein has also been shown to contain a heparin-binding domain. Thus the heparin-binding activities of extracellular matrix molecules may be involved in adhesion processes through cell surface heparan sulphate proteoglycans mediating interactions with underlying cytoskeletal components. In fact, recent data (Woods *et al.*, 1985) suggest that a hydrophobic heparan sulphate proteoglycan may link cytoskeleton to substratum matrix in rat embryo fibroblast cultures.

Materials and methods

Cells and culture conditions

Human embryo fibroblasts (a gift from Dr K.Hedman, Department of Virology, University of Helsinki, Finland) were used at passages 2 – 10 from frozen stocks, cultured in alpha modification of Eagle's medium (αMEM) supplemented with 5% fetal bovine serum (USA source) in 75 cm^2 tissue culture flasks (Corning).

Reagents

Fetal bovine serum, αMEM, glutamine, 0.05% trypsin/0.02% EDTA, phosphate-buffered saline with or without Ca^{2+} and Mg^{2+} (PBS or PBS^-) were all from Flow Laboratories. Cycloheximide, BSA and soybean trypsin inhibitor were from Sigma. Antiserum to chicken gizzard actin and methods for immunofluorescent labelling have been previously described (Badley *et al.*, 1978, 1980). The goat anti-rabbit IgG conjugated to fluorescein isothiocyanate used to visualise rabbit antiserum to actin was from Miles Laboratories Ltd and was used at 1:30 dilution in PBS.

Purification of fibronectin and fragments

The procedures for isolation of human plasma fibronectin, trypsin-generated 85-kd cell-binding fragment and plasmin-generated 29-kd amino-terminal fragment have been previously described (Vuento and Vaheri, 1979; Johansson, 1985; Fröman *et al.*, 1984, respectively). The purification protocols for the 85- and 29-kd fragments resulted in preparations which showed single bands on analysis by polyacrylamide gel electrophoresis (Johansson, 1985; Fröman *et al.*, 1984). Reduction and alkylation of the 29-kd fragment was as described by Doolittle *et al.* (1977). The other fibronectin fragments used in this study were a 105-kd cell-

binding fragment, a 31-kd heparin-binding fragment and fragments of 135 and 165 kd which contain both the 105- and 31-kd domains. These fragments were generated by digestion of human plasma fibronectin (3 mg/ml in PBS) at 22°C with chymotrypsin (Sigma, Type II) either at a concentration of 10 μg/ml for 7 min or at a concentration of 30 μg/ml for 90 min. The digestions were terminated by addition of phenylmethylsulphonyl fluoride (1 mM) to the incubation mixtures. The 105- and 31-kd fragments were isolated after passage of the 90 min digest through a column of gelatin – Sepharose and subsequent fractionation on a column of heparin – Sepharose as described (Johansson, 1985). The 105-kd fragment, which did not bind to the heparin – Sepharose column, was finally purified by chromatography on DEAE – Sephacel as previously described for the 85-kd tryptic cell-binding fragment (Johansson, 1985). The 31-kd fragment which was retained on the heparin – Sepharose column was eluted with a linear gradient of NaCl ranging from 0.1 to 0.5 M in 50 mM Tris-HCl buffer, pH 7.0. The 31-kd fragment was then purified free from other peptides by chromatography on a Mono S column (Pharmacia) using a gradient from 0.1 M to 0.25 M NaCl in 10 mM phosphate buffer, pH 7.4.

The 135- and 165-kd fragments were purified from the 7 min digest by affinity chromatography on heparin – Sepharose as described above for the 31-kd peptide (without the preceding gelatin – Sepharose step), followed by ion-exchange chromatography on Mono S. The sample was applied to the Mono S column in 10 mM phosphate buffer, pH 7.4 containing 50 mM NaCl and eluted with a linear gradient of NaCl from 0.05 to 0.25 M in a total volume of 60 ml. By this procedure, the 165-kd fragment eluted well before the 135-kd fragment. All preparations of fragments used in adhesion assays showed one band when analysed by polyacrylamide gel electrophoresis.

Antibodies against the heparin-binding fragments

The 29-kd fragment was isolated after preparative polyacrylamide gel electrophoresis in SDS under non-reducing conditions of plasmin-digested fibronectin (Fröman *et al.*, 1984). 100 μg of peptide in Freund's adjuvant was injected into the breast muscle of a hen three times at 2 week intervals. IgG was obtained from the egg yolks by sequential precipitation with polyethylene glycol according to Polson and von Wechmar (1980). The IgG was further purified by chromatography on DEAE – Sephacel. The sample was applied to the column in 10 mM Tris-HCl buffer pH 8.0, containing 50 mM NaCl and 0.02% NaN_3 and eluted with a linear gradient of NaCl from 50 to 250 mM in the same buffer. The IgG fraction was passed through a column of 85-kd fragment coupled to Sepharose 4B in order to remove any antibodies having affinity for this cell-binding fibronectin domain. The purified antibodies reacted with the 29-kd fragment in immunoblotting and enzyme-linked immunosorbent (ELISA) assay. In the same assays, there was no reactivity against 85-kd or 105-kd fragments, but there was recognition of the 31-kd heparin-binding fragment. This cross-reactivity probably reflects the presence of 31-kd fragment in the original 29-kd preparations used for immunisation. Since it clearly recognised both 29- and 31-kd heparin-binding fragments, we termed these antibodies anti-heparin-binding fragment IgGs (αHBF) and used these to monitor the combined effects of both 29- and 31-kd domains in fibronectin.

Cell attachment and spreading experiments

Fibronectin or its fragments were dried from PBS onto 10 mm diameter glass coverslips (Chance Propper Ltd.) for spreading assays or the wells of tissue culture multi-well plates (2.0 cm^2 surface area/well, Linbro, Flow Laboratories) for attachment assays. The amounts of fibronectin or its fragments used in the attachment assays are noted in the legends. For spreading assays, the coverlips were coated with 5 or 10 μg of fibronectin or its fragments or a mixture of 5 μg 'cell-binding' + 5 μg heparin-binding fragments. Control, non-adhesive substrates were coated with BSA (50 μg/well or coverslip). Before use the substrates were rehydrated with distilled water (30 min), washed (3 × 15 min) with PBS, incubated with 1 mg/ml heat-treated (80°C, 10 min) BSA in PBS^-, and rinsed (3 × 5 min) with MEM without serum (αMEM^-). In some experiments, coated substrata were incubated with antibodies (200 μg/ml) to the heparin-binding fragments for 30 min at 37°C, prior to final rinses with αMEM.

Human embryo fibroblasts were pre-treated (2 h) with 25 μg/ml cycloheximide to prevent protein synthesis (Couchman *et al.*, 1983), rounded and detached with trypsin – EDTA and suspended in αMEM^- containing trypsin inhibitor (100 μg/ml). Cells were centrifuged, washed with 20 ml αMEM^- and aliquoted onto pre-coated coverslips in tissue culture plates or onto the coated wells directly. In some experiments, cells were treated (30 min, 37°C) after detachment but prior to seeding with antibodies (200 μg/ml) to the heparin-binding fragments. Cycloheximide (25 μg/ml) was present at all stages of detachment, attachment and spreading.

Attachment assays were performed in multi-well plates using cells pre-labelled with 0.5 μCi/ml [^{35}S]methionine (Amersham International) as previously described (Couchman *et al.*, 1983). For spreading assays, cells on coated coverslips were fixed after 5 h with 3% glutaraldehyde in αMEM^- (30 min, 37°C) for interference reflection microscopy or 3.5% paraformaldehyde in PBS^- for immunofluorescent staining (Badley *et al.*, 1978, 1980). In some experiments, cells were allowed to spread on coats of 85 kd fibronectin fragment for 4 h prior to addition of other fibronectin fragments.

Microscopy

Cells were viewed on a Leitz Ortholux II microscope fitted with epi-illumination, phase contrast and interference reflection objectives. Photographs were taken on Ilford HP 5 film. For measurements of spread cell areas, the area of 50 individual cells was measured for each substrate using a microcomputer with light pen attached to the microscope. Spread cell areas for each substrate were compared by Duncan's multiple range test ($P = 0.05$).

Acknowledgements

We would like to thank Mrs A.Stones for photographic assistance, Mrs C.Foulger for cell culture help, Mr K.W.Davies and Mr T.C.Hughes for help with measurement and statistical analysis of spread cell areas. The work was supported by Public Health Service grant AM 27807, by Swedish Medical Research Council Grant 06525 and by the Atherosclerosis Research Unit at the University of Alabama at Birmingham. M.H. is a recipient of an Established Investigator Award from the American Heart Association.

References

Atherton,B.T. and Kanwar,Y.S. (1984) *J. Cell Biol.*, **99**, 77a.
Badley,R.A., Lloyd,C.W., Woods,A., Carruthers,L., Allcock,C. and Rees,D.A. (1978) *Exp. Cell Res.*, **117**, 231–244.
Badley,R.A., Woods,A., Carruthers,L. and Rees,D.A. (1980) *J. Cell Sci.*, **43**, 379–390.
Beyth,R.A. and Culp,L.A. (1984) *Exp. Cell Res.*, **155**, 537–548.
Couchman,J.R. and Rees,D.A. (1979) *J. Cell Sci.*, **39**, 149–165.
Couchman,J.R., Rees,D.A., Green,M.R. and Smith,C.G. (1982) *J. Cell Biol.*, **93**, 402–410.
Couchman,J.R., Höök,M., Rees,D.A. and Timpl,R. (1983) *J. Cell Biol.*, **96**, 177–183.
Doolittle,R.F., Cassman,K.G., Cottrell,B.A., Frienzer,S.J., Hucko,J.T. and Takagi,T. (1977) *Biochemistry*, **16**, 1703–1709.
Fransson,L.-Å., Carlstedt,I., Cöster,L. and Malmström,A. (1983) *Eur. J. Cell Biol.*, **1**, 18.
Fröman,G., Świtalski,L., Faris,A., Wadström,T. and Höök,M. (1984) *J. Biol. Chem.*, **259**, 14899–14905.
Gibson,W.T., Couchman,J.R., Badley,R.A., Saunders,H.J. and Smith,C.G. (1983) *Eur. J. Cell Biol.*, **30**, 205–213.
Gold,L.I., Frangione,B. and Pearlstein,E. (1983) *Biochemistry*, **22**, 4113–4119.
Grinnell,F. (1978) *Int. Rev. Cytol.*, **53**, 65–144.
Grinnell,F. and Feld,M.K. (1979) *Cell*, **17**, 117–129.
Heath,J.P. and Dunn,G.A. (1978) *J. Cell Sci.*, **29**, 197–212.
Hynes,R.O. and Yamada,K.M. (1982) *J. Cell Biol.*, **95**, 369–377.
Johansson,S. (1985) *J. Biol. Chem.*, **260**, 1557–1561.
Kjellén,L., Pettersson,I. and Höök,M. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 5371–5375.
Laterra,J., Silbert,J.E. and Culp,L.A. (1983) *J. Cell Biol.*, **96**, 112–123.
McKeown-Longo,P.J. and Mosher,D.F. (1985) *J. Cell Biol.*, **100**, 364–374.
Pierschbacher,M.D. and Ruoslahti,E. (1984a) *Nature*, **309**, 30–33.
Pierschbacher,M.D. and Ruoslahti,E. (1984b) *Proc. Natl. Acad. Sci. USA*, **81**, 5985–5988.
Polson,A. and von Wechmar,N.B. (1980) *Immunol. Commun.*, **9**, 475–493.
Rapraeger,A.C. and Bernfield,M. (1983) *J. Biol. Chem.*, **258**, 3632–3636.
Sekiguchi,K. and Hakomori,S.-I. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 2661–2665.
Thom,D., Powell,A.J. and Rees,D.A. (1979) *J. Cell Sci.*, **35**, 281–305.
Vuento,M. and Vaheri,A. (1979) *Biochem. J.*, **183**, 331–337.
Woods,A., Smith,C.G., Rees,D.A. and Wilson,G. (1983) *Eur. J. Cell Biol.*, **32**, 108–116.
Woods,A., Höök,M., Kjellén,L., Smith,C.G. and Rees,D.A. (1984) *J. Cell Biol.*, **99**, 1743–1753.
Woods,A., Couchman,J.R. and Höök,M. (1985) *J. Biol. Chem.*, **260**, 10872–10879.
Yamada,K.M. (1983) *Annu. Rev. Biochem.*, **52**, 761–799.

Received on 25 October 1985; revised on 21 January 1986