Localization of Cell Surface Sites Involved in Fibronectin Fibrillogenesis

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Abstract. Fibronectin binding sites on cultured human fibroblasts were localized by high voltage electron microscopy using either 5- or 18-nm colloidal gold beads (Au5 or Au18) bound to intact fibronectin, the 70-kD amino-terminal fragment of fibronectin that blocks incorporation of exogenous fibronectin into extracellular matrix, or 160–180-kD fragments of fibronectin with cell adhesion and heparin-binding activities. Binding sites for Au18-fibronectin on the cell surface were localized to specific regions along the edge of the fibroblast and on retraction fibers. Au18-fibronectin complexes at these sites were initially localized in clusters that co-aligned with intracellular microfilament bundles. With longer incubations, Au18-fibronectin complexes were arranged into long fibrillar networks on the cell surface and in the extracellular space. The appearance of Au18-fibronectin in these fibrillar networks and disappearance of clusters of Au18-fibronectin suggest that Au18-fibronectin complexes are arranged into matrix at specific regions of the cell surface. Au18-70-kD fragment complexes initially had a similar distribution to Au18-fibronectin complexes. With longer incubations, Au18-70-kD fragment complexes were found in long linear arrangements on the cell surface. Double labeling experiments using Au18-70-kD fragment and Au160-180-kD fragments showed that the 70-kD fragment and the 160–180-kD fragments bind to different regions of the cell.

Fibronectin is a connective tissue glycoprotein that consists of two structurally similar but not necessarily identical disulfide-bonded subunits (Schwarzbauer et al., 1985; Sekiguchi et al., 1985). There are two principle types of fibronectin: (a) plasma fibronectin, which appears to be produced by hepatocytes (Tamkun and Hynes, 1983) and is found in the circulation, and (b) cellular fibronectin, which is synthesized by a variety of cell types (for recent reviews see Mosher, 1984; Hynes, 1985). Both types of fibronectin are structurally similar, except for an additional type III homology sequence found in cellular fibronectin (Kornblitt et al., 1984).

Both types of fibronectin can be found in a soluble form in blood, other body fluids, and culture medium, and in an insoluble, usually fibrillar, form in the extracellular space of connective tissues, basement membranes, and cultured cells. Fibronectin found in fibrils can come from either exogenously added plasma fibronectin or cellular fibronectin (Hayman and Ruoslahti, 1979; Oh et al., 1981).

It is not clearly understood how fibronectin and other macromolecules of the extracellular matrix are assembled into an extracellular matrix. In cultured fibroblasts and embryonic tendon and corneal fibroblasts it has been shown that fibronectin (Hedman et al., 1978) and collagen (Tratlad and Hayashi, 1979) fibril formation occur in close association with the fibroblast cell surface. These observations suggest that cells may play a role in regulation of the formation of the extracellular matrix. One way cells can influence the formation of the extracellular matrix is by control of the stoichiometry and sequential appearance of secreted extracellular matrix components. Another way is by events mediated at the cell surface. Recently, a presumptive cell surface receptor that mediates the fibrillogenesis of exogenous fibronectin into extracellular matrix fibrils has been described (McKeown-Longo and Mosher, 1983; 1985). The receptor appears to interact primarily with the 70-kD amino-terminal gelatin- and heparin-binding region of fibronectin, rather than with the site on fibronectin that mediates cell adhesion (McKeown-Longo and Mosher, 1985).

In the present study, we have used high voltage electron microscopy (HVEM)1 to identify sites in the cell layer where exogenous fibronectin and fragments of fibronectin bind, and to study the relationship between cellular structures and extracellular matrix fibrils during fibrillogenesis. For this, human skin fibroblasts in culture were incubated with 18-nm gold beads (Au18) coupled to either fibronectin or the 70-kD fragment of fibronectin that contains the type I and II homology units important for binding to the presumptive matrix assembly receptor. Fragments of fibronectin containing the majority of the type III homology units and such cell binding domains as the cell adhesion and heparin-binding sites (Pierschbacher et al., 1981; McKeown-Longo and Mosher, 1985) were bound to 5-nm colloidal gold beads (Au5), and were used to distinguish other binding sites on fibronectin (Pytel et al., 1985; Akiyama et al., 1986) from the presumptive matrix assembly receptor binding sites.

1 Abbreviations used in this paper: Au5, 5-nm colloidal gold beads; Au18, 18-nm colloidal gold beads; HVEM, high voltage electron microscopy.
Contaminating 40-kD fragment on a Sephadex G-100 column (94 × 3.5 cm) of fibronectin were eluted with 3 M guanidine-HCl in 20 mM Tris-HCl, 0.15 M sodium chloride, pH 7.4. An extra 20% was slowly added to the digestion mixture (which was in 10 mM Tris-HCl, 0.15 M sodium chloride, pH 7.4) and titration to pH 7.4 with 0.5 M Tris base. The digest was then placed in water (10% wt/vol) was bound to colloidal gold sol at pH 9.0. Colloidal gold beads 18 nm in diameter (Au88) were prepared by adding 0.5 ml of 4% gold chloride to 200 ml deionized distilled water (Geoghegan and Ackerman, 1977). To this, 5 ml of freshly prepared 1% trisodium citrate salt solution and placed in fresh media containing 0.2% bovine albumin and Au88-fibronectin (7.0 µg/ml), Au88-70-kD fragment (13 µg/ml), Au88-cell adhesion fragments (90 µg/ml), or Au88-bovine albumin (15 µg/ml). The fibroblasts were incubated for 0.25, 0.5, 1.5, 3, or 5 h at 37°C.

Preparation of Colloidal Gold Fibronectin or Fibronectin Fragments

Colloidal gold beads 18 nm in diameter (Au88) were prepared by adding 0.5 ml of 4% gold chloride to 200 ml deionized distilled water (Geoghegan and Ackerman, 1977). To this, 5 ml of freshly prepared 1% trisodium citrate salt solution was added, and the mixture was boiled under reflux for 30 min until the color turned from brown to red. The colloidal gold sol was filtered through a 0.45-µm millipore filter and stored at 4°C. Colloidal gold beads 5 nm in diameter (Au23) that had been prepared using white phosphorus were kindly provided by Dr. Ralph Albrecht, University of Wisconsin.

Colloidal gold beads were added to fibronectin, fibronectin fragments, or bovine albumin using a modification of the Geoghegan and Ackerman (1977) procedure. Colloidal gold sol (Au23) was adjusted to pH 7.0 with 0.2 N potassium carbonate and filtered through a 0.45-µm millipore filter. The minimum concentration of fibronectin needed to stabilize 1 ml of the gold sol was determined with an adsorption isotherm (Horsiburger and Rosset, 1977) and found to be between 5 and 10 µg of plasma fibronectin in 2 mM Tris-HCl, 15 mM sodium chloride, pH 7.4. This minimum concentration of plasma fibronectin plus an extra 20% was slowly added to the adjusted gold sol and incubated for 5 min at 23°C. Unbound areas on the gold beads were blocked with 1% polyethylene glycol (final concentration 0.05%) filtered through a 0.2-µm filter. Au23-fibronectin was centrifuged at 28,000 g for 30 min at 4°C. The protein-gold complex was resuspended into assay buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.4, containing 0.05% saponin and 0.2% tannic acid) and 25I-fibronectin bound to gold. The 25I-fibronectin-gold complex retained >60% of the cell-binding activity of uncomplexed 25I-fibronectin. Colloidal gold complexed to fibronectin fragments were prepared in a similar manner except that the pH of the colloidal gold sol was 7.2. Albumin in water (80% w/v) was bound to colloidal gold sol at pH 9.0.

Preparation of Fibronectin and Fibronectin Fragments

Plasma fibronectin was purified from a commercial by-product of human plasma Factor VIII production (Mosher and Johnson, 1983). The 70-kD fragment of fibronectin (Balian et al., 1979) was prepared by digesting fibronectin (2 mg/ml) with cathepsin D (3 µg/ml) in 50 mM sodium acetate buffer, pH 3.5, containing soybean trypsin inhibitor (3.2 µg/ml), and phenylmethylsulfonyl fluoride (8 µg/ml) for 16 h at 37°C. The digestion was initiated by forcefully injecting a concentrated fibronectin solution (which was in 30 mM Tris-HCl, 0.15 M sodium chloride, pH 7.4) under the surface of the acetate buffer containing the cathepsin D, taking care to avoid foaming. The digestion was stopped with pepstatin A (0.2 µg/ml) and titration to pH 7.4 with 0.5 M Tris base. The digest was then placed on a gelatin-Sepharose column equilibrated with 20 mM Tris-HCl, 0.15 M sodium chloride, pH 7.4, and the gelatin-binding 70- and 40-kD fragments of fibronectin were eluted with 3 M guanidine-HCl in 20 mM Tris-HCl, 0.15 M sodium chloride, pH 7.4. The 70-kD fragment was purified free of the contaminating 40-kD fragment on a Sephadex G-100 column (94 × 3.5 cm) equilibrated with 20 mM Tris-HCl, 0.15 M sodium chloride, pH 7.4. Polyacrylamide slab gel electrophoretic analysis in the presence of sodium dodecyl sulfate showed that the purified fragment migrated as a single band with an apparent size of 70 kD (data not shown).
Figure 1. A stereo micrograph of a whole mount of an embryonic human skin fibroblast (S132) incubated with Au₁₈-fibronectin for 15 min at 37°C. Clusters of Au₁₈-fibronectin and single beads of Au₁₈-fibronectin on the dorsal surface of the fibroblast (arrowheads) and on the ventral surface of the cell facing the substratum (white arrow) appear to co-align with intracellular microfilaments. Au₁₈-fibronectin clusters are also seen wrapping around the edge of the cell (large arrows) and on the dorsal surface connected to filaments of Au₁₈-fibronectin (small arrow). Stereo tilt 7.4° from horizontal. Bar, 0.5 μm.

The cell surface labeling pattern was examined in 3–4-d-old cultures of fibroblasts by conventional transmission electron microscopy. Fig. 3 shows a longitudinal section of an Al-F fibroblast incubated with Au₁₈-fibronectin for 30 min. Clusters of Au₁₈-fibronectin are found on the cell surface above microfilament bundles (Fig. 3, A and C, arrows). The Au₁₈-fibronectin on the cell surface does not appear to be associated with any filamentous cell surface material, but rather appears to be bound directly to the cell membrane. Au₁₈-fibronectin complexes bound to the cell surface could be competitively inhibited with excess unlabeled fibronectin (data not shown). Since growth and labeling conditions for the cells studied by transmission electron microscopy were identical to conditions for the fibroblasts studied in the HVEM as whole mounts, it seems likely that the Au₁₈-fibronectin complexes observed in the whole mounts by HVEM were also directly bound to the cell surface.

### Table I. Patterns of Labeling of Human Skin Fibroblasts with Au₁₈-Fibronectin

| Labeling conditions | No. of cells | Labeled cells |
|---------------------|-------------|---------------|
|                     |             | Clusters | Clusters and fibrils | Fibrils | No label |
| Au₁₈-fibronectin    |             | %        | %                   | %       | %        |
| (15–30 min)        | 27          | 63       | 15                  | 15      | 7        |
| Au₁₈-fibronectin    | 19          | 11       | 63                  | 16      | 11       |
Figure 2. (A) A whole mount of an A1-F skin fibroblast incubated with Au$_8$-fibronectin and a 100-fold excess of plasma fibronectin for 30 min at 37°C. Arrows show scattered Au$_8$-fibronectin complexes on the dorsal surface. (B) A whole mount of a human skin S132 fibroblast incubated with Au$_8$-albumin complexes for 15 min at 37°C. Arrows show scattered Au$_8$-albumin complexes on the cell surface. Bars, 0.5 μm.

Under transmission electron microscopy, some Au$_8$-fibronectin was found to be associated with filamentous material on the cell surface and in some instances in the extracellular matrix above the fibroblast (Fig. 3, B and C, small arrows). Au$_8$-fibronectin complexes bound to the extracellular matrix could not be competitively inhibited with excess unlabeled fibronectin (data not shown) and may represent one source of the large nonspecific binding fraction reported earlier (McKeown-Longo and Mosher, 1983). Some endocytosis of the Au$_8$-fibronectin complexes was observed (Fig. 3 D), but the amount of Au$_8$-fibronectin taken up by endocytosis represented <1% of the complexes. Au$_8$-fibronectin complexes are seen bound to the substratum in a random distribution (Fig. 3 E).

When confluent fibroblast cultures containing numerous extracellular fibrils were labeled with Au$_8$-fibronectin for 30 min and examined by transmission electron microscopy, Au$_8$-fibronectin was observed on the cell surface as well as in matrix fibrils (data not shown). Binding of Au$_8$-fibronectin to cell surfaces of these confluent cultures was specific and could be blocked with unlabeled fibronectin. Matrix bound Au$_8$-fibronectin, on the other hand, could not be competitively inhibited with excess unlabeled fibronectin.

Reorganization of Au$_8$-Fibronectin Complexes with Time

To see if Au$_8$-fibronectin could be organized into fibrils after binding and if fibril formation occurred on the cell surface, subconfluent cultures of fibroblasts that contained little pre-existing matrix were incubated with Au$_8$-fibronectin for 1.5–5 h. These longer incubations resulted in the Au$_8$-fibronectin on the cell surface being arranged into linear arrangements or networks of extracellular fibrils. The fibrillar arrangements varied in length and density from cell to cell and from culture to culture. The areas ranged from 0.04 to 90 × 10$^{-1}$ μm$^2$. The average area of a fibrillar arrangement was 12.5 × 10$^{-1}$ μm$^2$. 16% of the fibroblasts contained only fibril-like arrangements of Au$_8$-fibronectin, 63% of the fibroblasts contained clusters of Au$_8$-fibronectin as well as fibril-like arrangements of the Au$_8$-fibronectin, and only 11% had clusters exclusively (Table I). In comparison, in cultures incubated with Au$_8$-fibronectin for 15–30 min, 63% of the fibroblasts contained only clusters of Au$_8$-fibronectin. In fibroblasts incubated with Au$_8$-fibronectin for 1.5–5 h, the fibrillar arrangements represented up to 11.3% of the area observed (Table II). The clusters of Au$_8$-fibronectin represented ~0.2% of the area observed. This is a two- to three-fold increase in the area occupied by the fibrillar arrangements of Au$_8$-fibronectin in the cells incubated for the longer times. The area represented by clusters of Au$_8$-fibronectin remained relatively unchanged with time.

Fig. 4 A shows Au$_8$-fibronectin arranged in a fibrillar network on the upper surface of an S132 fibroblast incubated with Au$_8$-fibronectin for 3 h. The Au$_8$-fibronectin is found in large linear clusters arranged over bundles of intracellular microfilament bundles (large arrows). The fibrillar networks of Au$_8$-fibronectin (small arrow) are frequently connected to filapods. These networks are also frequently observed to be connected to retraction fibers (data not shown). The filopodia appeared to pull the networks of Au$_8$-fibronectin off the cell surface and into the extracellular space. Au$_8$-fibronectin complexes underneath the cell are arranged in clusters, and fibrillar arrangements and networks of Au$_8$-fibronectin on the lower surface of the fibroblast are not observed.

Fig. 4 B shows a fibroblast (Detroit 551) incubated with Au$_8$-fibronectin for 3 h. The Au$_8$-fibronectin is found in large linear clusters arranged over bundles of intracellular microfilaments (large arrow). There do not appear to be fibrils connecting the Au$_8$-fibronectin in these clusters as there are along the edge of the cell (small arrows) and on the
Figure 3. Conventional transmission electron micrograph of an A1-F human skin fibroblast incubated with Au₁₅-fibronectin for 30 min at 37°C. (A) Clusters of Au₁₅-fibronectin bound to the cell surface (large arrow). G, Golgi complex. (B) Clusters of Au₁₅-fibronectin bound to filamentous material on the dorsal cell surface (small arrow). CP, coated pit. (C) Cluster of Au₁₅-fibronectin bound to the cell surface (large arrows) and to filamentous material on the dorsal cell surface (small arrows). (D) Au₁₅-fibronectin complexes endocytosed by the cell (arrowheads). (E) Au₁₅-fibronectin complexes bound to filamentous material on the ventral surface of the fibroblast (arrow). Numerous vesicles are observed on the ventral surface (V). Au₁₅-fibronectin complexes are also observed bound to the substrate. (F) Longitudinal section of fibroblast shown in Figs. A-E. Bars: (A-E) 0.25 μm; (F) 1.0 μm.

cell in Fig. 4 A. These findings suggest that these linear, fibril-like arrangements may be bound to the cell surface. The appearance of these linear arrangements with time and the disappearance of small clusters of Au₁₅-fibronectin on top of the cell suggest that the Au₁₅-fibronectin is rearranged at specific regions of the upper surface of the fibroblast. That is, the linear arrangements observed in Fig. 4, A and B may represent intermediate stages of fibril formation, in which

Table II. Areas of the Cell Surface Containing Au₁₅-Fibronectin Clusters and Fibrils

| Labeling conditions       | Area          | Area (percent of total) | Classification of cell according to distribution of label | Area          | Area (percent of total) |
|---------------------------|---------------|-------------------------|--------------------------------------------------------|---------------|-------------------------|
| Au₁₅-fibronectin (15-30 min) |               |                         | Clusters                                | 0.03-18.5       | Clusters 0.18          |
|                           |               |                         | Clusters and fibrils                      | 0.03-20.1       | Clusters 0.14          |
|                           |               |                         | Fibrils                                  | 0.36-7.3        | Fibrils 5.0            |
| Au₁₅-fibronectin (1.5-5 h) |               |                         | Clusters                                | 0.03-10.7       | Clusters 0.24          |
|                           |               |                         | Clusters and fibrils                      | 0.03-15.9       | Clusters 0.20          |
|                           |               |                         | Fibrils                                  | 0.56-5.0        | Fibrils 7.9            |

The cells analyzed in Table I were further analyzed as described in Materials and Methods. The total area is a minimum inasmuch as the calculations did not take into account the ventral surface of the cell nor the curvature of the cell sides.
fibronectin molecules are being brought together, disulfide-bonded, and translocated into the extracellular space.

**Cell Surface Binding of Au₁₇₀-70-kD Fragment and Au₁₇₀-160–180-kD Fragments**

To distinguish binding sites on the cell surface that are involved in matrix formation from other binding sites on fibronectin, fibroblasts were incubated with the 70-kD fragment that contains the amino-terminal gelatin- and heparin-binding sites located in the type I and II homology sequences and the 160–180-kD fragments that are lacking the amino and carboxyl type I homology sequences. The 160–180-kD fragment contains a second heparin-binding site (Sekiguchi et al., 1985) and the region of fibronectin that binds to a 140-kD...
Figure 5. (A) A stereo micrograph of an Al-F human skin fibroblast incubated with Au10-70-kD fragments for 30 min at 37°C. Clusters of Au10-70-kD fragments on the dorsal surface of the cell co-align with intracellular bundles of microfilaments (large arrows). Small arrow shows Au10-70-kD fragments in extracellular matrix fibrils. Stereo tilt 7° from horizontal. (B) A stereo micrograph of an Al-F skin fibroblast incubated with Au10-70-kD fragments for 30 min. Linear arrangements of Au10-70-kD fragments that co-align with intracellular microfilaments are observed along the edge of the cell (arrows). Stereo tilt 7° from horizontal. (C) A stereo micrograph of an Al-F skin fibroblast. A linear arrangement of Au18-70-kD fragments is observed when Al-F skin fibroblasts are incubated with Au10-70-kD fragments for 5 h at 37°C (large arrow). Small arrows show clusters of Au10-70-kD fragments. Stereo tilt 8° from horizontal. (D) A whole mount of an Al-F human skin fibroblast incubated with Au10-70-kD fragment complexes and a 100-fold excess of the 70-kD fragment for 30 min at 37°C. Arrows show scattered Au10-70-kD fragments on the cell surface. Bars: (A) 0.25 μm; (B–D) 0.5 μm.
cell surface receptor involved in cell adhesion and spreading (Pytel et al., 1985; Akiyama et al., 1986).

Fibroblasts labeled with Au\textsubscript{70-kD} fragment showed a labeling pattern similar to that observed for cells labeled with Au\textsubscript{fibronectin}. Fibroblasts (A1-F) incubated with Au\textsubscript{70-kD} fragment for 30 min had clusters and some linear arrangements of complexes on the upper surface of the cell (Fig. 5, A and B). Some of the clusters and many of the linear arrangements co-aligned with intracellular microfilaments, as was the case with Au\textsubscript{fibronectin}. A small percentage of the Au\textsubscript{70-kD} complexes were arranged in clusters on the lower surface of the fibroblast. Linear arrangements of Au\textsubscript{70-kD} fragment were not observed on the lower surface of the fibroblasts. Incubation of A1-F fibroblasts with Au\textsubscript{70-kD} fragment for 5 h resulted in an increased number of linear, fibril-like arrangements and fewer clusters of the Au\textsubscript{70-kD} fragment (Fig. 5 C). Some of the areas along these linear arrangements appear to co-align with intracellular microfilaments. The binding of Au\textsubscript{70-kD} fragment was specific and could be blocked by incubating fibroblasts with excess 70-kD fragment. Fig. 5 D shows the cell surface of a fibroblast labeled with excess 70-kD fragment and Au\textsubscript{70-kD} fragment for 30 min at 37°C. Clusters of Au\textsubscript{70-kD} fragment and Au\textsubscript{70-kD} fragment in linear arrangements are not present.

When A1-F fibroblast cultures were incubated with both Au\textsubscript{70-kD} fragment and Au\textsubscript{160-180-kD} fragments, the two gold complexes did not colocalize on the cell surface (Fig. 6). Binding of Au\textsubscript{160-180-kD} fragments to cells was sparse. Many of the Au\textsubscript{160-180-kD} fragments bound in small clusters predominantly on the lower surface of the cell near the ruffling edge of the fibroblast. Linear arrangements and fibrillar networks of the Au\textsubscript{160-180-kD} fragments were not observed. In some cells however, discrete patches of Au\textsubscript{160-180-kD} fragments were distributed along a diagonal line.

**Discussion**

The incorporation of fibronectin into the extracellular matrix of cultured human skin fibroblasts reportedly is a receptor-mediated process (McKeown-Longo and Mosher, 1983, 1984, and 1985). These previous studies showed that exogenously added I\textsuperscript{125}-plasma fibronectin binds to the cell layer of cultured human fibroblasts in two distinguishable pools. In pool I, binding of fibronectin is reversible, and bound fibronectin is soluble in deoxycholate. Pretreatment of cells with low concentrations of trypsin to remove pre-existing matrix has no effect on pool I binding, and pretreatment of cells with cycloheximide to block synthesis of endogenous fibronectin does not decrease pool I binding (McKeown-Longo and Mosher, 1985). More recently, Allen-Hoffmann and Mosher (1985 and unpublished observations) have shown that pool I binding can be down-regulated with cholera toxin or up-regulated with transforming growth factor beta within 30 min after exposure to the agent. Such a rapid effect is more consistent with labile binding sites on the cell surface than with static binding sites in pre-existing matrix.

The properties of fibronectin in pool I, together with fluorescence microscopic studies (McKeown-Longo and Mosher, 1983; 1985), suggest that fibronectin in pool I is binding to a cell surface receptor rather than matrix fibrils. It should be noted that studies by Chernousov et al. (1985) suggest that intact fibronectin and the 70-kD amino-terminal fragment bind to pre-existing matrix. However, the shortest incubation time used in these studies was 3 h and the concentration of the ligands used was 500 \(\mu\text{g/ml} (7 \times 10^{-6} \text{ M})\) for the 70-kD fragment and \(10^{-6} \text{ M}\) for intact fibronectin. These concentrations are much higher than the \(K_D\) for the binding to the matrix assembly receptor (3 \(\times 10^{-8} \text{ M}\)). When McKeown-Longo and Mosher (1983) attempted to saturate pool I binding, there was a considerable nonsaturable component, so that for fibronectin concentrations >35 \(\mu\text{g/ml} (7 \times 10^{-8} \text{ M})\), more than 50% of total binding was nonsaturable. We noted that binding of Au\textsubscript{fibronectin} to pre-existing matrix was not blocked by excess unlabeled fibronectin. Thus, we suspect that most of the binding noted by Chernousov et al. (1985) was to lower affinity or nonsaturable sites in the extracellular matrix, e.g., to collagen, glycosaminoglycan, or already assembled fibronectin fibrils.
In the present studies we examined whole mounts of subconfluent cultures of fibroblasts by HVEM to localize fibronectin in pool I for possible cell surface interactions involved in the assembly of fibronectin fibrils. Subconfluent cultures were primarily studied rather than confluent cultures because the subconfluent cultures actively produced an extracellular fibronectin matrix. We found that AUa-fibronectin initially bound to the cell surface in clusters above bundles of microfilaments. These clusters were primarily located along the edge of the fibroblast. The clustering pattern of AUa-fibronectin found in these micrographs is reminiscent of the short straia of fibronectin observed in immunofluorescence studies of young cultures of NIL8 cells (Hynes and Destree, 1978). In the NIL8 cells, the observed straia aligned with actin bundles by double antibody immunofluorescence. Such tiny fibrils and patches were also observed by fluorescence microscopy of confluent fibroblast cultures labeled with FITC-fibronectin for 20 min (McKeown-Longo and Mosher, 1983). These fibrils and patches of FITC-fibronectin were thought to represent soluble fibronectin bound to the cell layer but not disulfide cross-linked into insoluble matrix fibrils.

Conventional transmission electron microscopy showed that specifically bound AUa-fibronectin was not associated with filamentous cell surface matrix components but attached to the cell surface. Therefore, AUa-fibronectin is initially not bound to cell surface collagen, despite the fact that fibronectin has been shown to co-distribute with procollagen in the pericellular matrix (Bornstein and Ash, 1977; Vaheri et al., 1978). To establish this point further, we have done fluorescence microscopy and HVEM on cultures pretreated with collagenase and then incubated with exogenous FITC-fibronectin or AUa-fibronectin and found patterns of binding similar to those of untreated cells (unpublished observations).

Some of the AUa-fibronectin binding after 15–30 min was found in fibrillar structures on the cell surface or in extracellular matrix in ~15% of the fibroblasts. Fibrillar AUa-fibronectin was much more extensive after longer incubations. Fibrillar membrane-associated material was patchy in appearance and ~19 nm from the cell surface in transmission electron micrographs. Hedman et al. (1978) observed that in young cultures ferritin-antifibronectin complexes localized 25 nm or less from the cell surface. This AUa-fibronectin may therefore represent membrane-associated fibronectin that has started to be assembled into matrix fibrils.

Insoluble rod-like fibronectin complexes can be formed in the absence of cells if the fibronecin is precipitated with heparin (Jilek and Hörmann, 1979) or polyamines (Vuento et al., 1980). However, ultrastructural studies on fibronectin fibril formation in vitro using collagen gels, heparin, hyaluronic acid, chondroitin sulfate, or chondroitin sulfate proteoglycan have not recapitulated the assembly of fibronectin fibrils in vivo (Turley et al., 1985). In addition, we observed that fibronectin fibrils were not formed under culture conditions similar to those used in this study unless cells were present. Thus, it seems reasonable to conclude that a preassembled matrix alone is not sufficient to assemble fibronectin fibrils and that some aspects of fibronectin fibril assembly are controlled by cells.

AUa-fibronectin was bound to cells over a 5-h period to visualize possible cell surface events involved in the assembly of fibronectin fibrils (pool II). With longer incubations, AUa-fibronectin on the upper surface of the fibroblast became arranged into linear arrangements as well as into fibrillar networks. A linear rearrangement of AUa-fibronectin on the cell surface was also observed when the cultures were incubated with AUa-fibronectin for 15 min, washed, and incubated for an additional hour without conjugate (unpublished observation). The linear arrangements were frequently found near retracting fibers and filopods. The arrangements may represent intermediate stages of fibrillogenesis. Fibronectin fibrillogenesis may therefore occur when the fibronectin molecules bound to the matrix assembly receptor on the cell surface are brought into contact with other fibronectin-receptor complexes on the cell surface. The intracellular microfilaments observed underneath the cell surface fibronectin may be responsible for bringing together and aligning the fibronectin molecules in such a way that disulfide exchange can occur to form a fibril.

This rearrangement of fibronectin into fibrillar structures on the cell surface appears to involve interactions between the fibroblast cell surface and the 70-kD amino terminal type I and II homology units of fibronectin (McKeown-Longo and Mosher, 1985). Cultures double-labeled with the amino-terminal 70-kD fragment and the 160–180-kD fragments (which lack Type I and II homology units), showed that the amino-terminal 70-kD fragment and the 160–180-kD fragments did not colocalize. Because the 160–180-kD fragments contain the cell adhesion site of fibronectin and a strong heparin-binding site, the matrix assembly sites for fibronectin on cells must be distinct from the RDGS and heparin binding sites. Incubation of the AUa-70-kD fragment for longer periods of time resulted in the rearrangement of AUa-70-kD fragments into linear patterns along the edge of the fibroblast. These linear arrangements may represent the intermediate fibrill-like arrangements discussed above, since the 70-kD fragment does not form disulfide multimers and does not enter the extracellular matrix (McKeown-Longo and Mosher, 1985).

Our finding that little of the 160–180-kD fragments bound to cells is consistent with the findings of Chernousov et al. (1985), who noted that two FITC-60-kD fragments of fibronectin that contained the cell adhesion site of fibronectin and a heparin-binding site bound poorly to cell layers. Many of the cell adhesion sites presumably are occupied with pre-existing fibronectin. Monoclonal antibodies against the cell adhesion receptor showed that the cell adhesion receptor was localized to areas surrounding the termini of actin in stress fibers (Damsky et al., 1985) and colocalized with vinculin and α-actin at extracellular matrix contacts (Duband et al., 1986; Chen et al., 1985). The 140-kD cell adhesion receptor was not localized within focal contacts at the cell periphery or on the upper (dorsal) surface of cells (Chen et al., 1985), nor did it colocalize with bundles of actin along the lateral edges of the cell (Duband et al., 1986).

However, the regions along the lateral edges of the fibroblast where fibronectin fibrillogenesis appears to take place and AUa-70-kD fragment binds are rich in intracellular microfilament bundles that appear to be actin filaments based on their size and location. The close association among AUa-fibronectin or AUa-70-kD complexes and microfilament filaments along the lateral edges of the cell and the absence of AUa-160–180-kD fragments in these regions suggest
that there are two modes of fibronectin–microfilament interactions, one mediated by the cell adhesion receptor and one mediated by the matrix assembly receptor. The existence of two fibronectin–microfilament interactions raises the question as to whether the fibronexus described by Singer (1979) may represent regions on the cell surface other than sites of cell adhesion where fibronectin fibrillogenesis is occurring. Further studies using culture conditions identical to those used by Singer would be needed to answer this question.

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References

Allen-Hoffmann, B. L., and D. F. Mosher. 1985. Fibronectin matrix assembly receptor number is decreased in normal human fibroblasts after treatment with cholera toxin. J. Cell Biol. 101(5, Pt. 2):59a. (Abstr.)

Akiyama, S. K., S. S. Yamada, and K. M. Yamada. 1986. Characterization of a 140-kD avian cell surface antigen as a fibronectin-binding molecule. J. Cell Biol. 102:442-448.

Balins, G. E., E. M. Clich, E. Crouch, J. M. Davidson, and P. Bornstein. 1979. Isolation of a collagen-binding fragment from fibronectin and cold-insoluble globulin. J. Biol. Chem. 254:1429-1432.

Bornstein, P., and J. F. Ash. 1977. Cell surface-associated structural proteins in connective tissue cells. Proc. Natl. Acad. Sci. USA. 74:2480-2484.

Chernousov, M. A., M. L. Metiis, and V. E. Koteliansky. 1985. Studies of extracellular fibronectin matrix formation with fluoresceinated fibronectin and fibronectin fragments. FEMS (Fed. Eur. Biochem. Soc.) Lett. 183:365-369.

Damsky, C. H., K. A. Knudsen, D. Bradley, C. A. Buck, and A. F. Horwitz. 1985. Distribution of the cell substratum attachment (CSAT) antigen on myogenic and fibroblastic cells in culture. J. Cell Biol. 100:1528-1539.

Duband, J.-L., S. Rocher, W.-T. Chen, K. M. Yamada, and J. P. Thiery. 1986. Cell adhesion and migration in the early vertebrate embryo: location and possible role of the putative fibronectin receptor complex receptor complex. J. Cell Biol. 102:160-179.

Engwall, E., and E. Ruoslahti. 1977. Binding of soluble form of fibronectin into fibronectin to collagen. Int. J. Cancer. 20:1-5.

Geoghegan, W. D., and A. Ackerman. 1977. Absorption of horseradish peroxidase, ovalbumin, and anti-immunoglobulin to collagen bovine gold for the indirect detection of concanavalin A, wheat germ agglutinin and goat anti-human immunoglobulin G on cell surfaces at the electron microscopic level: a new method, theory and application. J. Histochem. Cytochem. 25:1187-1200.

Hayman, E. G., and E. Ruoslahti. 1979. Distribution of fetal bovine serum fibronectin and endogenous rat cell fibronectin in extracellular matrix. J. Cell Biol. 83:255-259.

Hedman, K., A. Vaheri, and J. Warthiovaara. 1978. External fibronectin of human fibroblasts is predominently a matrix protein. J. Cell Biol. 76:748-760.

Horsburger, M., and J. Rosset. 1977. Colloidal gold, a useful marker for transmission and scanning electron microscopy. J. Histochem. Cytochem. 25:295-305.

Hynes, R. O. 1985. Molecular biology of fibronectin. Annu. Rev. Cell Biol. 1:67-90.

Hynes, R. O., and A. T. Destree. 1978. Relationship between fibronectin (LETS protein) and actin. Cell. 15:875-886.

Jieck, F., and H. Hermann. 1979. Fibronectin (cold insoluble globulin VI) Influence of heparin and hyaluronic acid on the binding of native collagen. Hoppe-Seyler's Z. Physiol. Chem. 360:597-603.

Kornblihtt, A. R., K. Vibe-Pedersen, and P. E. Baralle. 1984. Human fibronectin: molecular cloning evidence for two mRNA species differing by an internal segment coding for a structural domain. EMBO (Eur. Mol. Biol. Organ.) J. 3:221-226.

Maupin, P., and T. D. Pollard. 1983. Improved preservation and staining of Hela cell actin filaments, clathrin-coated membranes, and other cytoplasmic structures by tannic acid-glutaraldehyde-saponin fixation. J. Cell Biol. 96:51-62.

McKeown-Longo, P. J., and D. F. Mosher. 1983. Binding of plasma fibronectin to cell layers of human skin fibroblasts. J. Cell Biol. 97:466-472.

McKeown-Longo, P. J., and D. F. Mosher. 1984. Mechanisms of formation of disulfide-bonded multimers of plasma fibronectin in cell layers of cultured human fibroblasts. J. Biol. Chem. 259:12210-12215.

McKeown-Longo, P. J., and D. F. Mosher. 1985. Interactions of the 70,000-mol-wt amino-terminal fragment of fibronectin with the matrix assembly receptor of fibroblasts. J. Cell Biol. 100:364-374.

Mosher, D. F. 1984. Physiology of fibronectin. Annu. Rev. Med. 35:561-575.

Mosher, D. F., and R. B. Johnson. 1985. In vitro formation of disulfide-bonded fibronectin multimers. J. Biol. Chem. 258:6595-6601.

Oh, E., M. Pierschbacher, and E. Ruoslahti. 1981. Deposition of plasma fibronectin in tissues. Proc. Natl. Acad. Sci. USA. 78:3218-3221.

Pierschbacher, M. D., E. G. Hayman, and E. Ruoslahti. 1981. Location of the cell attachment site in fibronectin with monoclonal antibodies and proteolytic fragments of the molecule. Cell. 26:259-267.

Pytsela, R., M. D. Pierschbacher, and E. Ruoslahti. 1985. Identification and isolation of a 140 kD cell surface glycoprotein with properties expected of a fibronectin receptor. Cell. 40:191-198.

Ris, H. 1985. The cytoplasmic filamentous system in critical point-dried whole mounts and plastic-embedded sections. J. Cell Biol. 100:1474-1478.

Schwarzbauer, J. E., J. I. Paul, and R. O. Hynes. 1985. On the origin of species of fibronectin. Proc. Natl. Acad. Sci. USA. 82:1424-1428.

Sekiguchi, K., A. Sirl, L. Zardi, and S.-I. Hakomori. 1985. Differences in domain structure between human fibronectins isolated from plasma and from culture supernatants of normal and transformed fibroblasts. J. Biol. Chem. 260:5105-5114.

Singer, J. I. 1979. The fibronexus: a transmembrane association of fibronectin-containing fibers and bundles of 5 nm microfilaments in hamster and human fibroblasts. Cell. 16:675-685.

Tamkun, J. W., and R. O. Hynes. 1983. Plasma fibronectin is synthesized and secreted by hepatocytes. J. Biol. Chem. 258:5728-5736.

Trelstad, R. L., and K. Hayashi. 1979. Tendon fibrillogenesis: intracellular collagen subassemblies and cell surface changes associated with fibril growth. Dev. Biol. 71:228-242.

Turley, E. A., C. A. Erickson, and R. P. Tucker. 1985. The retention and ultrastructural appearances of various extracellular matrix molecules incorporated into three-dimensional hydrated collagen lattices. Dev. Biol. 109:347-369.

Vaheri, A., M. Kurkinen, V. P. Lehto, E. Linder, and R. Timpl. 1978. Contribution of pericellular matrix proteins in cultured fibroblasts and loss in transformation: fibronectin and procollagen. Proc. Natl. Acad. Sci. USA. 75:4944-4948.

Williams, E. C., R. B. Johnson, and D. F. Mosher. 1983. Fibronectin: effect of disulphide bond reduction on its physical and functional properties. J. Biol. Chem. 258:5911-5914.

Wolosewick, J. J., and K. R. Porter. 1976. Stereo high voltage electron microscopy of whole cells of the human diploid line, WI-38. Am. J. Anat. 147:303-324.