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Cell Biology

Fibronectin is not present in the focal adhesions formed between normal cultured fibroblasts and their substrata

(cell adhesion/cryoultramicrotomy/immunoelectron microscopy/transmembrane interactions)

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ABSTRACT **Fibronectin is an extracellular matrix protein that has been implicated in the spreading and adhesion of cultured fibroblasts to their substrata. In this paper, double immunoelectron microscopic labeling experiments for fibronectin and for concanavalin A-binding proteins on the cell surface were carried out on ultrathin frozen sections of cultures of embryonic chicken heart fibroblasts. On cross sections through the focal adhesions of the cell to the substratum there was substantial labeling for concanavalin A-binding proteins but no detectable labeling for fibronectin, whereas both the binding proteins and fibronectin were extensively labeled elsewhere on the cell surface and substratum. These results demonstrate that fibronectin is not present within the sites of focal adhesions. Therefore, the functions of fibronectin in cell spreading and adhesion are not directly mediated through its binding at focal adhesion sites. An alternative model is presented which can account for such fibronectin functions.**

The molecular structures at the sites where cultured fibroblasts adhere to one another and to the substrata on which they grow are subjects of much current interest. One reason for this interest is that the adhesive properties of normal fibroblasts *in vitro* are markedly reduced upon transformation by oncogenic viruses and other agents, and the inference has been drawn that this decreased adhesiveness may be associated with the release and subsequent invasiveness of malignant cells *in vivo* (1). When well spread on their substrata, normal fibroblasts adhere to such surfaces at two types of discrete sites (2–6). One type, called focal adhesions, is characterized by a set of small punctate regions of intimate approach (10–15 nm) of the ventral cell surface to the substratum. The focal adhesions are probably responsible for the strong adhesion of the cell to its substratum and are the sites where, inside the cell, bundles of microfilaments appear to terminate at the cell membrane (5, 7). The second type of adhesive sites, called close contacts, is characterized by a set of broader areas, often surrounding or immediately adjacent to focal adhesions, where the cell surface is somewhat further separated from the substratum (≈30 nm) than at the focal adhesions. Close contacts are thought also to contribute to cell adhesiveness, but less strongly than the focal adhesions.

In this paper we confine ourselves to the question: What role does the protein fibronectin play in these adhesion sites? Fibronectin is an extracellular matrix protein (for a review, see ref. 8) that, by a variety of experiments, has been implicated in the adhesion of cultured cells to their substrata (see *Discussion*). The specific suggestion has arisen that fibronectin may mediate cell adhesion by its presence between the cell surface and the substratum at the focal adhesions. In preliminary experiments in this laboratory to test this suggestion, B. Geiger (personal communication) studied the distribution of fibronectin on the ventral cell surfaces of cultured fibroblasts by immunofluorescence microscopy; the same cells were also immunofluorescently labeled for the protein vinculin (9, 10), which served as an intracellular marker for the focal adhesion sites. In addition, these sites were independently identified by interference reflection microscopy (2–6). The findings were that immunofluorescent labeling for fibronectin was observed adjacent to the focal adhesion sites, but was absent from the sites themselves. This result, however, suffered from a crippling ambiguity: the molecules of the antibody reagents used to immunolabel the fibronectin might be too large to have penetrated the narrow gap between the cell surface and the substratum at the sites of the focal adhesions, and any fibronectin that was present within those sites might therefore have remained unlabeled in such experiments. Other studies were therefore undertaken to investigate this problem (11).

The approach described in this paper was to use double-labeling methods in immunoelectron microscopy to investigate the focal adhesion sites in cross section. If immunolabeling for fibronectin was always absent in the region of the focal adhesion sites but some other cell surface components were always immunolabeled within the same sites, it could be concluded that fibronectin was not present in those sites. For our present purposes, we chose as the other cell surface components those glycoproteins with a binding affinity for concanavalin A (Con A). The Con A-binding sites of the glycoproteins are located on the exterior face of the cell surface membrane (12). The experiments were then carried out as follows. The method of cryoultramicrotomy (13) was adapted to prepare ultrathin frozen sections of fixed fibroblasts in monolayer culture. These sections were then immunolabeled for observation by transmission electron microscopy by use of an indirect double-labeling technique with ferritin–antibody and Imposil–antibody reagents (14) to visualize both fibronectin and Con A-binding proteins (CBP) in the same section. In such ultrathin sections, where the plane of sectioning was nearly perpendicular to the plane of the substratum, the focal adhesions could be identified as regions of very close approach of the cell surface to the substratum. Focal adhesions, seen in cross section, were found to be regularly and extensively immunolabeled for CBP but showed no labeling for fibronectin. Substantial immunolabeling of fibronectin and CBP was seen, however, elsewhere on the ventral cell surface. We can therefore conclude that fibronectin is not present in the focal adhesions. The molecular basis of cell

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Abbreviations: Con A, concanavalin A; CBP, Con A-binding proteins; ECH fibroblast, embryonic chicken heart fibroblast.

adhesion to substrata and the role of fibronectin in that adhesion are briefly considered in the light of this result.

MATERIALS AND METHODS

For use as an antigen, plasma fibronectin was purified from fresh chicken plasma by affinity chromatography on columns of gelatin-Sepharose 4B (15). Plasma fibronectin in complete Freund's adjuvant was used to immunize rabbits and guinea pigs. Rabbit antibodies to Con A, rabbit and guinea pig antibodies to fibronectin, goat antibodies to rabbit IgG and to guinea pig IgG, and guinea pig antibodies to goat IgG were prepared and each was purified by affinity chromatography by standard procedures (9). Ferritin-conjugated antibodies and Imposil-conjugated antibodies were prepared as described (14). Rhodamine-conjugated goat antibodies to guinea pig IgG was a standard preparation (9).

Embryonic chicken heart (ECH) fibroblasts were derived from ventricle explants from 10-day-old chicken embryos by a sandwich method (16). Fibroblasts between three and seven passages were used. Specimens were prepared for cryoultramicrotomy by growing the cells on carbon-coated crosslinked gelatin films on glass coverslips. These films were prepared by coating the coverslips with a thin layer of 7% (wt/vol) gelatin solution, which was then dried at 37°C. The gelatin film was then coated with a 10- to 20-nm thick carbon film in a vacuum evaporator. This was followed by treatment with 4% (vol/vol) glutaraldehyde overnight at 4°C. Some of these coated coverslips, to be used for immunofluorescence studies, were then treated exhaustively with $NaBH_4$ (5 mg/ml) to eliminate the autofluorescence introduced by the glutaraldehyde. After thorough washing of the coverslips with Dulbecco's modified Eagle's medium, followed by washing with the medium containing 10% (vol/vol) fetal calf serum, the substrate were ready for cell culture.

After 1–12 hr in culture, the ECH fibroblasts were fixed with a two-stage procedure (17). They were first treated with 60 mM ethylacetimidate/3% (vol/vol) formaldehyde/2% (wt/vol) sucrose in phosphate-buffered saline (pH 7.8) for 5 min at room temperature and then with 2% glutaraldehyde/3% formaldehyde/2% sucrose in phosphate-buffered saline for 30 min. For immunoelectron microscopy, fixed cells were infused with 0.6 M sucrose in 1% agarose/0.02% NaN_3 in phosphate-buffered saline for 15 min at 37°C. The crosslinked gelatin film with the fixed cells attached to it was then peeled off the coverslip with a forceps and rolled up into a cylindrical roll in the presence of the infusion solution. The roll was cut into 2-mm blocks; the blocks were then frozen in liquid N_2 and cut into ultrathin sections in the frozen state (−90°C) (13).

The thawed ultrathin frozen sections were immunolabeled either singly for fibronectin or for CBP or doubly for both components by indirect labeling procedures. Only the double-labeling experiments are presented in this paper. In these experiments, the fibronectin was always first treated with its specific antibodies to avoid any problems of steric interference from the other labeling reagents. A typical sequence of successive labeling reactions, with intervening washes, was as follows: (*i*) guinea pig antibodies to fibronectin (20 μg/ml); (*ii*) Con A (1 μg/ml); (*iii*) goat antibodies to guinea pig IgG (20 μg/ml); (*iv*) rabbit antibodies to Con A (10 μg/ml); (*v*) Imposil-conjugated guinea pig antibodies to goat IgG (100 μg/ml); and (*vi*) ferritin-conjugated goat antibodies to rabbit IgG (60 μg/ml). This procedure labeled fibronectin with Imposil particles and Con A (hence CBP) with ferritin. Alternatively, in some labeling experiments, the first four steps were the same as above, but in step *v* ferritin-conjugated guinea pig antibodies to goat IgG were used and in step *vi* Imposil-conjugated goat antibodies to rabbit IgG were used. In such experiments, the labeling was reversed: ferritin labeling for fibronectin and Imposil labeling for Con A. Several types of control experiments were performed. For example, an excess of glutaraldehyde-crosslinked fibronectin was added together with the antibodies to fibronectin or 0.1 M methyl α-D-mannopyranoside was added together with Con A to compete specifically for the labeling of fibronectin or CBP, respectively.

After such immunoelectron microscopic labeling, the ultrathin sections were stained and stabilized by the absorption staining method (18). Specimens were examined in a Philips EM-300 electron microscope at 60 kV.

For immunofluorescence observations, the fixed cells on the $NaBH_4$-treated coverslips were treated with the guinea pig antibodies to fibronectin followed by rhodamine-conjugated goat antibodies to guinea pig IgG and examined as in previous studies (9).

RESULTS

ECH fibroblasts cultured on the carbon-coated crosslinked gelatin films ($\approx$1–5 μm thick) on glass showed cell shapes and patterns of fibronectin deposition on the cell surface (Fig. 1) similar to those of cells grown directly on glass. In particular, by 6 hr after plating there was a substantial amount of immunofluorescent labeling of fibronectin on the ventral surfaces of the cells (Fig. 1).

The rolled-up sheets of cells on the coated gelatin films used in the electron microscopy experiments packed together numerous cells in similar orientations such that ultrathin sections through the rolls contained a large number of cells sectioned in the same plane. In this way, in sections that were nearly perpendicular to the plane of the substratum, we could clearly and regularly discern focal adhesions in cross section, defined as limited regions of closest approach between the outer cell surface and the substratum (Fig. 2). By about 6 hr after plating, filamentous densities were often observed inside the cells (Fig. 2 *C* and *D*) close to the membrane at the sites of these focal adhesions, which most likely corresponded to bundles of microfilaments that are known to terminate at those sites (5, 7).

The double immunolabeling of such morphologically identified focal adhesions regularly showed substantial labeling for

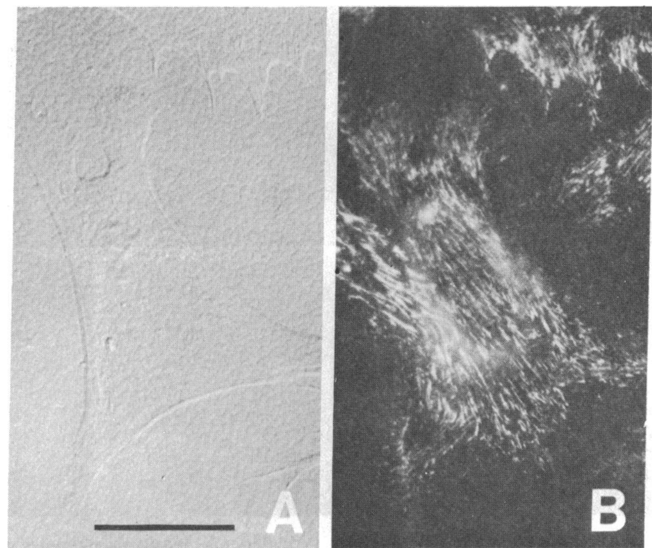

FIG. 1. Fixed ECH fibroblast 6 hr after it was plated on a film of carbon-coated crosslinked gelatin on top of a glass coverslip. (*A*) Nomarski image; (*B*) indirect rhodamine immunofluorescent labeling pattern of the fibronectin on the ventral surface of the same cell. (Bar = 25 μm.)

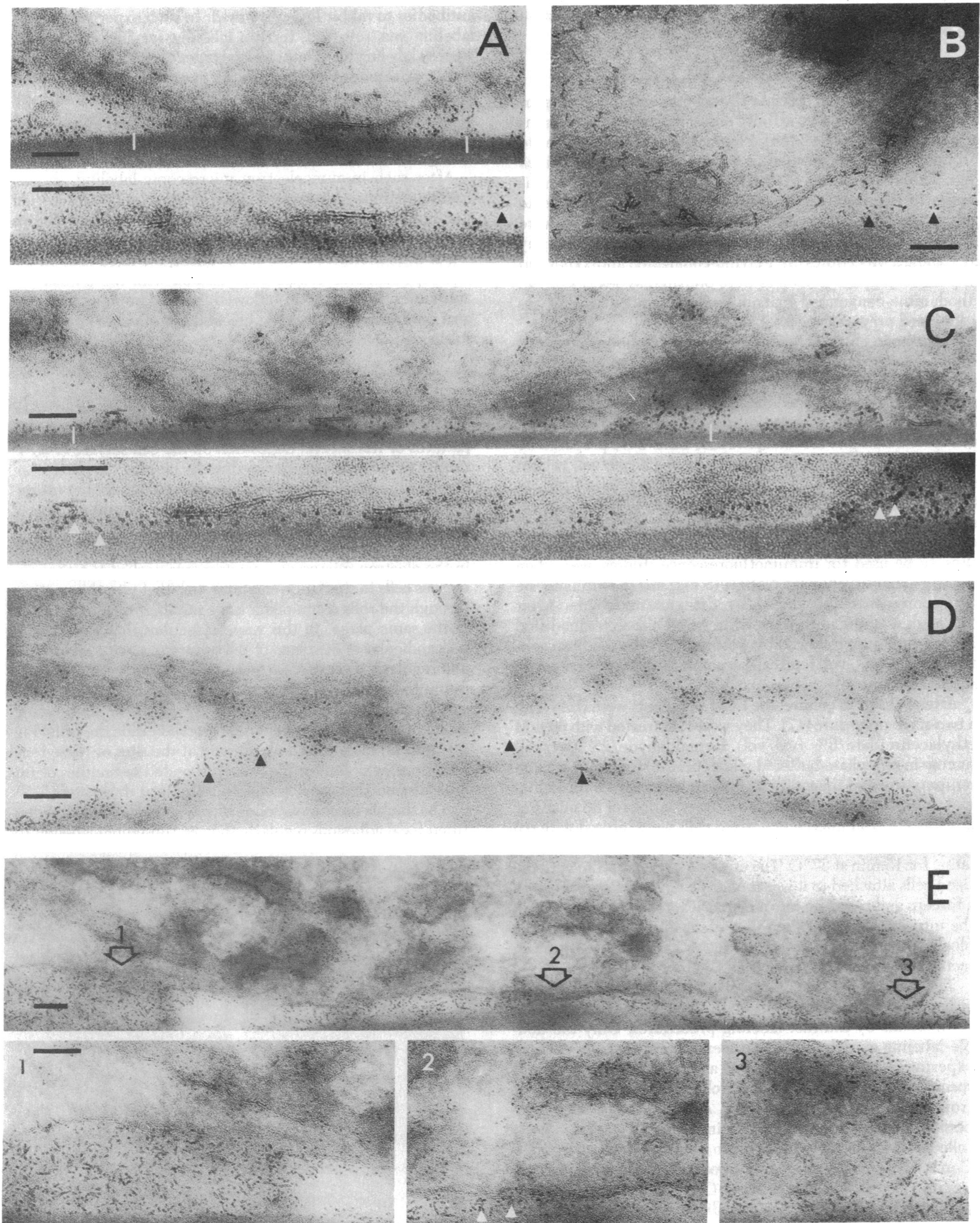

FIG. 2. *(Legend appears at the bottom of the next page.)*

CBP but not for fibronectin. (It should again be emphasized that the fibronectin was always immunolabeled first, so this result cannot be attributed to a prior blocking of fibronectin antigenic sites by Con A.) Close to 100 such sites were examined with clear-cut results. For example, in those experiments in which ferritin conjugates were used to label the CBP and Imposil conjugates to label the fibronectin, only the isometric ferritin particles were observed within the regions of the focal adhesions (Fig. 2 *A*, *C*, *D*, and *E*). Anisometric Imposil particles (small black arrowheads in Fig. 2 *A* and *D*; small white arrowheads in Fig. 2 *C* and *E2*) could often be seen at the edges of the focal adhesion sites, where an increase in separation of the cell surface and substratum was observed, but not within the focal adhesion sites themselves. At sites well removed from the focal adhesions, both ferritin and Imposil labels were plentifully apparent, associated both with the cell surface and the surface of the substratum (Fig. 2*D*). Similar results were obtained if the labels were reversed, with Imposil conjugates used to label CBP and ferritin conjugates to label fibronectin (Fig. 2*B*), demonstrating that both types of conjugates had about the same accessibility to the CBP within the focal adhesion sites. Control experiments indicated that each type of immunolabeling was specific (not shown).

The density of labeling for CBP appeared to be somewhat reduced within the focal adhesion sites compared to regions outside those sites (Fig. 2*D*). There are a number of factors that could account for this difference. The most important of these is that the labeling of CBP within the focal adhesion sites probably occurred only superficially (that is, only close to the surface of the section) because of limited penetrability of the labeling reagents; on the other hand, the labeling of CBP on the cell membrane and on the substratum, which were freely accessible outside of the focal adhesion sites, probably extended through the entire depth of the section. Therefore, in projection, the density of labeling would appear to be smaller within the focal adhesion sites than elsewhere.

Single immunolabeling for either fibronectin or CBP on similar ultrathin sections yielded results that were closely parallel to those obtained with the double immunolabeling experiments; substantial labeling for CBP was observed in the regions within focal adhesion sites, but no significant labeling for fibronectin was detected at such sites in other specimens.

DISCUSSION

Fibronectin is a protein component of the extracellular matrix that is associated with the surfaces of fibroblasts and other cells in culture. Many lines of evidence strongly suggest that fibronectin is somehow involved in determining the shape, cytoskeletal structure, adhesiveness, and other properties of these cells (for review, see ref. 8). Briefly, this evidence is as follows: (*i*) fibronectin is present on the surfaces of normal fibroblasts, which are flat and strongly adherent cells, but is usually absent from transformed fibroblasts, which are more rounded-up and less adherent than the normal cells (1, 8). (*ii*) The addition of fibronectin to certain transformed fibroblasts partially restores the normal shape, cytoskeletal structure, and adhesiveness of these cells (19, 20). (*iii*) Fibronectin in the medium or absorbed to substrata appears to mediate the attachment and spreading of fibroblasts and other cells on the substrata (21–23). (*iv*) A transmembrane association of cell surface fibronectin with intracellular bundles of actin-containing microfilaments has been directly demonstrated by immunofluorescence (24, 25) and electron microscopic (26) observations. These lines of evidence, particularly the last, have led to the suggestion that fibronectin might be present on the cell surface within the sites of focal adhesion formed between the cell and its substratum. However, no direct evidence for this suggestion has been obtained. In this paper, we have found no detectable immunoelectron microscopic labeling for fibronectin on cross sections through the focal adhesions formed by fibroblasts and their substrata, although considerable labeling for fibronectin was found on the cell surfaces (Fig. 1), including regions near the focal adhesions (Fig. 2). This absence of immunolabeling for fibronectin within the focal adhesions cannot be an artifact due to the inaccessibility of the antibody reagents to the narrow gap between the cell surface and substratum at those sites because Con A-binding glycoproteins (either on the outer cell surface or absorbed to the substratum) are immunolabeled within the same sites (Fig. 2). We therefore conclude that fibronectin is not present in significant amounts on the cell surface or on the substratum within the focal adhesions.

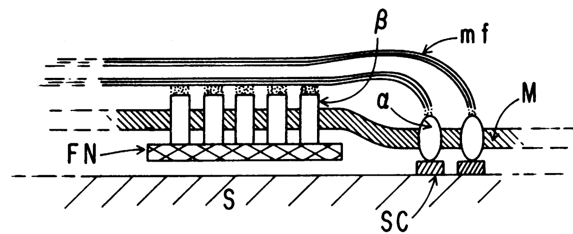

FIG. 3. Highly schematic representation of the possible molecular architecture at two different types of microfilament (mf)–membrane (M) linkages on the ventral surface of a fibroblast in contact with its substratum (S). At the right side of the figure, the end-on type of microfilament–membrane linkage is depicted; at the left, the lateral type is shown. α and β represent two different hypothetical integral membrane proteins associated with the two types of linkage. SC denotes possible serum component(s), and FN, fibronectin molecules or molecular complexes containing fibronectin. Fibronectin is shown as linked (directly or indirectly) to the β integral proteins where the lateral type of microfilament–membrane linkages exists. It may also be directly or indirectly linked to the substratum (not shown). For further details, see text.

It is possible to attribute functions to fibronectin in regulating cell morphology and cell adhesion and to reconcile all of the evidence discussed so far by a scheme that involves two distinct types of microfilament–membrane linkages inside cultured fibroblasts, one end-on and the other lateral (Fig. 3). The end-on type, which occurs at the sites of focal adhesions (5, 7), involves

FIG. 2 (*on preceding page*). Double immunolabeling for fibronectin and CBP on ultrathin frozen sections of ECH fibroblasts cut perpendicularly to the plane of the substratum. (*A–C*) Cells 6 hr after plating; (*D* and *E*) cells 12 hr after plating. In *A*, *C*, *D*, and *E*, ferritin conjugates (seen as circular black dots) were used to label CBP, and Imposil conjugates (seen as elongated rods) were used to label fibronectin. In *B*, the labels were reversed. Immediately beneath *A*, *C*, and *E* are regions of the same figures (between the white lines in *A* and *C* and as indicated by the numbers and open arrows in *E*) that are enlarged to permit better visualization of the labels. In each figure there are regions of intimate approach of the cell surface to the substratum and, within these regions, densities that appear to be filamentous abut the membrane (most clearly seen in *D*). In these regions, taken to be focal adhesions, only CBP labeling (ferritin in *A*, *C*, *D*, and *E*; Imposil in *B*) is observed. The small black arrowheads in *A* and *D* and the white arrowheads in *C* and *E2* point to Imposil particles, which represent the labeling of fibronectin close to the edges of the focal adhesions. Similarly, the small black arrowheads in *B* point to ferritin particles designating fibronectin close to the edge of a focal adhesion. In *E*, area *1* is densely labeled for fibronectin and probably represents a thick cable of fibronectin fibers in cross section, area *2* contains a focal adhesion, and area *3* is a region of close cell–substratum contact at the edge of the cell. (Bars = 100 nm.)

an association of the termini of microfilaments with the membrane. We have suggested that vinculin (9, 10) may participate in this type of linkage as a membrane peripheral protein. In addition to end-on linkages, however, we propose that lateral linkages, all along the length of the microfilaments, can be formed to membranes. Such lateral linkages might be mediated by intracellular peripheral proteins different from vinculin (perhaps including α-actinin). Different transmembrane integral membrane components, say α and β, would be involved in the two types of microfilament–membrane linkages. In the end-on type, the molecules of the specific integral protein(s) α, at their outside-facing regions, might have a strong affinity for certain serum components (22) that were attached to the substratum. Such a set of components and interactions would result in a focal adhesion (Fig. 3, right side). Fibronectin would not be involved. In the lateral type of microfilament–membrane association, specific transmembrane integral protein(s) β in the membrane would be linked (directly or indirectly) to sites along the microfilaments closely apposed to the cytoplasmic surface of the membrane, whereas on the outside-facing surface, β molecules would have an affinity for fibronectin molecules or for some other extracellular matrix component to which fibronectin was bound. Extracellular fibronectin would thereby be linked, through β molecules, to microfilaments inside the cell that were laterally associated with the membrane (Fig. 3, left side). Such fibronectin linkages to β molecules could occur at either the ventral or dorsal surfaces of the fibroblasts. On the ventral surfaces, fibronectin or an extracellular matrix component to which fibronectin was bound might, in addition, have attachment sites to the substratum (see Fig. 2*E1*) that promoted the adhesion of the cell to the substratum at sites that were different from the focal adhesions. This association of fibronectin with the lateral type of microfilament–membrane attachments could account for the colinear distributions of fibronectin and actin-containing microfilaments that have been observed at the surfaces of cultured fibroblasts (24–26).

Any of several possible extensions of this minimal scheme would then assign a role to fibronectin in the regulation of cell morphology and in cell adhesion. For example, the increased concentration and attachment of extracellular fibronectin to β molecules might promote the extent of the lateral type of microfilament–membrane linkages so that any individual microfilament would become attached to the membrane at multiple sites. This could have the morphological effect of flattening the cell membrane because of the rigor imparted by the extended scaffolding of attached microfilaments on the inner surface of the membrane. On the ventral surfaces of the cell, the fibronectin-mediated increase in the lateral type of microfilament–membrane linkages might, by tethering the microfilaments to the membrane as well as the membrane to the substratum, potentiate the formation of nearby end-on microfilament–membrane linkages (i.e., promote the formation of focal adhesions). In such a scheme, fibronectin could therefore induce fibroblast flattening and spreading, as well as increased adhesion to the substratum, without direct participation in the focal adhesions themselves. These and other possibilities are discussed in more detail elsewhere (27).

During these same experiments, we also examined sites of close cell–cell contacts in sections that were doubly labeled by immunoelectron microscopy for fibronectin and CBP (not shown). Sites of close cell–cell contact in cross section showed substantial labeling for CBP but no significant labeling for fibronectin, much as did the sites of focal adhesions at cell–substratum contacts. Fibronectin, therefore, is not present either within close cell–cell contacts or within focal adhesion sites.

As this manuscript was being prepared, the studies of Birchmeier *et al.* (28) were published. By immunofluorescent labeling of cultured WI-38 fibroblasts for fibronectin combined with interference reflection microscopy to locate the focal adhesions, they observed labeling for fibronectin close to, but not at, the sites of focal adhesions. However, as was pointed out in the Introduction, unless it is demonstrated that the antibody reagents used can gain access to and label components that are present within the narrow gap between the cell surface and the substratum at the sites of focal adhesions, the absence of labeling for fibronectin at those sites is an ambiguous result.

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