

# Focal Adhesion Sites and the Removal of Substratum-bound Fibronectin

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**Abstract.** Fibronectin was not removed from the substratum beneath focal adhesion sites when fibroblasts spread in serum-free medium on adsorbed fibronectin substrata, or when fibroblasts spread in serum-containing medium on covalently cross-linked fibronectin substrata. Under these conditions, there was colocalization between 140-kD fibronectin receptors and focal adhesion sites. It was concluded that removal of adsorbed fibronectin from beneath focal adhesion sites was a mechanical process that required serum. The effect of serum was nonspecific since serum could be replaced by equivalent concentrations of serum albumin, ovalbumin, or gamma globulins. Quantitative measurements indicated that the presence

of proteins in the incubation medium weakens the interaction of fibronectin with the substratum, thereby allowing the adsorbed protein to be removed from the substratum at sites of high stress. After removing fibronectin from the substratum, cells reorganized this material into patches and fibrils beneath cells, and the reorganized fibronectin colocalized with fibronectin receptors. Some of the patches of fibronectin were phagocytosed. The fibronectin fibrils were observed to be in register with actin filament bundles and sometimes translocated to the upper cell surfaces. It is proposed that removal of fibronectin from beneath focal adhesion sites is an example of how cells can modify their extracellular matrices through contractile activity.

**F**OCAL cell adhesions are sites of closest cell-substratum contact. They were visualized first by electron microscopy (1) and later by interference reflection microscopy (27). Although focal adhesions are not essential for cell attachment and spreading (reviewed in reference 20), their presence correlates with increased strength of cell attachment (34) and restricted cell motility (13, 29).

Several different cytoskeletal proteins have been found on the cytoplasmic side of focal adhesion sites (31). Geiger et al. (17) proposed that these cytoskeletal proteins associate at focal adhesions through a cascade mechanism that is initiated after formation of cell-substratum attachments. This cascade involves clustering of membrane receptors, binding of soluble vinculin (and possibly talin) on the cytoplasmic membrane surface, and assembly of actin filament bundles that insert into the cytoplasmic side of the focal adhesion sites.

There have been some puzzling findings concerning cell-substratum attachments involved in focal adhesions. On one hand, it has been shown that fibroblasts require both the cell and heparin binding domains of fibronectin to form focal adhesions (28, 30, 40), and immunofluorescence experiments indicated that the 140-kD fibronectin receptor complex is associated with focal adhesion sites (10, 14, 19). On the other hand, fibroblasts were observed to remove fibronectin from beneath focal adhesion (3), and immunoelectron microscopic studies revealed that fibronectin is absent from these sites (11).

Considering the evidence above, we became interested in examining in greater detail the ability of fibroblasts to remove and reorganize substratum-adsorbed fibronectin during adhesion. The results of our studies are reported herein.

## Materials and Methods

### Cells

Early passage human foreskin fibroblasts were grown at 37°C in DME (Gibco, Grand Island, NY) supplemented with 20% FBS (Gibco) in a humidified incubator with 5% CO<sub>2</sub>. Cultures were harvested with 0.05% trypsin-0.6 mM EDTA (Gibco), washed, and resuspended at a concentration of  $2 \times 10^5$ /ml in DME containing 20 mM Hepes buffer (Sigma Chemical Co., St. Louis, MO) and other additions as indicated. Test substrata (see below) were placed in 35-mm tissue culture dishes (Costar, Cambridge, MA) and incubated with 1.0 ml of cells at 37°C in a humidified incubator for the time periods designated.

### Fibronectin and Other Proteins

Human plasma fibronectin (Fn)<sup>1</sup> was obtained from the New York Blood Center. Fibronectin was conjugated to FITC (Sigma Chemical Co.) as described by Chernousov et al. (12). Briefly, 2 mg of fibronectin were incubated with 0.1 mg of FITC for 2 h at 22°C in 1.0 ml of 0.1 M carbonate/bicarbonate buffer (pH 9). Unreacted dye was removed by gel filtration on Sephadex G-25 (Pharmacia Inc., Piscataway, NJ). Fluorescein-conjugated

1. *Abbreviations used in this paper:* DPBS, Dulbecco's PBS; FFn, fluorescein-conjugated fibronectin; Fn, human plasma fibronectin; FnR, fibronectin receptor; PS, phosphate saline (10 mM sodium phosphate, 150 mM NaCl, pH 7.2).

fibronectin (FFn) contained about four FITC molecules per fibronectin molecule and retained complete biological activity based on cell spreading assays (data not shown).

<sup>3</sup>H-Fibronectin (<sup>3</sup>H-Fn) was prepared by reductive alkylation without loss of biological activity as previously described (32). The specific radioactivity was 3,400 cpm/μg. Radioactive samples to be measured were mixed with 10 ml Budget Solve (RPI Corp., Mount Prospect, IL) and counted in a Nuclear Chicago Mark II scintillation spectrophotometer (Tracor Analytic Inc., Elk Grove Village, IL). BSA (crystalline) and ovalbumin (crystalline) were obtained from Sigma Chemical Co. Human gamma globulins (Fraction II) were obtained from Miles Laboratories Inc. (Naperville, IL).

### Substrata

Glass coverslips (22 mm, No. 1½, American Scientific Products, McGraw Park, IL) were incubated for 15 min at 22°C with 0.05 ml of Dulbecco's PBS (DPBS; 150 mM NaCl, 3 mM KCl, 6 mM Na<sub>2</sub>HPO<sub>4</sub>, 1 mM CaCl<sub>2</sub>, 0.5 mM MgCl<sub>2</sub>, pH 7.2) containing 2 μg of Fn or FFn, or with 0.25 ml of DPBS containing 10 μg of <sup>3</sup>H-Fn. These amounts of fibronectin, which are much higher than necessary for cell spreading, facilitated observation of removal and reorganization of Fn from the substratum. The amount of solution used to treat substrata with <sup>3</sup>H-Fn insured uniform adsorption of fibronectin over the entire substrata surfaces, which was important for making quantitative measurements. The treated coverslips were rinsed thoroughly with DPBS before use.

Fn, FFn, and <sup>3</sup>H-Fn were covalently cross-linked to glass coverslips using the method of Aplin and Hughes (2). Briefly, coverslips (directly from the box) were incubated for 20 min at 22°C with 0.25 ml of γ-aminopropyltriethoxysilane (Pierce Chem. Co., Rockford, IL). After rinsing with deionized H<sub>2</sub>O, they were then treated for 30 min at 22°C with 0.25% glutaraldehyde in phosphate saline (PS) (10 mM sodium phosphate, 150 mM NaCl, pH 7.2). The activated coverslips were rinsed again and then incubated for 60 min at 22°C with 0.05 ml of DPBS containing 2 μg of Fn or FFn or with 0.25 ml of DPBS containing 10 μg of <sup>3</sup>H-Fn. Finally, they were rinsed with 1% SDS (Sigma Chemical Co.) to remove loosely bound protein and then with DPBS.

### Microscopy

Mouse anti-vinculin (chicken gizzard) antibody was a generous gift from Dr. Ben Geiger (Weizmann Institute, Rehovot, Israel). Affinity purified rabbit anti-140-kD fibronectin receptor (human) antibody was a generous gift from Dr. Erkki Ruoslahti (La Jolla Cancer Research Foundation). Rabbit anti-actin (chicken gizzard) and mouse anti-fibronectin (human) antibodies were prepared and characterized as specific by immunoblotting (data not shown). Rhodamine and fluorescein-conjugated goat anti-mouse and goat anti-rabbit IgG antibodies were obtained from Cappel Worthington Biochemicals.

Samples for immunofluorescence analysis were fixed with 3% formaldehyde (Electron Microscopy Services Co.) in PS or simultaneously fixed/permeabilized with 3% formaldehyde, 0.5% Triton X-100 (Sigma Chemical Co.) in PS for 5 min at 22°C. Specimens were rinsed with PS containing 1% glycine and 1% bovine serum albumin. Incubations with first and second antibodies were for 5 min at 22°C. The glass coverslips were mounted on microscope slides using 90% glycerol, 0.1% *p*-phenylene diamine in PS adjusted to pH 8. Observations and photographs were made with a Zeiss IM-35 microscope.

Interference reflection microscopy was performed as previously described (21) with a Zeiss Universal microscope equipped with an antiflex system using an Antiflex Neofluar 63/1.25 oil immersion objective.

## Results

### Cellular Reorganization of Substratum-bound Fibronectin after 30 min in 10% Serum-containing Medium

Fluorescein-conjugated fibronectin was adsorbed on glass coverslips, and these substrata were incubated with human fibroblasts in DME containing 10% fetal bovine serum. After 30 min, dark streaks against the bright fluorescent background indicated regions where FFn was removed from the substratum (Fig. 1 *A*, arrows), and fluorescent spots indi-

cated the accumulation of patches of FFn along the cell periphery (Fig. 1 *B*, arrows).

To learn more about the localization of FFn, samples were fixed and analyzed by indirect immunofluorescence using anti-Fn antibodies and rhodamine-conjugated second antibodies. We anticipated a difference in the staining pattern depending upon whether cells were intact or permeabilized. FFn located either in excluded regions beneath the cell body or inside cells would be stained by anti-pFn only if the cells were permeabilized.

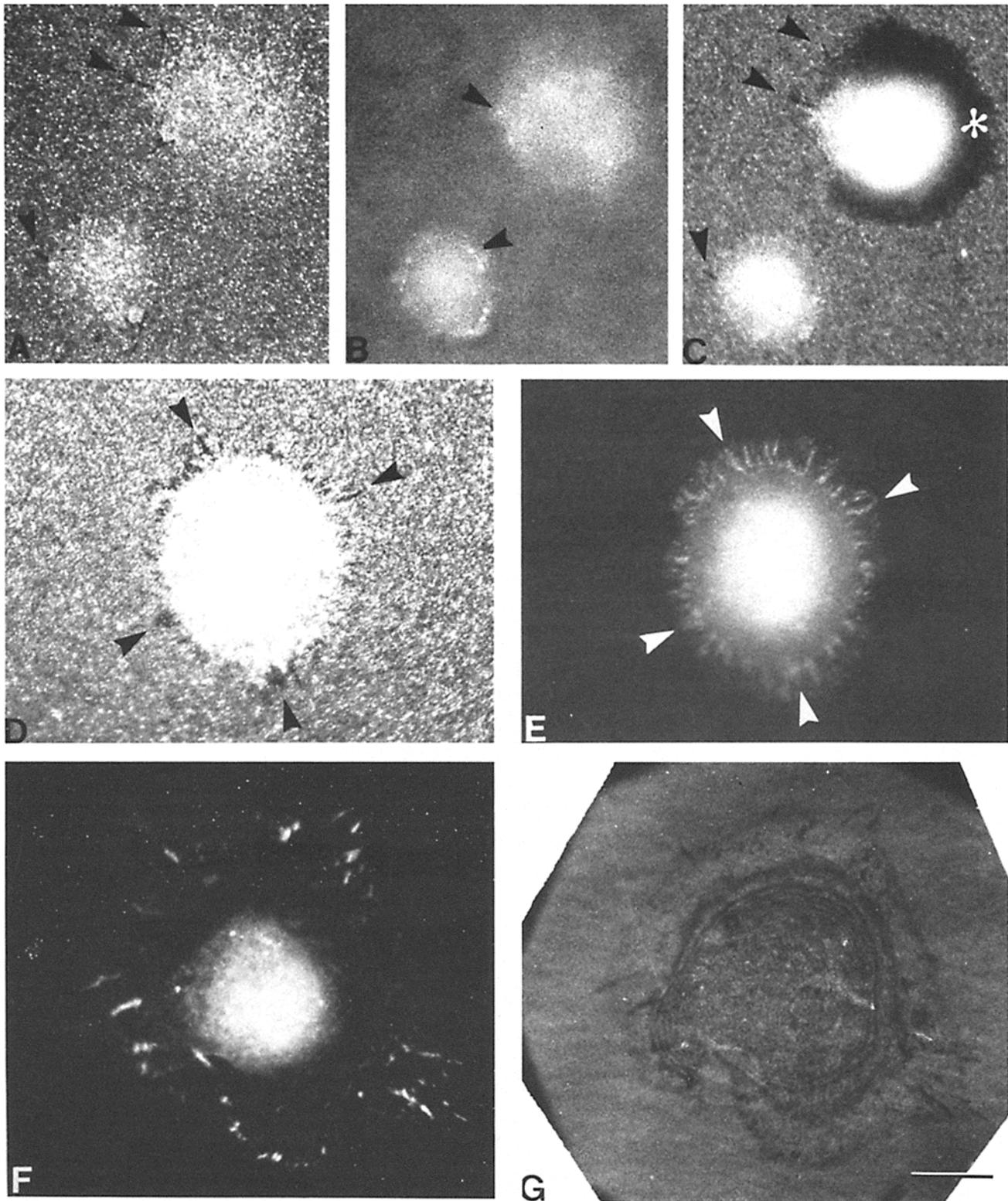
When intact specimens were stained with anti-pFn, most of the substratum appeared uniformly fluorescent. There was no staining, however, in the areas from which FFn had been removed (Fig. 1 *C*, arrows; compare with 1 *A*). In addition, dark regions of nonstaining (Fig. 1 *C*, asterisk) revealed the space beneath spreading cells from which anti-Fn antibodies were excluded. These regions were not observed if cells were permeabilized (data not shown).

To determine the relationship between areas of FFn removal and focal adhesion sites, cells spread on FFn for 30 min were fixed/permeabilized, and the focal adhesion sites were detected by indirect immunofluorescence staining for vinculin (16). A very close correspondence was found between the areas from which FFn was removed (Fig. 1 *D*, arrows) and fluorescence streaks associated with vinculin (Fig. 1 *E*, arrows), which agrees with the previous findings of Avnur and Geiger (3). It should be noted that in these cells most vinculin-containing adhesion sites (Fig. 1 *F*) corresponded to focal adhesions sites observed by interference reflection microscopy (Fig. 1 *G*).

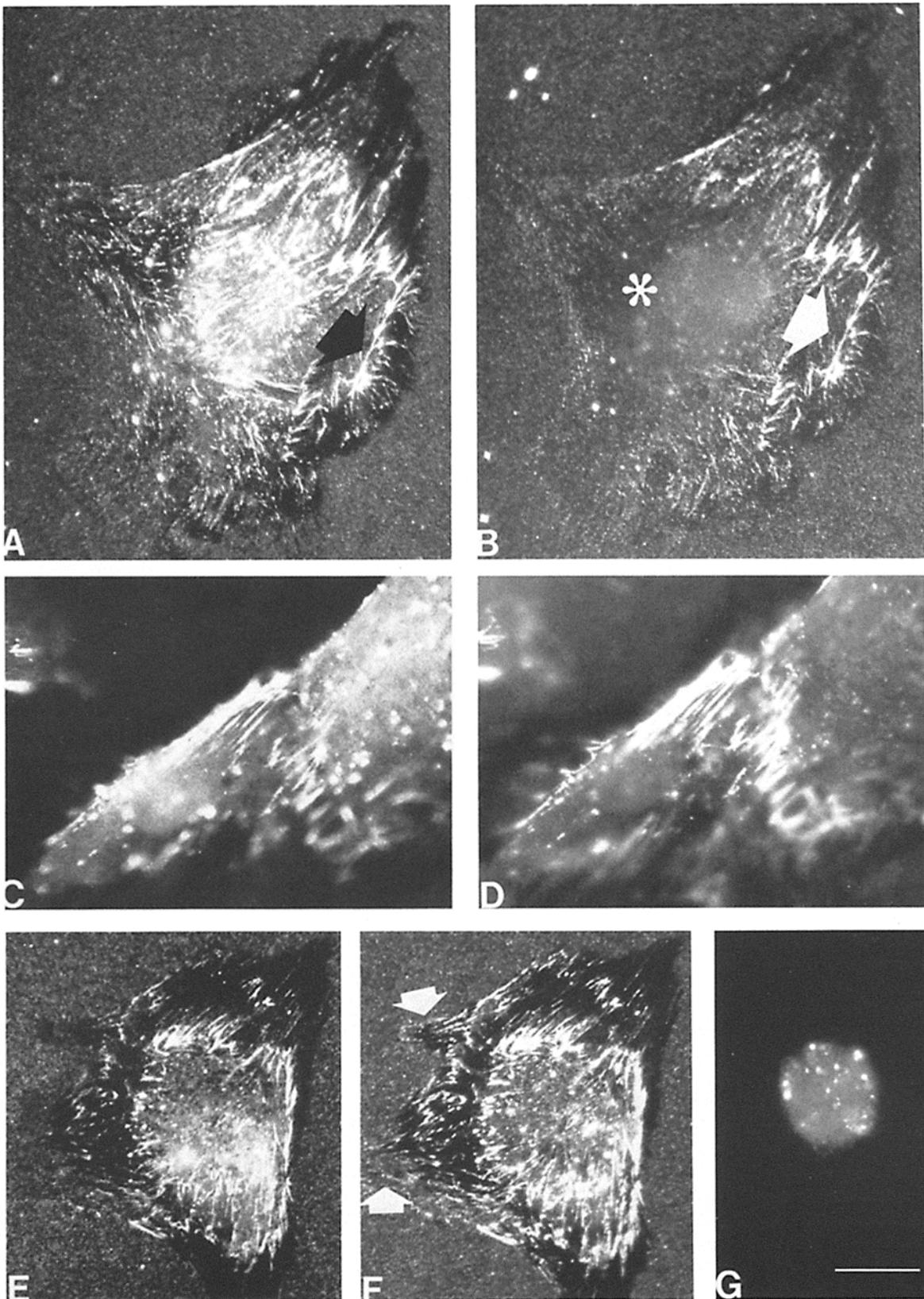
### Cellular Reorganization of Substratum-bound FFn after 4 h in 10% Serum-containing Medium

After longer incubation times, the extent of FFn removal from the substratum was increased, and the reorganized FFn appeared in patches and fibrils (Fig. 2 *A*). The patches of FFn and some of the fibrils were in the excluded space beneath cells since anti-Fn could not detect this material in intact cells (Fig. 2 *B*, asterisk). In addition, however, some of the FFn fibrils in the same plane of focus were accessible to anti-Fn (Fig. 2, *A* and *B*, arrows). Therefore, only part of the space beneath the cells was excluded.

By adjusting the plane of focus, it was evident that many of the FFn fibrils detected by anti-Fn staining of intact cells were located at the upper cell surfaces. This material might have been bound originally beneath cells and then translocated along their surfaces. Alternatively, FFn might have been released from the substratum and then rebound by the cells. To distinguish between these possibilities, two control experiments were carried out. First, cells were cultured on nonfluorescent Fn in 10% serum-containing medium to which 3 μg FFn was added. This amount of FFn was greater than that used to treat the substrata in the other experiments (2 μg). Under these conditions, little fluorescence became associated with cells (data not shown). In addition, experiments were carried out with cells cultured on FFn in the presence of a 500-fold excess of nonfluorescent Fn (1 mg) added to the medium. Reorganization of FFn under these conditions occurred similarly as in the absence of added Fn, and FFn fibrils were observed at the upper cell surfaces (Fig. 2, *C* and *D*). Consequently, it seems likely that FFn located at the upper cell surface was translocated there after binding



**Figure 1.** Cellular reorganization of substratum-adsorbed FFn after 30 min in 10% serum-containing medium. Human fibroblasts were incubated for 30 min in DME containing 10% FBS on substrata coated with FFn (A-E) or Fn (F and G). In A-C the sample was fixed and stained with mouse anti-Fn. In D and E and in F and G, the samples were fixed/permeabilized and stained with mouse anti-vinculin. The second antibody was rhodamine-conjugated goat anti-mouse IgG. A, B, and D were viewed with fluorescein optics; C, E, and F were viewed with rhodamine optics; G was viewed with interference reflection optics. Dark streaks in the bright background revealed where FFn was removed from the substratum (A, arrows), and at a higher plane of focus, FFn patches were observed (B, arrows). Anti-Fn detected where FFn was removed from the substratum (C, arrows) and also showed a region of antibody exclusion beneath the intact cells (C, asterisk). Regions from which FFn was removed initially (D, arrows) corresponded to focal adhesions detected with anti-vinculin (E, arrows). Vinculin-containing adhesion sites (F) corresponded to focal adhesion observed by interference reflection microscopy (G). Other details are in Materials and Methods. Bar, 10  $\mu$ m.



**Figure 2.** Cellular reorganization of substratum-adsorbed FFn after 4 h in 10% serum-containing medium. Human fibroblasts were cultured on FFn-coated substrata for 4 h in DME containing 10% FBS. In some cases the medium was supplemented with 1 mg/ml Fn (C and D). In A and B and in C and D, the samples were fixed and stained with mouse anti-Fn. In E and F the sample was fixed/permeabilized and stained with mouse anti-Fn. The second antibody was rhodamine-conjugated goat anti-mouse IgG. In G, the sample was treated with 0.05% trypsin/0.6 mM EDTA for 5 min at 37°C and then fixed. A, C, E, and G were viewed with fluorescein optics, and B, D, and F were viewed with rhodamine optics. FFn reorganized into streaks and patches (A) that either were beneath or inside the cells since they

to the lower cell surface, not released from the substratum and then rebound.

If the cells were permeabilized before staining with anti-Fn, then all of the reorganized FFN (Fig. 2 *E*) could be detected by the antibodies (Fig. 2 *F*). Most of the regions from which anti-Fn was removed were unstained by anti-Fn, indicating that cellular fibronectin had not replaced FFN in these areas. There were some areas along the cell margins, however, where cellular Fn fibrils could be detected by anti-Fn in the absence of FFN (Fig. 2 *F*, *arrows*).

As indicated above, the reorganized FFN that was detected by anti-Fn in permeabilized cells but not in intact cells might have been either in the excluded region beneath cells or inside cells. To distinguish between these possibilities, cells were exposed briefly to trypsin, which we have shown previously to remove cell surface-bound, but not phagocytosed, fibronectin-coated particles (32). This treatment destroyed all of the substratum-bound fluorescence (data not shown) and fibrillar FFN associated with the cells (Fig. 2 *G*). Patches of FFN, however, appeared to resist trypsin treatment (Fig. 2 *G*). These patches could be stained by anti-Fn if the cells were permeabilized but not in the absence of permeabilization (data not shown). It could be concluded, therefore, that some of the patches of FFN, but not the fibrils, were internalized by the cells.

### **Reorganization of FFN in Relationship to Fn Receptors and Actin**

It was of interest to determine whether FFN removed from the substratum was bound by cell surface Fn receptors. After various incubation times, cells cultured on FFN were fixed/permeabilized and then stained with an antibody directed against the human 140-kD fibronectin receptor (anti-FnR) (35, 36). After 1 h, FFN that had reorganized into patches (Fig. 3 *A*, *arrow*) was observed to colocalize with Fn receptors (Fig. 3 *B*, *arrow*) based upon the coincident fluorescence patterns. Along marginal cell processes where there was marked accumulation of fibronectin receptors (Fig. 3 *B*), some FFN also was observed (Fig. 3 *A*). As was the case with most of the reorganized FFN (Figs. 1 and 2), most of the reorganized fibronectin receptors could not be detected by anti-FnR if the cells were intact.

After 4 h, reorganized FFN (Fig. 3 *C*, *arrows*) still appeared to colocalize with Fn receptors (Fig. 3 *D*, *arrows*). At this time, some fibrillar accumulations of FFN were observed to stain positively with anti-FnR (data not shown). It seemed likely, therefore, that anti-FnR could detect receptors bound to Fn as long as they were not in the excluded space beneath the cells. In addition, there were regions along the cell periphery where Fn receptors were evident (Fig. 3 *D*, *asterisks*) but FFN no longer could be observed (Fig. 3 *C*). Sometimes, but not always, cellular fibronectin was detected in these areas (data not shown).

Experiments were also carried out to determine whether

there was any relationship between reorganized FFN and the actin cytoskeleton. No correspondence between FFN patches and actin cables was detected up to 4 h of incubation (data not shown). Fibrillar FFN, however, often appeared to be aligned with actin cables, at least in part (Fig. 3, *E* and *F*, *arrows*).

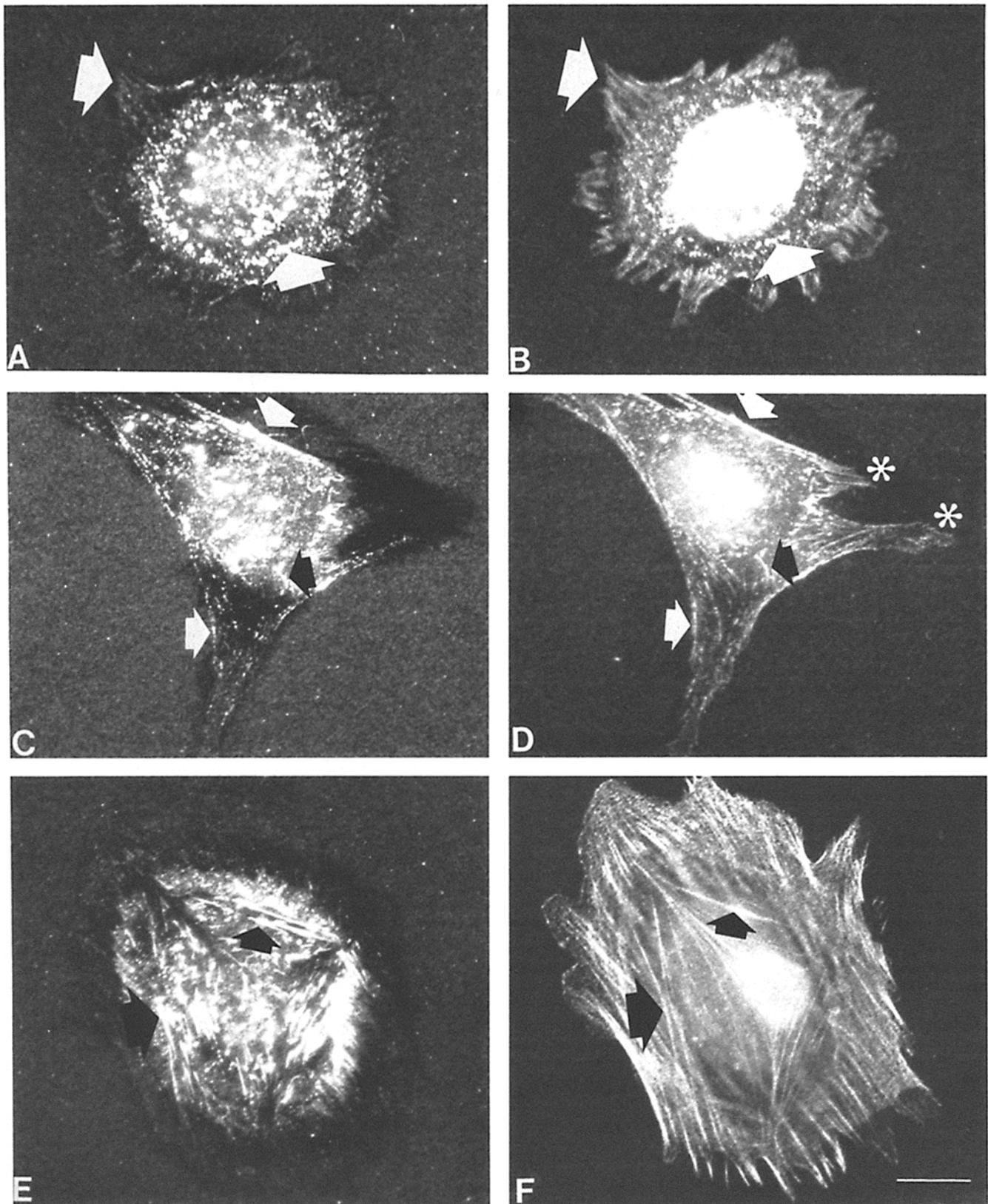
### **Lack of Cellular Reorganization of Substratum-bound FFN in Serum-free Medium**

Previously, we reported that fibroblasts incubated on fibronectin-coated substrata formed focal adhesions in the presence or absence of serum in the incubation medium, but that the focal adhesion sites were larger and more numerous in the presence of serum (23). The studies demonstrating that focal adhesion sites did not contain Fn were carried out in serum-containing medium (e.g., 3, 11). Cells cultured in low serum, on the other hand, were reported to have ventral fibronexus junctions (37), which may be similar to focal adhesions. It was of interest, therefore, to determine whether removal of substratum-bound fibronectin beneath focal adhesions occurred differently in serum-free medium compared with serum-containing medium. Fibroblasts cultured on FFN substrata in serum-free medium attached and spread, and actin filament bundles detected with anti-actin antibodies appeared normal (Fig. 4 *A*). Under these conditions, however, essentially no removal of FFN from the substratum could be detected (Fig. 4 *B*).

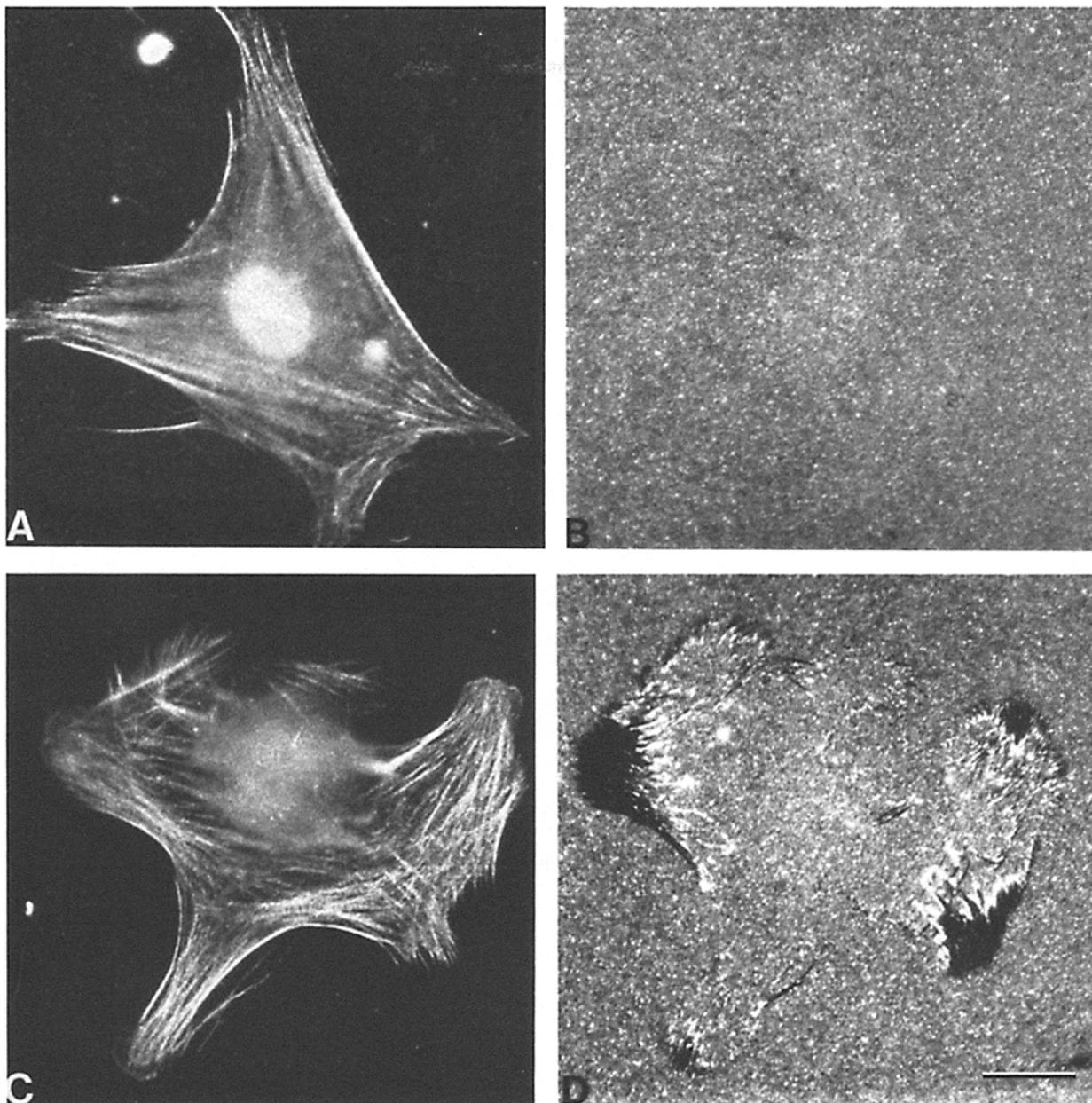
The above result suggested that cell adhesion in serum-free medium occurred without removal of substratum-bound fibronectin. It was of interest, therefore, to determine the mechanism of the serum effect. One possibility was that fibronectin added in the serum specifically promoted loss of substratum-bound fibronectin. To test this possibility, experiments were carried out using serum-free medium supplemented with 40  $\mu$ g/ml of human Fn, which approximates the fibronectin levels found in 10% serum. The samples with added human Fn behaved similarly as samples in serum-free medium in that there was no removal of substratum-bound FFN (data not shown).

Another possibility was that bulk serum protein, present in high concentration compared with the amount of FFN bound to the substratum, nonspecifically weakened the interaction of fibronectin with the substratum. This weakened binding might permit cells to remove Fn from the cell-substratum binding sites under tension (i.e., focal adhesions). To test this possibility, experiments were done using serum-free medium supplemented with 5 mg/ml BSA, which approximates the overall protein concentration of 10% FBS. Under these conditions, cells spread normally as shown by staining of the actin filament bundles (Fig. 4 *C*), and FFN was removed from the substratum (Fig. 4 *D*), results similar to those with serum-containing medium. The size and appearance of focal adhesion formed under these conditions

could not be detected with anti-Fn in intact cells (*B*, *asterisk*). Some FFN fibrils were accessible (*A*, *arrow*) to anti-Fn (*B*, *arrow*). By adjusting the plane of focus, it was evident that some of the FFN appeared at the upper cell surface (*C*) and this material could be detected by anti-Fn in intact cells (*D*). All of the reorganized fibronectin (*E*) could be detected by anti-Fn if the cells were permeabilized (*F*), and cellular Fn was observed along cell margins (*F*, *arrows*) although reorganized FFN no longer was present in these areas. After brief trypsinization, all of the fibrillar FFN was removed from the cells, but some patches of FFN appeared to have been phagocytosed (*G*). Other details are in Materials and Methods. Bar, 10  $\mu$ m.



**Figure 3.** Reorganization of FFn in relationship to Fn receptors and actin. Human fibroblasts were cultured on FFn-coated substrata in DME containing 10% FBS for 1 h (*A* and *B*), 2 h (*E* and *F*), or 4 h (*C* and *D*). Samples were fixed/permeabilized and stained with rabbit anti-FnR (*A–D*) or rabbit anti-actin (*E* and *F*) followed by rhodamine-conjugated goat anti-rabbit IgG. *A*, *C*, and *E* were viewed with fluorescein optics, and *B*, *D*, and *F* were viewed with rhodamine optics. After 1 h, patches of FFn (*A*, arrows) appeared to colocalize with Fn receptors (*B*, arrows) and FFn also was observed along cell processes where Fn receptors were concentrated. After 4 h, reorganized FFn (*C*, arrows) maintained its association with Fn receptors (*D*, arrows), but some regions of Fn receptors (*D*, asterisks) were not associated with FFn. Some fibrillar FFn (*E*, arrows) appeared to be organized along actin cables (*F*, arrows). Other details are in Materials and Methods. Bar, 10  $\mu$ m.



**Figure 4.** Cellular reorganization of substratum-adsorbed FFn in serum-free medium. Human fibroblasts were cultured on FFn-coated substrata in DME (*A* and *B*) or DME containing 5 mg/ml BSA (*C* and *D*) for 2 h. Samples were fixed/permeabilized and stained with rabbit anti-actin followed by rhodamine-conjugated goat anti-rabbit IgG. *A* and *C* were viewed with rhodamine optics, and *B* and *D* were viewed with fluorescein optics. Cells spread in serum-free medium and showed a normal reorganization of the actin cytoskeleton (*A*) but did not remove or reorganize FFn on the substratum (*B*). Cells spread in serum-free medium supplemented with 5 mg/ml BSA and also demonstrated normal cytoskeletal reorganization (*C*), and in addition, FFn on the substratum was removed and reorganized (*D*). Other details are in Materials and Methods. Bar, 10  $\mu$ m.

were similar to those observed in serum-free medium (data not shown).

#### **Desorption of Substratum-bound $^3\text{H}$ -Fn**

The above results were consistent with the idea that fibronectin was bound to the substratum less tightly in the presence of serum than in its absence. Proteins bind tightly to material surfaces, and protein desorption from material surfaces into physiological salt solutions is a very slow process. An increased turnover rate of substratum-bound protein has been reported to occur, however, when there is a high concentra-

tion of protein molecules present in the incubation medium (7, 9, 25). Experiments were therefore carried out to determine if serum or purified proteins promoted the loss of Fn from the substratum.

To quantitate the amount of Fn on the substrata, glass coverslips were treated with  $^3\text{H}$ -Fn under the same conditions used to coat these substrata with FFn. After incubation with 40  $\mu\text{g/ml}$   $^3\text{H}$ -Fn, the amount ( $\pm$ SD) bound to the glass coverslips was  $219 \pm 40$  ng/cm $^2$ , which is similar to the results of our previous studies on saturation binding of  $^3\text{H}$ -Fn to polystyrene surfaces (22). Desorption studies (Table I) indi-

**Table I. Release of Noncovalently Adsorbed <sup>3</sup>H-Fibronectin into Various Incubation Media\***

Additions to serum-free medium (DME)	% Release (± SD)
None	15.7 ± 0.1
10% FBS	70.7 ± 5.6
1% SDS	83.8 ± 2.3
7 mg/ml BSA	44.5 ± 2.1
7 mg/ml ovalbumin	36.8 ± 4.5
7 mg/ml gamma globulins	36.3 ± 0.4

\* Duplicate substrata coated with <sup>3</sup>H-Fn were incubated for 1 h with 1.0 ml of DME and other additions as indicated. At the end of the incubations, radioactivity released into the medium and remaining on the glass coverslips (which were cut in half and placed directly into scintillation vials) was determined. Percent release was calculated based on total radioactivity bound. Other details are in Materials and Methods.

cated that after 1 h in serum-free medium, only a small portion of <sup>3</sup>H-Fn (~15%) was lost from the substratum. Addition of serum resulted in a marked increase in the loss of <sup>3</sup>H-Fn from the substratum, ~70% in 1 h. By comparison, treatment with 1% SDS resulted in ~85% <sup>3</sup>H-Fn release during the same time period. Purified protein solutions (BSA, ovalbumin, and human gamma globulins) added to serum-free medium at concentrations similar to the protein concentration of 10% serum-containing medium promoted release of <sup>3</sup>H-Fn from the glass coverslips ~60% as well as serum (Table I).

#### Cell Adhesion to <sup>3</sup>H-Fn and FFn Covalently Cross-linked to Glass Coverslips

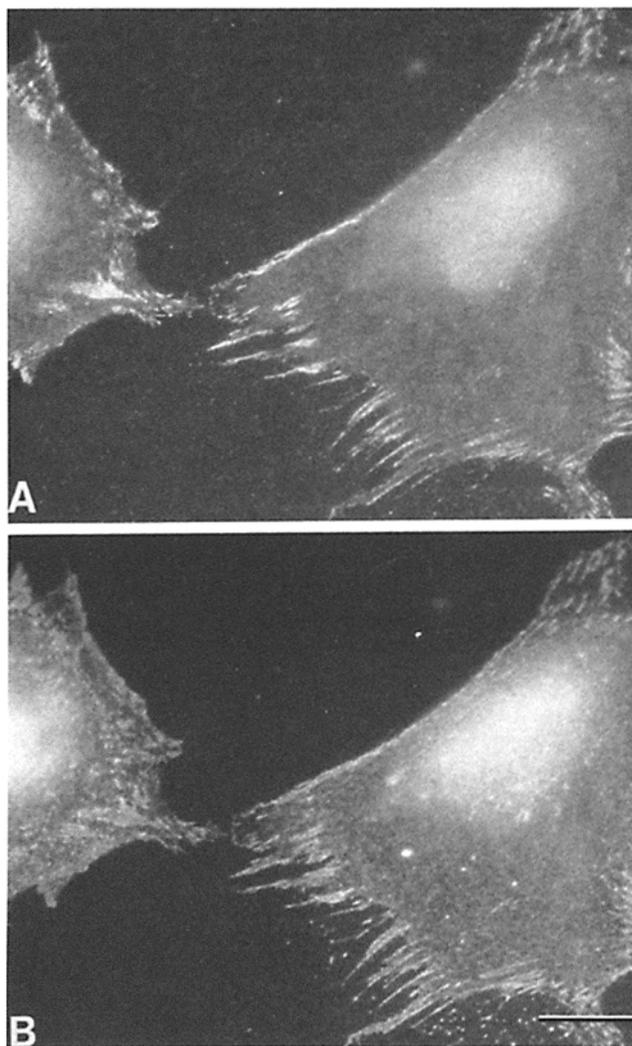
Based on the experiments in serum-free medium, it seemed reasonable to conclude that removal of fibronectin from focal adhesion sites required serum. To confirm that the cells removed FFn mechanically and not by another mechanism, e.g., proteolysis, experiments were carried out under conditions that prevented mechanical removal of Fn from the substratum.

Glass coverslips were modified with  $\gamma$ -aminopropyltriethoxysilane, treated with glutaraldehyde, and covalently coupled to <sup>3</sup>H-Fn or FFn. The amount of <sup>3</sup>H-Fn that bound to these substrata was  $186 \pm 42$  ng/cm<sup>2</sup>. After a 1-h incubation of covalently cross-linked <sup>3</sup>H-Fn in serum-free medium, <1% of the radioactivity was lost from the substratum (Table II). With serum present there was only ~1% loss. Addition of 1% SDS, which resulted in 85% removal of adsorbed <sup>3</sup>H-Fn (Table I), caused only ~5% loss of <sup>3</sup>H-Fn from the covalently cross-linked substrata (Table II).

**Table II. Release of Covalently Bound <sup>3</sup>H-Fibronectin into Various Incubation Media\***

Additions to serum-free medium (DME)	% Release (± SD)
None	0.21 ± 0.16
10% FBS	1.2 ± 0.91
1% SDS	5.0 ± 3.2

\* Duplicate substrata covalently cross-linked to <sup>3</sup>H-Fn were incubated for 1 h with 1.0 ml of DME and other additions as indicated. At the end of the incubations, radioactivity released into the medium and remaining on the glass coverslips (which were cut in half and placed directly into scintillation vials) was determined. Percent release was calculated based on total radioactivity bound. Other details are in Materials and Methods.



**Figure 5.** Organization of focal adhesion sites and fibronectin receptors of human fibroblasts spread on covalently cross-linked Fn substrata. Human fibroblasts were cultured on <sup>3</sup>H-Fn covalently cross-linked substrata in DME containing 10% FBS for 2 h. Only ~1% of the radioactivity was lost into the medium during the incubation. Samples were fixed/permeabilized and stained with mouse anti-vinculin and rabbit anti-fibronectin receptor followed by a mixture of fluorescein-conjugated goat anti-mouse IgG and rhodamine-conjugated goat anti-rabbit IgG. The cells demonstrated a normal pattern of marginal focal adhesions detected with anti-vinculin (A, fluorescein optics) and these corresponded closely with the distribution of fibronectin receptors (B, rhodamine optics). In control experiments, it was determined that there was no interspecies cross-reactivity between the first and second antibodies (data not shown). Other details are in Materials and Methods. Bar, 10  $\mu$ m.

Cells incubated on substrata with covalently cross-linked FFn attached and spread similarly as cells cultured on substratum with noncovalently adsorbed FFn. Under these conditions, the distribution of FFn appeared to be similar to that shown in Fig. 4 B, i.e., there was no detectable removal of FFn from the substratum even though the medium contained 10% serum (data not shown). Thus covalent cross-linking prevented the serum-dependent removal of FFn as expected. Also, when cells spread on covalently cross-linked <sup>3</sup>H-Fn

substrata in 10% serum-containing medium, only ~1% of the radioactivity was released into the incubation medium, similar to the result presented in Table II. Staining of these cells with anti-vinculin antibodies revealed a normal distribution of focal adhesion sites along the cell margins (Fig. 5 A), and the focal adhesion sites were coincident with Fn receptors detected with anti-FnR (Fig. 5 B). Clusters of Fn receptors like those seen under conditions of removal and reorganization of FFn (Fig. 3 B) were not observed.

## Discussion

Based on the studies reported in this paper it can be concluded that removal of fibronectin from the substratum is a mechanical process that requires serum. Fibronectin was found beneath focal adhesions under two different conditions, either when fibroblasts attached and spread in serum-free medium on substrata coated with adsorbed fibronectin, or when fibroblasts attached and spread in serum-containing medium on substrata coated with covalently bound fibronectin.

The present findings clarify differences previously reported regarding the relationship between fibronectin and focal adhesion sites. Studies that demonstrated an absence of fibronectin from focal adhesion sites used serum-containing incubation media (3, 4, 6, 11, 15). On the other hand, studies in which fibronectin was found beneath focal adhesion sites used serum-free or low serum-containing medium (21, 37).

Analysis of cells spread on substrata coated with covalently cross-linked fibronectin revealed colocalization of the 140-kD fibronectin receptor and focal adhesion sites. Therefore, this receptor may be important in focal adhesion formation. Previously it has been reported that the 140-kD Fn receptors are associated with focal adhesion sites (10, 14, 19).

The effect of serum on removal of fibronectin from focal adhesion sites appears to be nonspecific since serum could be replaced by equivalent concentrations of serum albumin, ovalbumin, or gamma globulins. Based on the quantitative measurements, the presence of protein in the incubation medium weakened the interaction of fibronectin with the substratum and promoted its desorption. Our studies confirm the previously reported increase in turnover of substratum-adsorbed Fn in the presence of serum (25). It is significant that protein desorption from substrata depends not only on bulk phase protein concentration (7, 9), but can also be promoted by shearing (7). Since focal adhesions are the sites of greatest stress between cells and the substratum (8), the highest mechanical force would be expected to be applied to the substratum at these locations, which can account for the removal of fibronectin.

Once the fibronectin was removed from the substratum, it appeared to be reorganized into patches and fibrils. Avnur and Geiger (3) reported that some of the fibronectin removed from the substratum by chick embryo fibroblasts was phagocytosed. Their criterion for internalization, however, was inaccessibility to anti-Fn in intact cells. By subjecting the cells to trypsinization it was evident that only fibronectin patches, not fibrils, were phagocytosed. The fibronectin fibrils that were inaccessible to anti-Fn staining in intact cells were in the excluded region that develops beneath cells during spreading, a possibility not considered previously. It seems likely that fibrils of reorganized fibronectin that connect cells

to the substratum correspond to the so-called "extracellular matrix adhesions" that were shown to contain fibronectin (11).

Reorganization of fibronectin probably involves the 140-kD fibronectin receptor since the reorganized fibronectin co-localized with these receptors. In addition, when the fibronectin could not be removed from the substratum, the patched reorganization of fibronectin receptors was not observed. Several other laboratories have shown colocalization of cellular fibronectin and fibronectin receptors (10, 14), but this is the first instance in which colocalization has been demonstrated between fibronectin receptors and fibronectin that was adsorbed on the substratum to which the cells were attached.

With time the fibronectin removed from the substratum and organized into fibrils became aligned with actin filament bundles, as was shown previously for cellular fibronectin (26). Some fibrillar fibronectin appeared to translocate to the upper cell surface, after which it could be detected by anti-Fn antibodies in intact cells. These fibrils also were stained by anti-FnR antibodies applied to nonpermeabilized cells, indicating that the fibronectin maintained its association with fibronectin receptors during translocation.

The ability of cells to reorganize substratum fibronectin is an example of how cells modify their extracellular matrices through contractile activity. Another example of this activity is the reorganization of loose, hydrated collagen gels into condensed, dermal-like collagen matrices (5, 24). Transformed cells that have a poorly organized actin cytoskeleton are much less proficient compared with normal cells at removing fibronectin from their substrata (18) and reorganizing hydrated collagen gels (38). It may be that such contractile modifications of extracellular matrices are important in embryonic cell migration (33) and connective tissue morphogenesis (39).

I am grateful to Drs. George Bloom, Bill Snell, and Richard Anderson for their helpful advice and to Cheryl Lamke-Seymour and Jannet Marshall for their expert technical assistance.

These studies were supported by grant CA14609 from the National Institutes of Health.

Received for publication 31 May 1986, and in revised form 27 August 1986.

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