HeLa CELLS FORM FOCAL CONTACTS THAT ARE NOT FIBRONECTIN DEPENDENT

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SUMMARY

HeLa cells cultured on glass substrata produce numerous prominent focal contacts, which reside at the termini of actin microfilament bundles. However, very few of the cells stain for fibronectin with specific anti-fibronectin antibody. Moreover, the cells form focal contacts in fibronectin-depleted medium, in the presence of high concentrations of anti-fibronectin immunoglobulin G and in the presence of monensin. No fibronectin synthesis can be detected by [35S]methionine-labelling and immunoprecipitation. The possibility that HeLa cell focal contacts are independent of fibronectin in their formation is discussed in relation to the controversy about the relationship between fibronectin and focal contacts.

INTRODUCTION

Interference reflection microscopy has revealed discrete areas beneath cultured cells where the separation between cell and substratum is about 10-15 nm (Curtis, 1964; Izzard & Lochner, 1976; Abercrombie & Dunn, 1975; Heath & Dunn, 1978). These are called focal contacts and represent regions of adhesion between the cell and the substratum. They occur in both fibroblasts (see above references) and epithelial cells (Heath, 1982; Billige et al. 1982). Focal contacts have been shown to occur at the peripheral ends of actin microfilament bundles or stress fibres (Heath & Dunn, 1978) and also to contain the cytoplasmic proteins α-actinin and vinculin (Geiger, 1979; Geiger, Tokuyasu, Dutton & S. J. Singer, 1980; I. Singer, 1982).

The molecular nature of the extracellular components of focal contacts is a problem of great interest and some controversy. In particular, there has been considerable debate concerning the presence or absence of the high molecular weight matrix glycoprotein, fibronectin, in focal contacts. Fibronectin is believed to be of great importance in cell adhesion and has been shown to possess the capacity to bind both to cells and to other extracellular matrix components such as collagen and hyaluronic acid (Yamada & Olden, 1978; Hynes & Yamada, 1982). Thus it would seem to possess the necessary properties to mediate cell–substratum or cell–matrix interactions. Does it exert this action in focal contacts?

Extracellular fibronectin and cytoplasmic actin filaments have been shown to be closely associated in fibroblasts (Heggness, Ash & S. J. Singer, 1978; Hynes & Destree, 1978; 1. Singer, 1979). Staining of focal contacts for fibronectin in rat dermal fibroblasts has been reported by Rees et al. (1978) and coincidence between actin fibronectin, vinculin and focal contacts in NiL8 fibroblasts by I. Singer (1982). On
the other hand, some workers have reported that fibronectin distribution alternates with that of actin-containing focal contacts (Birchmeier et al. 1980), while others show that fibronectin appears to be associated with close contacts (regions with approx. 30 nm cell-substratum separation) rather than focal contacts (Chen & S. Singer, 1981, 1982; Norton & Izzard, 1982). Avnur & Geiger (1981) found that chicken gizzard fibroblasts in many cases actually removed adsorbed fibronectin from the substratum at many but not all focal contacts. We have found that focal contacts of chick embryo corneal epithelial cells lack fibronectin (Mattey & Garrod, 1983).

In this paper we report some studies on HeLa cells that have a bearing on the relationship between focal contacts and fibronectin. HeLa cells are from a human cervical carcinoma line (Scherer, Syverton & Gey, 1953) that has been maintained in culture for many generations. It may be that they should not be compared too closely with normal fibroblasts since they are of epithelial origin and highly transformed. However, on glass substrata they form large, distinct focal contacts that are positioned at the ends of actin microfilament bundles. These focal contacts do not appear to require fibronectin for their formation, nor do they appear to contain fibronectin.

MATERIALS AND METHODS

Cell culture

HeLa cell line was purchased from Flow Laboratories, Irvine, Scotland and maintained in monolayer culture at 37°C. Cells were cultured in Minimal Essential Medium with Earle's salts (EMEM) supplemented with 10% newborn bovine serum (NBS), 100 units penicillin, 100 µg streptomycin, 1 mM-glutamine and non-essential amino acids (Flow). The monolayers were trypsinized with 0.25% trypsin in Hanks' salts (Flow) plus 1 mM-EDTA. The resulting suspension was centrifuged, resuspended in a solution of soya bean trypsin inhibitor (Sigma) at 0.1 mg/ml then recentrifuged. Cells for microscopy were seeded onto 18 mmX18 mm coverslips (size 0, Chance Propper). Chick embryonic limb bud mesenchyme cells were obtained and cultured as described by Nicol & Garrod (1979). For treatment with anti-fibronectin immunoglobulin G (IgG) they were cultured in EMEM plus 10% fibronectin-depleted foetal calf serum.

Preparation of fibronectin and fibronectin-depleted serum

Fibronectin (FN) was prepared from citrated chicken plasma by affinity purification on a gelatin-Sepharose 4B column according to the method of Engvall & Ruoslhati (1977). NBS or foetal bovine serum (FBS) was depleted of fibronectin by passing samples three times through a gelatin-Sepharose 4B column. Fractions containing proteins that were not bound to the column were concentrated to the original volume.

Preparation of goat anti-fibronectin antibody

Fibronectin prepared by gelatin-Sepharose chromatography was electrophoresed on a preparative 15% (w/v) polyacrylamide gel (Laemmli, 1970). The fibronectin-containing band, as shown by Coomassie Blue staining, was cut from the gel, eluted, and used to immunize a goat. Fibronectin at approximately 300 µg/ml was mixed and emulsified with Freund's complete adjuvant for the first three injections and Freund's incomplete adjuvant for a fourth injection; 500 µl of the emulsion was injected at each of two intramuscular sites. Injections were given at 4-week intervals and a test bleed taken 7 days after the final injection was positive for anti-fibronectin; 500 ml of blood was taken on the eighth day and the antibody titre of the serum by fluorescent antibody staining of chick embryonic limb bud cells was found to be > 1/242,000. This anti-chick fibronectin antibody was found to recognize human fibronectin both on Ouchterlony plates and in immunoprecipitation from human plasma. Rabbit anti-human fibronectin was purchased from BRL Ltd.
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**Treatment of cultures with anti-fibronectin antibody**

The IgG fractions were prepared from pre-injection and anti-fibronectin goat sera; 2 ml of each IgG was dialysed for 24 h against 100 ml of EMEM containing penicillin, streptomycin, glutamine and Fungizone. After dialysis the IgGs were sterilized by filtration through a Swinnex filter (Millipore).

Trypsinized cells were suspended in EMEM containing 10% fibronectin-depleted calf serum. This cell suspension was then diluted to approximately $5 \times 10^5$ cells/ml with either pre-injection IgG or anti-fibronectin IgG. The final concentration of the IgG solution was 3-5 mg/ml. This treatment has already been shown to disrupt the extracellular matrix in fibroblasts (Yamada, 1978) and chick embryonic limb bud cells (see Results).

**Adsorption of proteins to coverslips**

Glass coverslips were washed in 1% Decon followed by distilled water and then sterilized. Solutions of bovine serum albumin (BSA), polylysine, concanavalin A (ConA) and fibronectin at 2 mg/ml, and 2% calf serum were applied to the coverslips, which were incubated at room temperature for 1 h to allow adsorption of the proteins before removing excess solution. HeLa cells were plated onto the coverslips and allowed to settle for 24 h at 37°C in serum-free EMEM, after which they were fixed and examined for the presence of focal contacts using interference reflection microscopy (IRM) and for fluorescent antibody staining.

**Fibronectin addition**

HeLa cells were cultured on glass coverslips for 24 h using serum-free EMEM. The medium was then removed and replaced with fresh serum-free EMEM containing 0.1, 0.25 or 0.5 mg/ml fibronectin for a further 24 h. Fluorescent antibody staining and IRM were used to detect fibronectin and focal contacts.

**Interference reflection microscopy**

This was carried out using a Zeiss photomicroscope III with a HBO50 mercury vapour light source, a green band pass filter (546 nm) and a ×63 Antiflex objective.

**Fluorescent antibody staining**

Fluorescent antibody staining for actin and fibronectin was carried out according to the method described by Billig et al. (1982) but using fluorescein (FITC)-conjugated sheep anti-goat immunoglobulin (Wellcome) for anti-fibronectin detection. Fluorescence was observed on a Zeiss photomicroscope III as described by Nicol & Garrod (1979).

**Monensin treatment**

HeLa cells were cultured on glass coverslips in EMEM containing 10% fibronectin-depleted serum plus the ionophore monensin (Calbiochem). Virtanen, Vartio, Badley & Lehto (1982) reported an effect using 1 μm monensin on human fibroblasts, so concentrations of 1, 2 and 5 μm were used here. Treated cells were fixed, stained with fluorescent antibodies and examined for the presence of fibronectin and focal contacts. To ensure that there was no toxic effect, treated coverslips were washed and the cells were cultured for a further 24 h in EMEM with fibronectin-depleted serum and examined for recovery of focal contact formation.

**Inhibition of protein synthesis**

Cycloheximide (Sigma) was used in a range of concentrations from 0.2 μg/ml to 20 μg/ml to attempt to inhibit protein synthesis. Trypsinized cells were cultured either in EMEM plus fibronectin-depleted serum and cycloheximide for 24 h or in serum-free EMEM for 24 h followed by addition of cycloheximide for a further 24 h. Some cells from each group were washed and cultured for a further period in medium without cycloheximide. All of the cells were fixed, stained and then examined for fibronectin and focal contacts.
Metabolic labelling of cells

The cells were seeded into plastic culture flasks (Nunc) at 2.5 × 10⁶/flask in complete medium containing either normal or fibronectin-depleted serum. [³⁵S]methionine (1350 Ci/mmol, Amersham International) was added to the medium at 10 μCi/ml and the cells were cultured for 48 h.

Immunoprecipitation of fibronectin

Samples were taken from the culture medium of labelled cells at 24 and 48 h. The cells were extracted after 48 h by a modification of the method of Yamada, Yamada & Pastan (1975). The monolayers were washed three times in phosphate-buffered saline (PBS), then extracted for 2 h in a freshly prepared solution containing 1 M urea, 2% dimethyl sulfoxide and 1 mM phenylmethylsulphonyl fluoride (PMSF) (Sigma) in PBS (pH 7.3). The extracted monolayer was washed with PBS and solubilized with 2% sodium dodecyl sulphate (SDS), 1% β-mercaptoethanol (Sigma). The cell extracts were dialysed against several changes of PBS for 2 days at 4°C; 450 μl of each sample of medium, cell extract or solubilized monolayer was added to 1 ml of 0.4 M NaCl, 5 mM-EDTA, 1% Triton X-100 in 25 mM-Tris-HCl (pH 8); 30 μl of goat anti-chicken plasma fibronectin was added to each sample and the mixture was incubated at 4°C overnight; 100 μl of a 50% suspension of protein A-Sepharose CL-4B (Sigma), pre-swollen in the Triton/Tris buffer, was added and the mixture was incubated for 2 h at 4°C on a blood mixer. The beads were spun down, washed twice in buffer solution and once in 10 mM-Tris-HCl (pH 6.8), then resuspended in sample buffer for gel electrophoresis. After boiling the samples for 5 min they were loaded onto 10% (w/v) polyacrylamide slab gels (Laemmli, 1970); following electrophoresis the gels were stained with Coomassie Brilliant Blue-R, destained and dried. The dried gels were autoradiographed for 4 weeks at −20°C using Fuji RX X-ray film.

RESULTS

After culture overnight (16–18 h) on glass coverslips in complete tissue-culture medium, HeLa cells formed numerous prominent focal contacts which were present in almost every cell and were positioned at the ends of actin microfilament bundles (Figs 1, 2). However, the majority of cells showed negligible staining for fibronectin, no matter whether anti-chicken fibronectin or anti-human fibronectin was used to detect it (Figs 3, 4). In fact, only 1–3% of the cells showed any indication of a
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Figs 1–8
detectable fibrillar fibronectin matrix and, even when they did, the pattern of staining did not correspond to that of the focal contacts (Figs 5, 6). This was true whether the cells were simply fixed in paraformaldehyde or, additionally, made permeable with acetone or 1% Triton X-100. It is important to note that treatment of cells with anti-fibronectin antibody rendered the fibronectin matrix visible by IRM (Figs 5, 6). These fine matrix fibrils should not be confused with focal contacts.

These results raised the possibility that HeLa cells are able to form focal contacts in the absence of fibronectin and a series of experiments were performed in order to test this. Firstly, cells were plated onto glass coverslips in serum-free EMEM to which 0.1% soya bean trypsin inhibitor had been added. After 24 h under these conditions the majority of cells had an appearance similar to that shown in Fig. 7. We interpret

Figs 9–14.
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such large dark areas at the cell periphery as thin lamellae (Gingell, 1981), which may or may not be closely applied to the substratum. The fine fibrillar processes at the cell periphery also may or may not be closely applied to the substratum. Very occasionally cells have been found to possess focal contacts under these conditions. They were extremely rare, however, and not found in every culture. Identical results were obtained when cells were plated in serum-free medium on coverslips coated with chicken plasma fibronectin, gelatin, concanavalin A, poly(l-lysine) and bovine serum albumin (Fig. 8). Thus cells that were cultured in serum-free medium were defective in focal contact formation, even when the substratum has been previously coated with protein and even when that protein was fibronectin. Is this situation reversible and under what conditions?

When complete medium was added to cells that had been cultured in serum-free medium for 24 h they recovered to form the normal complement of focal contacts. The first appearance of focal contacts in such cells occurred after 4—6 h of serum replacement and by 24 h their appearance by IRM was comparable to that of controls. Is this recovery dependent upon added fibronectin? Cells were next treated with medium containing fibronectin-depleted serum after culture for 24 h in serum-free medium. Once again the focal contacts returned, beginning at 4—6 h and appearing as in control cultures after 24 h (Fig. 9).

Although the fibronectin-depleted serum used in these experiments had been thrice passed down a gelatin-affinity column and appeared to be free of fibronectin on

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Fig. 9. Cells cultured for 24 h in serum-free medium followed by 24 h in medium containing fibronectin-depleted serum, showing abundant focal contacts. Bar, 20 μm.

Fig. 10. Four-day-old chick embryonic limb bud mesenchyme cells prepared as described by Nicol & Garrod (1979) and cultured for 24 h in medium containing 4 mg/ml goat IgG from pre-injection serum. The fibrillar fibronectin matrix has been revealed by fixing with paraformaldehyde, staining with specific anti-fibronectin antibody and viewing by u.v. light. Bar, 40 μm.

Fig. 11. A culture of limb bud cells parallel to that shown in Fig. 10, but treated for 24 h with specific anti-fibronectin IgG at 3.5 mg/ml. Staining reveals aggregates of fibronectin–IgG complexes but no fibrillar matrix. Magnification as Fig. 10.

Fig. 12. HeLa cells viewed by IRM after culture for 24 h in serum-free medium followed by 24 h in medium containing fibronectin-depleted serum and 3.5 mg/ml of specific anti-fibronectin IgG. Note abundant focal contacts. Magnification as Fig. 9.

Fig. 13. Immunoprecipitation experiments with specific anti-human fibronectin antibody after labelling HeLa cells for 48 h with [35S]methionine. Lanes a to d, polyacrylamide gel stained with Coomassie Blue; lanes e to h, autoradiographs of lanes a to d. A sample of medium shows very few staining bands (lane a) but, nevertheless, there are some heavily labelled bands (lane c), demonstrating that proteins are synthesized by the cells and released into the medium. Lane b shows specific immunoprecipitation of fibronectin (FN) from the medium. The intense band near the bottom of the gel is IgG heavy chain. This fibronectin is unlabelled, however (lane f), and is therefore entirely derived from the serum in the medium. Lane c showed a 1 M-urea extract of the monolayer, many of the bands being heavily labelled (lane g). Immunoprecipitation of the urea extract shows no fibronectin band either by Coomassie Blue staining (lane d) or by autoradiograph (lane h).

Fig. 14. HeLa cells after culture for 24 h in complete medium plus 1 μM-monensin, showing prominent focal contacts. Magnification as Fig. 9.
overloaded polyacrylamide gels, it is nevertheless extremely difficult to be certain that trace amounts of fibronectin are not added by this technique. In order to substantiate this result, therefore, the formation of focal contacts was titrated against concentrations of normal and fibronectin-depleted serum. If the result was dependent on added fibronectin, focal contacts would be expected to form at a lower concentration of normal serum than of fibronectin-depleted serum. In both normal and fibronectin-depleted serum control-like focal contacts were formed down to the rather low concentration of 0.05% after 24 h of culture. Below that concentration cells had an appearance comparable to that in serum-free medium.

\[ \text{Fig. 13. For legend see p. 139.} \]
The above results suggest that HeLa cells do not require the presence of fibronectin in the medium in order to form focal contacts. Does the addition of fibronectin to cells in serum-free medium, nevertheless, promote the formation of focal contacts? Chicken plasma fibronectin at concentrations of 100, 250 and 500 μg/ml was added to cells during culture for 24 h on a glass surface in serum-free EMEM. After this time cells were comparable in appearance to control cells in serum-free medium, i.e. focal contacts were extremely rare. There did, however, seem to be rather more association of fibronectin with the cells than with controls as judged by fluorescent antibody staining, suggesting that fibronectin in the medium can bind to the cell surface to some extent.

Added fibronectin does not therefore seem to be required for, or to promote, focal contact formation by HeLa cells. It is, however, just possible that the cells produce their own fibronectin and incorporate it into focal contacts, and that such fibronectin is not accessible to fluorescent antibody staining even in cells that have been made permeable by treatment with acetone or Triton X-100. The following experiments have been done in an attempt to exclude this possibility.

Addition of anti-fibronectin antibody to living cell cultures causes extensive and dramatic reorganization of the fibronectin extracellular matrix (Yamada, 1978; Billig et al. 1982). The effect of our goat anti-fibronectin IgG on the matrix formed by chick embryo limb bud cells is shown in Figs 10 and 11. In view of this, can HeLa cells recover to form focal contacts in the presence of fibronectin-depleted serum plus anti-fibronectin IgG? Presumably, the latter, being present in the culture during recovery, should complex with and disrupt any fibronectin produced by the cells themselves. It was found that cells cultured for 24 h in serum-free medium and then treated with medium containing fibronectin-depleted serum plus 3·5 mg/ml anti-fibronectin IgG recovered to produce a pattern and number of focal contacts identical to that of control cells (recovery in pre-injection IgG), with the first appearance of focal contacts being at 4–6 h (Fig. 12).

HeLa cells labelled for 48 h with [35S]methionine showed no incorporation of label into fibronectin (Fig. 13). Thus no labelled fibronectin was immunoprecipitated by anti-human fibronectin (Fig. 13) or anti-chick fibronectin (not shown) from the medium, the urea extract of the monolayer or the SDS—mercaptoethanol extract of the monolayer. That the antibody precipitated unlabelled fibronectin from serum-containing medium is shown in Fig. 13, lane b. Further positive controls for this experiment are given by Matthey & Garrod (1984), who showed that metabolically labelled fibronectin could be detected in chick embryo corneal epithelial cells using these techniques and reagents. We conclude that HeLa cells do not synthesize quantities of fibronectin detectable by these methods.

The monovalent ionophore, monensin, inhibits the secretion of fibronectin, as well as other proteins, from cells in culture (Ledger, Uchida & Tanzer, 1981). Furthermore, it has been shown to inhibit the formation of focal contacts by human fibroblasts (Virtanen et al. 1982). When HeLa cells were cultured in the presence of 1 μM monensin (the concentration used by Virtanen et al.) they developed a normal complement of focal contacts after 24 h (Fig. 14).
In order to determine whether formation of focal contacts after trypsinization was dependent upon protein synthesis, HeLa cells were seeded in cycloheximide at different concentrations. Cells formed focal contacts at concentrations up to 1 μg/ml. Focal contact formation was inhibited at 2 μg/ml but recovery occurred after replacement of cycloheximide-containing medium with normal medium. At 10 μg/ml irreversible inhibition of focal contact formation occurred.

**DISCUSSION**

The results of this study suggest that the focal contacts of HeLa cells do not depend upon fibronectin for their formation. Firstly, exogenous fibronectin is not necessary for the formation of focal contacts because they form comparably in serum-containing and fibronectin-depleted medium. Secondly, exogenous fibronectin does not promote focal contact formation either when absorbed to the substratum or added to the medium. Thirdly, the cells do not synthesize detectable amounts of fibronectin as judged by metabolic labelling and immunoprecipitation with specific anti-fibronectin antibody. Finally, the cells form focal contacts in the presence of high concentrations of anti-fibronectin IgG, concentrations that cause disruption of the extracellular matrix of cultured chick heart fibroblasts.

The controversy about the relationship between focal contacts and fibronectin, outlined in the Introduction, has largely been associated with work on cultured fibroblasts. HeLa cells, on the other hand, are highly transformed epithelial cells. We do not, therefore, claim that our observations are of general importance, but merely point out that there are certain similarities between the focal contacts of fibroblasts and those of HeLa cells. Thus, in addition to demonstrating that HeLa cell focal contacts are located at the ends of actin microfilament bundles, we should point out that we have obtained weak but positive staining of HeLa cell focal contacts with anti-chick vinculin antibody (kindly donated by Dr S. Kellie).

Localization of vinculin to focal contacts is a feature of fibroblasts (Geiger, 1979; Geiger et al. 1980; Burridge & Feramisco, 1980). In this connection it is pertinent to mention that the focal contacts of primary chick embryo corneal epithelial (CE) cells, which are essentially fibroblast-like (Billig et al. 1982), stain brightly for vinculin (D. L. Mattey, unpublished). Although the focal contacts of CE cells are broadly dependent on the establishment of a fibronectin-containing matrix, some of these focal contacts apparently do not contain fibronectin (Mattey & Garrod, 1984). While noting that there are differences in details of structure between the stress fibres of fibroblasts and epithelial cells (Sanger, Sanger & Jockush, 1983), we feel that there may be an essential similarity between the focal contacts of fibroblasts and epithelial cells so that one may be used as a model for the other.

Virtanen et al. (1982) showed that both focal contact formation and fibronectin secretion were inhibited by monensin in cultured human fibroblasts. Our result showing that HeLa cells form focal contacts in the presence of monensin could therefore be taken as supporting evidence that fibronectin is not required for focal contact formation. However, we do not interpret the result in this way, because monensin is
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not specific for fibronectin and affects the secretion of other proteins (Ledger et al. 1981). Our result seems to suggest one of two things: either the protein components necessary for focal contact formation are present on the cell surface even in trypsinized cells, or the exteriorization of these components is not inhibited by monensin. Because cycloheximide reversibly inhibits focal contact formation we tentatively favour the latter of these possibilities. It may be, however, that since we have not studied the effect of monensin on protein glycosylation quantitatively, we have not used the correct dose of monensin, even though this would mean that the crucial dose must fall within a very narrow concentration range, between 1 μg/ml, which is ineffective, and 5 μg/ml, which is toxic.

What other possible mechanisms of focal contact formation can be envisaged? Couchman, Höök, Rees & Timpl (1983) have demonstrated that human embryo fibroblasts form focal contacts with equal facility on fibronectin and laminin-coated substrata. We have therefore stained HeLa cells with anti-laminin antibody (kindly provided by Dr B. L. Hogan) but can detect no significant staining of the matrix of permeabilized cells. (An absence of anti-laminin staining was also found in the matrix of corneal epithelial cells in culture (Billig et al. 1983). In that case, significant fibronectin staining could be detected beneath the cells (see also Mattey & Garrod, 1984).)

Another exogenous factor that may be involved in the formation of focal contacts is the 70000 molecular weight spreading factor of Knox & Griffiths (1980), although little information is currently available on this.

Maupin & Pollard (1983) have shown the presence of an extensive area of clathrin basket-work adjacent to areas of cell–substratum adhesion that has been identified as focal contacts by electron microscopy. There is no indication as to whether the extracellular face of the membrane opposite to the clathrin plaques bears adhesive molecules, though this must be considered a possibility. If it is so, we suggest that this represents an adhesion mechanism supplementary to that provided by focal contacts.

Oesch & Birchmeier (1982) have obtained a monoclonal antibody that inhibits attachment of chick embryonic fibroblasts to the substratum and is localized to focal contacts. This antibody recognizes an antigen of 500000 molecular weight, which reduces to 60000. This molecule seems to be a surface component of focal contacts and it would be most interesting to know whether it is also present in the focal contacts of epithelial cells.

A final speculation is that focal contacts are specific membrane structures that depend upon some generalized property of the substratum, such as wettability, for their formation. It may therefore simply be coincidence that association between fibronectin and focal contacts is sometimes found. Fibronectin may be just one component of the extracellular matrix that provides a suitable substratum for focal contact formation, but is not an essential requirement for it.

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