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The Removal of Extracellular Fibronectin from Areas of Cell–Substrate Contact

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Summary

Immunofluorescent labeling for fibronectin was largely excluded from sites of closest contact between spreading chicken gizzard fibroblasts and the substratum. This was observed by double immunofluorescent labeling of fixed cells for fibronectin and vinculin, a smooth muscle intracellular protein that is specifically associated with focal adhesion plaques, in conjunction with interference-reflection microscopy. When the cells were plated on a fibronectin-coated substratum they adhered to its surface and rapidly spread on it. The immunofluorescent labeling for fibronectin in those cultures (after fixation and triton permeabilization) was usually absent from the newly formed, vinculin-containing focal adhesion plaques. We have found, however, that the accessibility to the cell–substrate gap at the focal adhesion plaques is limited and therefore a more direct approach was adopted. We have found that cells spreading on a substrate coated with rhodamine-labeled fibronectin progressively removed the underlying protein from the substrate. The removal of fibronectin involved at least two distinct mechanisms. Part of the substrate-associated fibronectin was removed from small areas and displaced toward the cell center. The arrowhead-shaped areas from which fibronectin was removed often coincided with vinculin-rich focal contacts. We observed, however, many areas where focal contacts were found over unperturbed fibronectin carpet, as well as fibronectin-free areas with no overlapping focal contacts. The possibilities that fibronectin is actively displaced from areas of cell–substrate contact, that the focal adhesion plaques are transiently associated with these areas and their implications on the dynamics of cell spreading and locomotion are discussed. The second route of fibronectin removal from the substrate was endocytosis. The rhodamine-labeled fibronectin was found in the cells in a partial or transient association with clathrin-containing structures.

Introduction

A well-recognized property of cultured fibroblastic cells is their capability to adhere to solid substrata and to spread on their surfaces. Although this phe- is a glycoprotein found on the surface and around cultured fibroblasts and is closely related to the plasma protein cold insoluble globulin (CIG) (Morrison et al., 1948; Ruoslahti and Vaheri, 1975). The addition of either protein to cultured cells has been shown to promote cell attachment to the substratum (Ruoslahti and Vaheri, 1975; Pearlstein, 1976; Höök et al., 1977; Yamada et al., 1978) and subsequently to induce cell spreading (Grinnell, 1978; Grinnell and Hays, 1978). In a related vein, the addition of fibronectin to transformed cells, which often exhibit rounded-up shape and reduced surface fibronectin levels (Hynes, 1973; Vaheri and Ruoslahti, 1974), partially restored flattened morphology and normal adhesiveness characteristic of the nontransformed phenotype (Yamada et al., 1976; Ali et al., 1977).

A different line of studies has indicated that the closest and probably the strongest adhesion of well-spread fibroblasts to a solid surface occurs through specialized areas at the ventral cell surfaces denoted focal contacts or focal adhesion plaques (Curtis, 1964; Izzard and Lochner, 1976; Abercrombie and Dunn, 1975). These areas can be recognized as small, arrowhead-shaped plaques by interference-reflection microscopy. Moreover, electron-microscopic and immunofluorescent studies have shown that the cytoplasmic faces of focal adhesion plaques are the sites where actin-containing stress fibers terminate (Abercrombie et al., 1971; Heath and Dunn, 1978; Geiger, 1979; Wehland et al., 1979).

Some insight into the molecular architecture of microfilament–membrane association at the adhesion plaques was recently obtained by the demonstration that a newly described smooth muscle protein, vinculin, (130 kd protein) was concentrated at the cytoplasmic surfaces of these focal sites (Geiger, 1979; Geiger et al., 1979). This was based on immunofluorescent labeling with antivinculin antibodies, combined with interference-reflection microscopy.

Another set of experiments that linked fibronectin to the process of cell adhesion involved the demonstration that fibronectin can be associated transmembranally with intracellular actin-containing microfilament bundles. By immunofluorescence experiments (Heggeness et al., 1978; Hynes and Destree, 1978) and by electron microscopy (Singer, 1979) fibronectin-containing structures on the external surfaces of fibroblasts were found in close juxtaposition, across the cell membrane, with actin-containing filament bundles.

This and other related information taken together have raised the possibility (Hynes and Destree, 1978; Grinnell and Feld, 1979) that fibronectin molecules may function in cell adhesion and spreading by di-

and C–D in Figure 1. Thus focal contacts could be found from which fibronectin staining was not excluded and vice versa.

The Spatial Distribution of Fibronectin, Vinculin and Focal Adhesions in Cells during Spreading on

nectin during cell spreading we plated chicken gizzard cells on a fibronectin-coated substratum. At various stages of spreading the cells were fixed with formaldehyde, permeabilized with Triton X-100 and stained with antibodies to both fibronectin and vinculin as described above. A typical result obtained with cells fixed 2 hr after replating is shown in Figures 2A and 2B. The fibronectin carpet in between the cells was immunolabeled uniformly as shown in Figure 2A. Under the cells, however, the labeling was missing from discrete elongated areas as indicated by the arrows in Figure 2A. Along the margins of many of those areas, we often observed an enrichment in fibronectin labeling in a form of small filamentous bundles. When the immunolabeling patterns for fibronectin and vinculin (Figure 2B) were compared, we found that most of the areas free of fibronectin labeling coincided with vinculin-containing focal adhesion plaques.

A major objection that could be raised against the interpretation of the results described here is that the

most closely associated with the substratum despite the Triton treatment.

To address this problem directly several lines of experiments were taken. We attempted to modify the fixation conditions to render the focal contacts more accessible to the antibodies. Cells were shortly treated with detergent (0.5% Triton X-100 in 50 mM MES buffer [pH 6.0]) then fixed and immunolabeled as above. The results of such treatment (Figure 2C) indicated that fibronectin labeling was still absent from significant areas under and near the spreading cells. As a rule, in the vicinity of the areas devoid of fibronectin labeling, an accumulation of fibrous fibronectin was noticed.

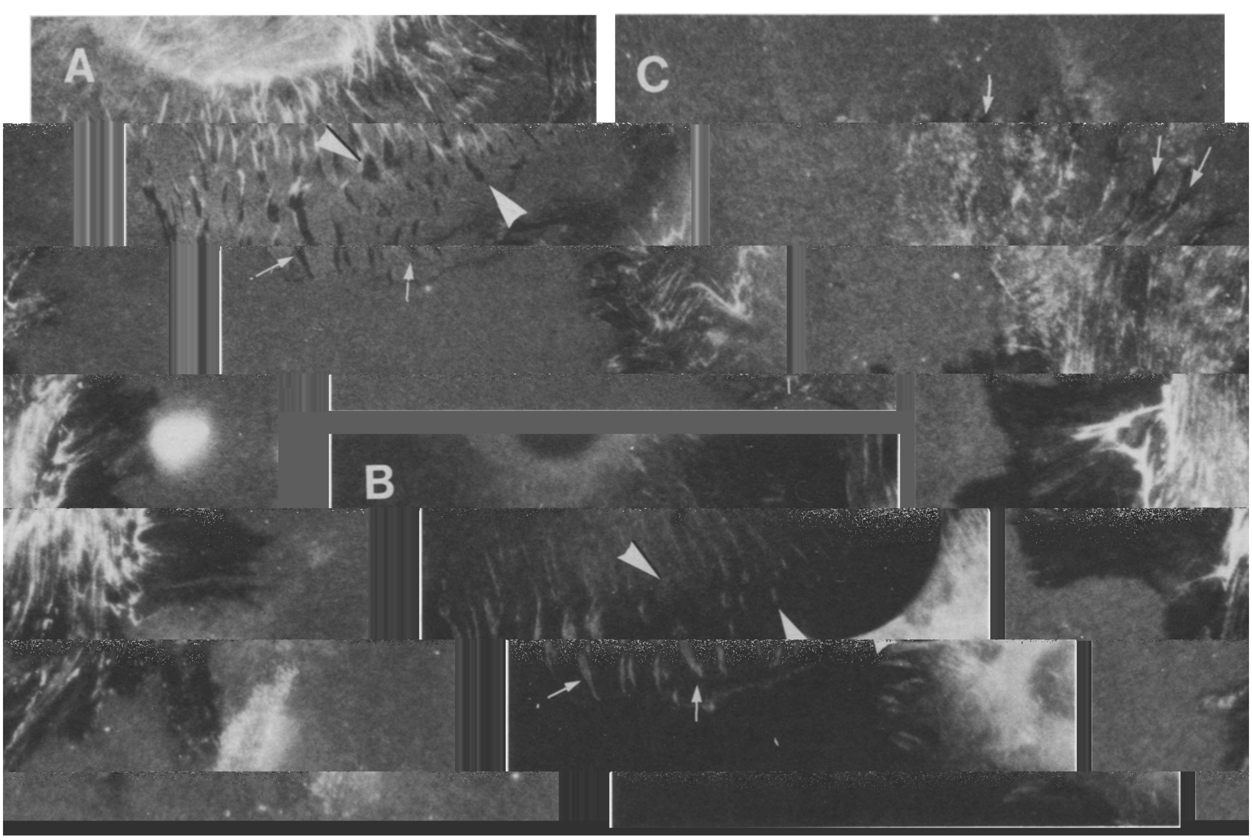


Figure 2. Immunofluorescent Labeling for Fibronectin (A and C) and Vinculin (B) of Chicken Gizzard Cells 2 Hr after Plating

(A) Cell fixed, permeabilized and immunolabeled with guinea pig antifibronectin and fluorescein-conjugated goat anti–guinea pig IgG.

(B) The same cell labeled with rabbit antivinculin and goat antirabbit rhodamine. The matched arrows in A and B point to vinculin-containing areas in which fibronectin labeling is missing. The arrowheads point to sites devoid of fibronectin labeling which do *not* coincide with vinculin-rich plaques. Notice the enrichment in fibrillary fibronectin near the edges of most of the vinculin-containing areas.

(C) Cell immunolabeled for fibronectin with rabbit antibodies and goat antirabbit IgG rhodamine. The cell was first permeabilized and then fixed and immunolabeled. Notice the absence of fibronectin from both restricted locations (arrows) and from large areas at the cell periphery. Bar indicates 10 μm.

To determine the fate of fibronectin directly we conjugated rhodamine to substrate-attached CIG (rh-CIG) and determined its distribution during cell attachment and spreading. Examination of such culture under the fluorescence microscope revealed a progressive removal of the rhodamine-labeled fibronectin. After about 30 min of spreading, the fibronectin carpet under the attached cells was still mostly uniform as may be appreciated from comparison of the fluorescent pattern and phase photomicrograph of the same area (Figures 3A and 3B, respectively). Some of the cells, however, showed at that stage a very limited removal of the substrate-attached fibronectin from restricted areas at the cell periphery, as indicated by the arrows in Figures 3A and 3B. The removal of fibronectin from the substratum at the cell periphery was often extensive, even when the cells were still in early stages of spreading as shown in Figure 3C. It was noted that an accumulation of fluorescently labeled fibrillary fibronectin was observed in the vicinity of the cleared area, proximal to the cell center. Careful examination of the fibronectin carpet revealed that it was also removed from restricted, elongated areas under the central regions of the cells. Longer incubation periods on the fibronectin carpet resulted in its extensive removal both from the cell periphery and the cell center. An example of cells growing for 60 min on rh-CIG carpet is shown in Figures 3D and 3E. The borders of the fibronectin-clear areas are not strictly related to the margins of the cells as can be concluded from the fluorescent pattern in Figure 3D and the combined fluorescence–phase micrograph in Figure 3E. Variations in the concentration of fibronectin used for coating the substrate (and hence in the thickness of the carpet) had an apparent effect on the process of fibronectin removal. Increasing the fibronectin concentrations over 30 μg/ml resulted in a poor and delayed removal of the carpet. In concentrations below 1.5 μg/ml the rhodamine labeling was weak, and the displacement of this thin carpet was difficult to document. The removal of substrate-attached fibronectin was not unique to either chicken fibroblasts or human CIG. A similar phenomenon was observed with other cell types, including human foreskin fibroblasts and mouse 3T3 cells, plated on either human or bovine CIG. An example of a human foreskin fibroblast spreading on rh-human CIG carpet is shown in Figure 3F. The addition of sodium azide in concentrations of up to 20 mM to the culture medium caused a delay in attachment, spreading and fibronectin removal by the cells, but did not prevent it.

The use of rh-CIG carpet enabled us to study the accessibility of the focal contacts to antibody reagents. When exposed to Triton X-100 (0.5% in 50 mM MES buffer, 5 mM $MgCl_2$, 3 mM EGTA [pH 6.0]) for 2 min, then fixed with formaldehyde and immunolabeled indirectly for fibronectin using fluorescein-conjugated antibodies, the two patterns obtained (Figures 3G and 3H, respectively) were essentially identical. When, however, fixation was carried out prior to detergent permeabilization we were able to find many regions in which the substrate-associated CIG was not disturbed, while antibody-labeling was missing from them. Figure 3I shows the fluorescent pattern of the fibronectin carpet after 1 hr of incubation with the cells. The immunolabeling of the same area (Figure 3J) indicated that the labeling was largely missing from many focal areas under the spreading cells although the fibronectin carpet in those areas appeared intact. We thus concluded that the accessibility of the antibodies to the focal contacts may indeed be very limited in prefixed cells. The implications of these findings on the interpretation of the results described in Figures 1 and 2 as well as the results obtained in other laboratories will be discussed later.

To study directly the correlation between cell con-

Figure 3. Fibroblastic Cells Plated on Rhodamine-CIG Carpet

(A) Rhodamine fluorescence in culture of chicken gizzard cells 30 min after spreading. The arrow points to the removal of rh-CIG from the substrate at the cell periphery.

(B) Phase photomicrograph of the same field. The cells are still radially spread and the fibronectin carpet under most of them is still intact or slightly perturbed.

(C) Rhodamine fluorescence as in A. The cells shown there exhibit a more extensive removal of fibronectin from the cell periphery and its accumulation proximally to the cell center.

(D) Rhodamine-CIG carpet 60 min after plating of chicken gizzard cells. The substrate-associated fibronectin is removed from both the periphery and central regions of the cell.

(E) Combined fluorescence–phase photomicrograph of the field shown in D. The fibronectin-free area at the periphery is often located outside the borders of the cells.

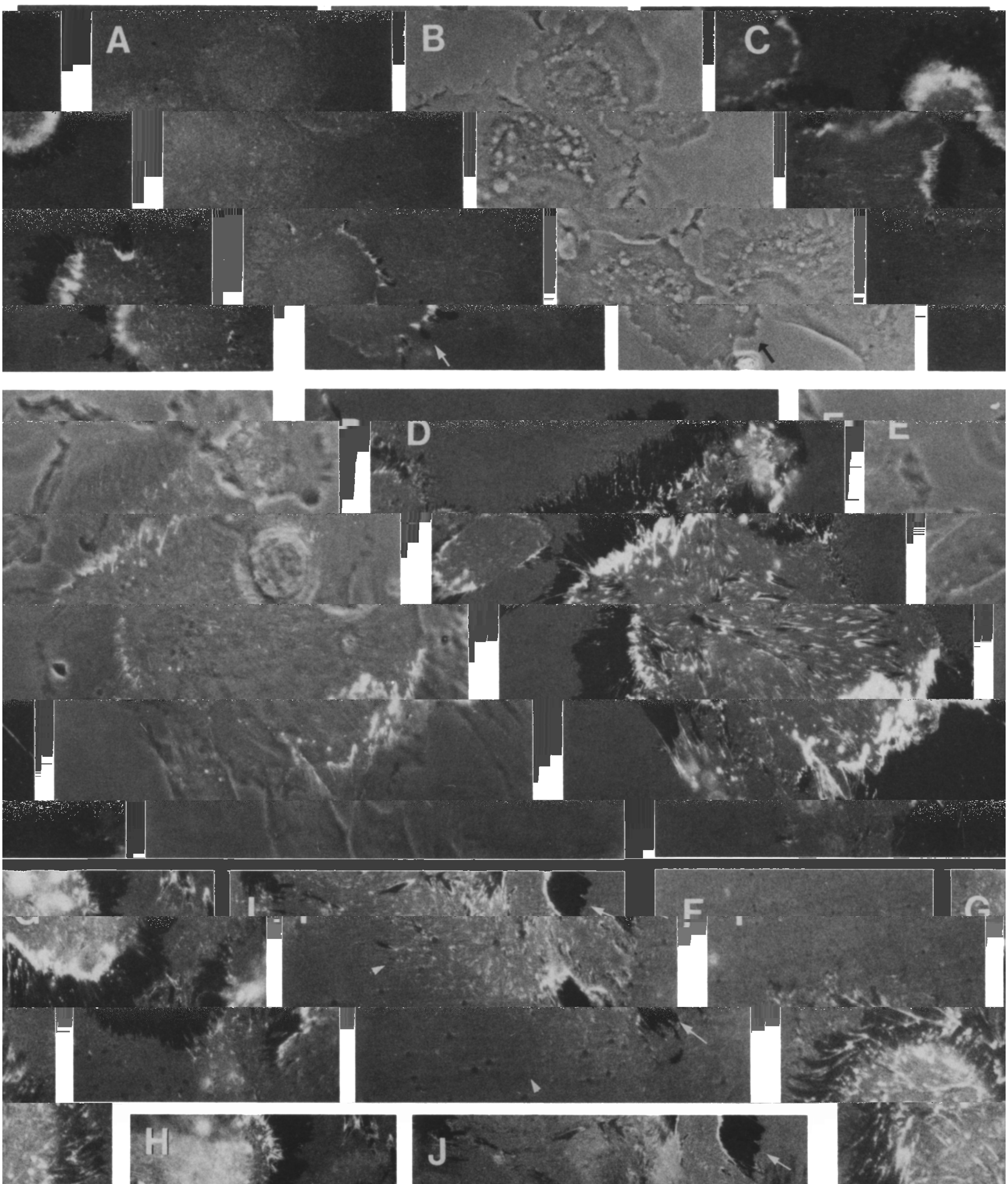
(F) Human foreskin fibroblast during early spreading on rhodamine-conjugated human CIG. The removal of rh-CIG is similar to that obtained with chicken cells.

(G) Rhodamine-CIG carpet 60 min after plating of chicken gizzard cells.

(H) The same field immunolabeled for fibronectin with rabbit antibodies to fibronectin and fluorescein-conjugated goat antirabbit IgG. The cells were *first* treated with Triton then fixed and immunolabeled. The substrate associated rhodamine and fluorescein-labeling patterns are essentially identical.

(I) Rhodamine-CIG carpet in cells 60 min after plating.

(J) Immunolabeling of the same cell for fibronectin (fluorescein). The cells were fixed first, and then permeabilized and immunolabeled. Notice the similar absence of rh-CIG and immunolabeling from the areas indicated by the arrows. There was, however, exclusion of fibronectin immunolabeling from many sites under the ventral cell membrane (arrowheads in J) though the rh-CIG in those areas appeared intact (matched arrowheads in I). Bar indicates 10 μm.


A
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J

tacts and the areas from which fibronectin was removed, cells growing on rh-CIG carpet were fixed either before or after detergent treatment and then immunolabeled for vinculin. As depicted in Figure 3 and in Figures 4A, 4D and 4E, fibronectin was no longer uniform under the cells and exhibited a complex pattern of distribution. Similarly, vinculin labeling inside the cells consisted of large number of randomly oriented patches (Figures 4B and 4E). The vinculin distribution was closely correlated to that of cell–substrate focal contacts as visualized by interference-reflection microscopy (compare, for example, the vinculin labeling in Figure 4B to the same cell observed by interference-reflection optics in Figure 4C). The fibronectin-free areas were partially related to the vinculin-containing focal contacts. Thus in many locations, such as those marked with matching arrows in Figures 4A–4C and in Figure 4D, fibronectin was removed from the area under the focal contact or its vicinity. This process was however not complete and the area under many of the vinculin-rich contact sites coincided with intact, or only slightly perturbed, fibronectin carpet. Similarly, many of the areas on the substrate from which fibronectin was extensively removed were not related to focal contacts and often were not even related to the borders of the cell itself (Figures 4E and 4F). In small areas where vinculin displayed a "fibrillary" or "streaky" appearance we have often observed an enrichment of fibronectin under those restricted areas as pointed out by the central

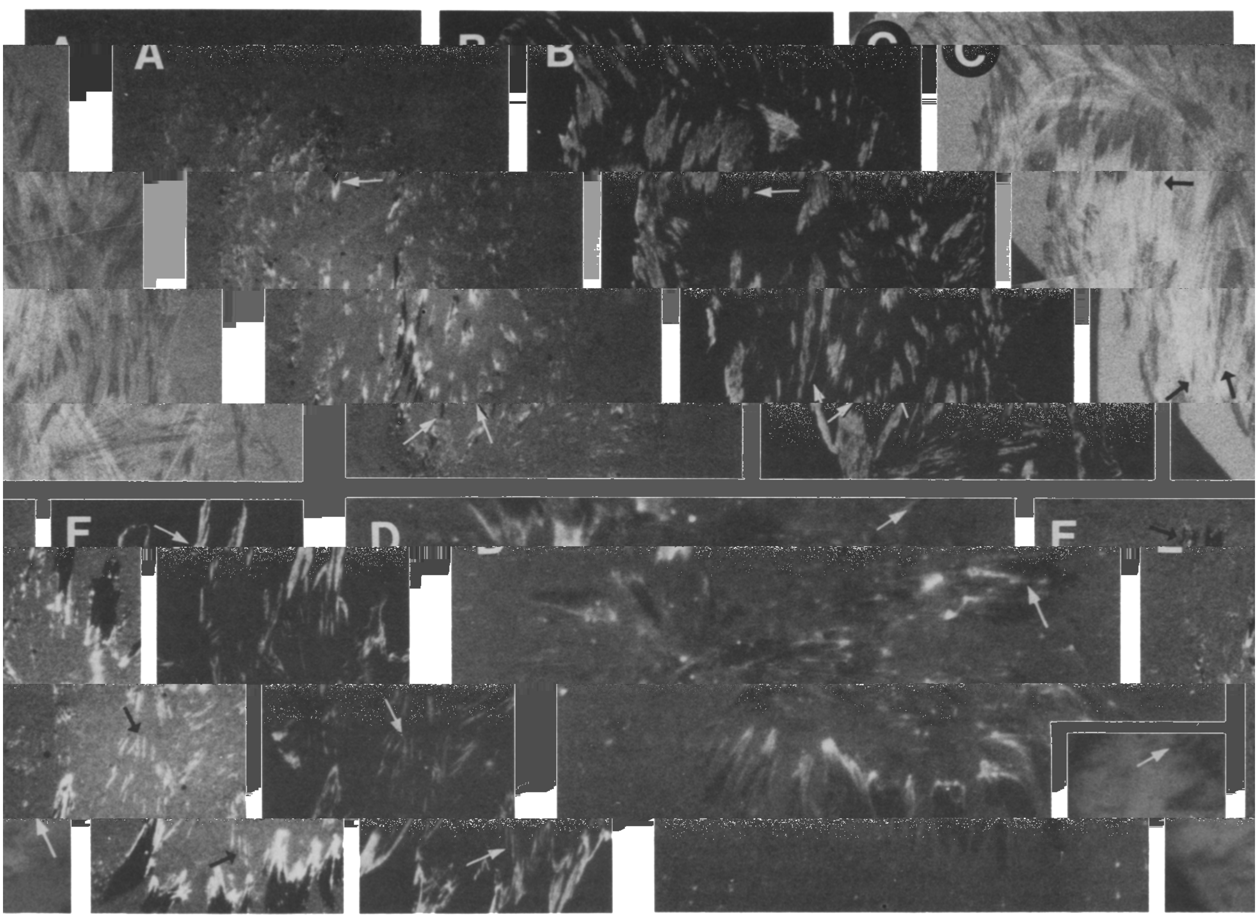

Figure 4. The Spatial Relationships between Fibronectin Removal, Vinculin and Interference-Reflection Patterns

(A) Rhodamine-CIG carpet 60 min after plating of chicken gizzard cells.

(B) The same cell, permeabilized, fixed and immunolabeled indirectly for vinculin using fluorescein-conjugated antibodies.

(C) The same cell examined with interference-reflection optics.

The arrows in A–C point to the areas in which fibronectin is apparently removed from vinculin-rich focal contacts. Many focal contacts are located over unperturbed fibronectin carpet.

(D) Rhodamine-CIG carpet 30 min after plating. The insert shows the interference-reflection image of the cell (arrows point to the same locations). The substrate associated fibronectin is apparently removed from the focal contact.

(E) Rhodamine-CIG carpet, 60 min after plating of chicken gizzard fibroblasts.

(F) The same cell immunolabeled for vinculin. Notice the partial and extensive removal of fibronectin from certain vinculin-rich areas (top arrow) while in many other locations fibronectin removal was not related to vinculin. The lower and central arrows point to sites in which fibronectin is accumulating in an apparent association with "fibrillary" vinculin labeling. Bar indicates 10 μm.

and bottom arrows in Figures 4E and 4F. The possible implications of these complex relationships between exogenous fibronectin and vinculin-containing focal contacts will be discussed later.

After incubation of chicken gizzard cells on a rh-CIG carpet much fluorescence was associated with intracellular vesicular structures as indicated by the arrows in Figures 5A, 5C and 5G. These fibronectin-containing structures were usually observed at a focal plane dorsal to the substrate attachment sites, as clearly seen in Figures 5E and 5G. The accumulation of these structures was progressive and closely re-

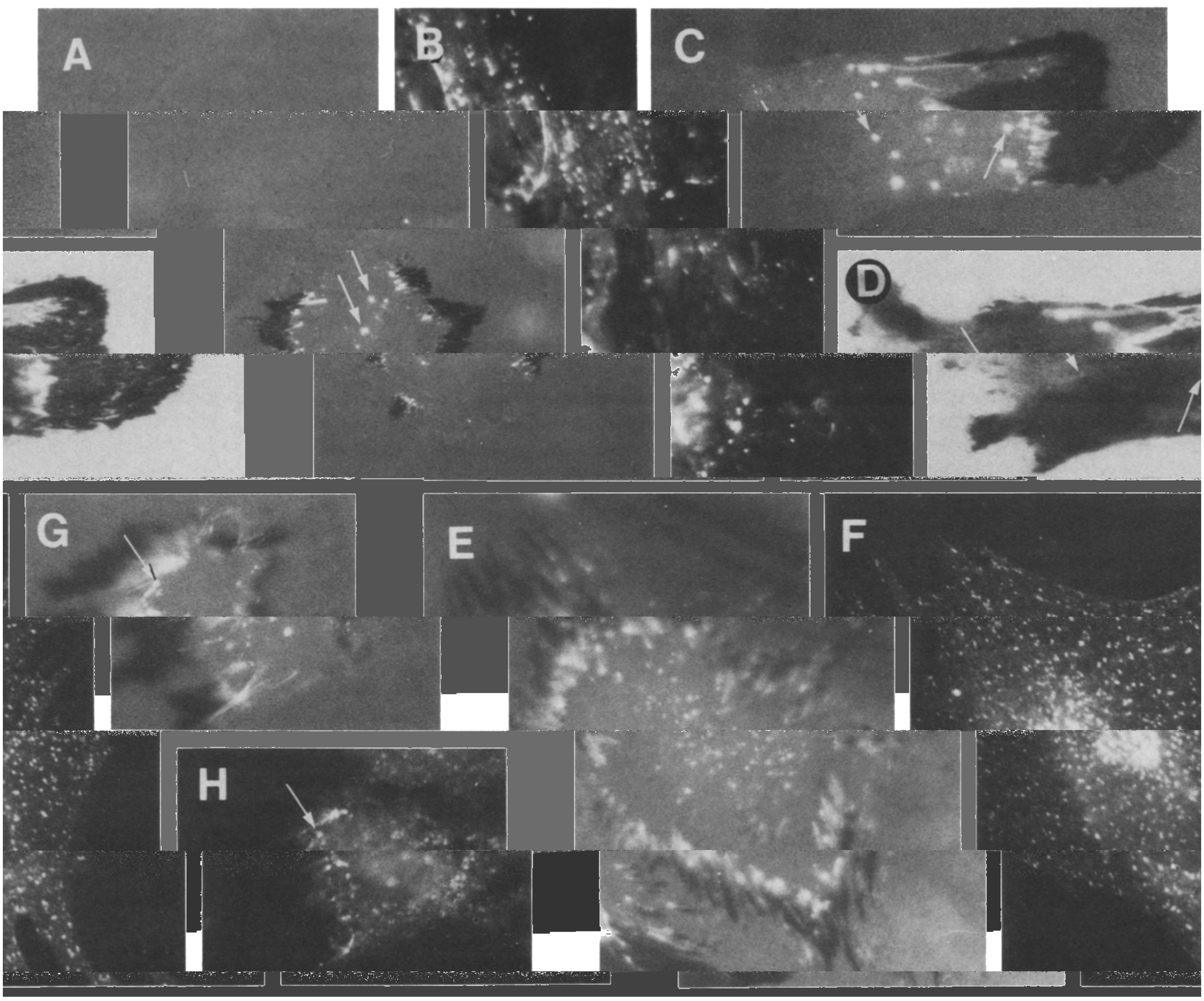

Figure 5. Endocytoses of Fibronectin by Chicken and Human Fibroblasts

(A) Chicken gizzard cell fixed 30 min after plating on rh-CIG carpet. Arrows point to particulated, nonfibrillary rh-CIG.

(B) Chicken gizzard cell as in A, fixed 2 hr after plating.

(C) Chicken gizzard cell fixed 60 min after plating on rh-CIG carpet.

(D) The same cell, after fixation and immunolabeling for fibronectin with fluorescein (no permeabilization was performed). The rhodamine-labeled fibronectin associated with granular structure (indicated by arrows) is not accessible to the antibodies and thus appears unlabeled. It is therefore [illegible] fibronectin was endocytosed by the cells.

(E) Human foreskin fibroblast, fixed 60 min after plating on rh-CIG carpet. Many rhodamine-labeled particulated structures are found in the perinuclear region, in a focal plane dorsal to the substrate.

(F) The same cell; fixed, permeabilized and immunolabeled indirectly with anticlathrin antibodies and fluoresceinated goat antirabbit IgG. The labeling is associated with numerous cellular structures, probably coated pits and coated vesicles as well as with tubular structure near the nucleus (possibly Golgi apparatus). The numbers of either fibronectin or clathrin-associated vesicles were usually too large to test for a direct correlation between the two.

(G) Chicken gizzard cell fixed 30 min after plating on rh-CIG carpet. This cell was selected for a limited amount of endocytosed fibronectin.

(H) The same cell, labeled after fixation and permeabilization for clathrin. Comparison of the two patterns indicates that significant number of the fibronectin-associated endocytic vesicles are apparently associated with clathrin (arrows point to the same locations). This correlation is only partial, namely, some of the endocytosed CIG is not associated with clathrin vesicles, and many of the coated vesicles do not contain CIG-detectable clathrin. Bar indicates 10 μm.

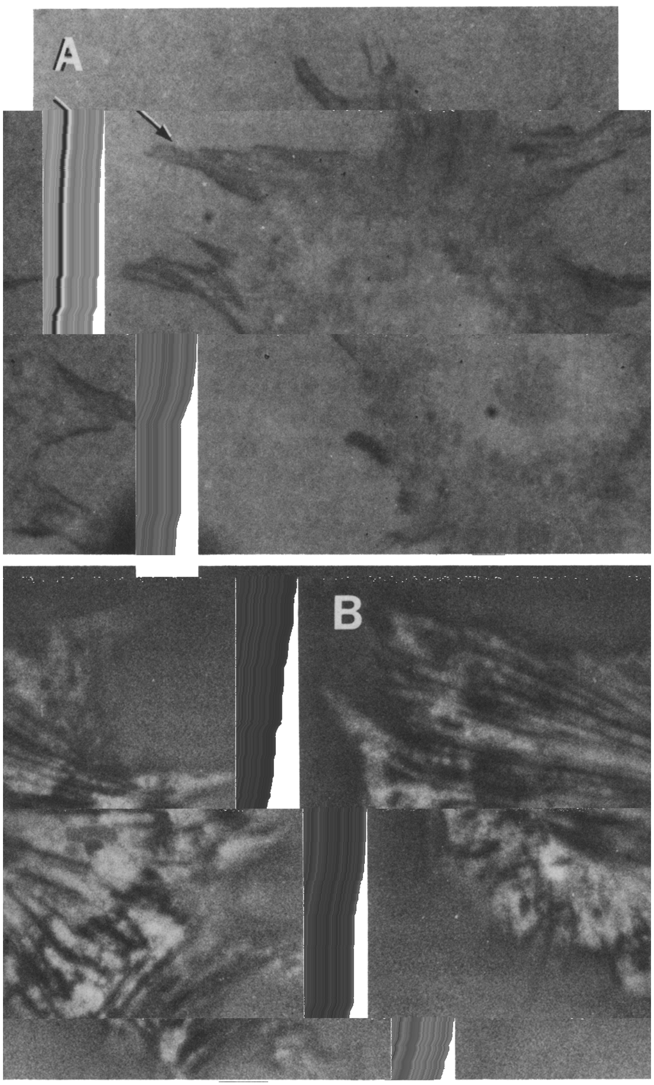

Figure 6. Chicken Gizzard Cells Plated on Substrate-Attached BSA.
(A) Chicken gizzard cell fixed 2 hr after plating on BSA carpet. The cell was fixed, Triton permeabilized and immunolabeled for BSA.
(B) Chicken gizzard cell fixed 60 min after plating on rh-BSA carpet. Notice the absence of fluorescence from elongated areas under the cells. Bar indicates 10 μm.

lated to the removal of the fibronectin carpet. After about 30 min of incubation (Figure 5A), only few such structures were visible, while after 2 hr, most of the fibronectin carpet was already removed and a very large number of fibronectin-containing particles was noted (Figure 5B). This fibronectin was most probably endocytosed by the cells because it was localized above the level of the substrate and it could not be labeled by antifibronectin antibodies unless the cells were permeabilized first with detergent. This is demonstrated by Figures 5C and 5D. The cells were growing on a rh-CIG carpet for 60 min, then they were fixed and immunolabeled with antifibronectin antibodies and fluoresceinated goat antirabbit IgG. The results indicated that the particulated rhodamine-conjugated fibronectin is not accessible to the antibodies and thus it is most probably inside the cells. When the cells were treated with Triton prior to the labeling, the intracellular rh-CIG was positively labeled (not shown).

To characterize further the fate of the endocytosed fibronectin, the cells (chicken or human fibroblasts) were fixed, permeabilized and labeled with anticlathrin antibodies, to visualize coated vesicles and coated pits. Usually a very large number of clathrin-containing structures were found (Figures 5F and 5H) and the correlation with the large number of fibronectin-containing vesicles (for example Figure 5E) was difficult. In many cells (of both human and chicken origin), however, areas were found in which the endocytosed fibronectin appeared closely associated with clathrin-labeled structure (compare the fluorescence in the region indicated by the arrow in Figures 5G (fibronectin) and 5H (clathrin).

The Redistribution of Bovine Serum Albumin (BSA) during Cell Spreading on BSA Coated Substratum

As another type of control a uniform carpet of BSA was prepared on glass coverslips and the cells, suspended in serum-containing medium were plated on it. After different periods the cells were fixed, permeabilized and labeled for BSA with rabbit anti-BSA and rhodamine-conjugated goat antirabbit IgG. The immunolabeling for BSA (Figure 6A) was largely absent from cell–substrate contacts, as verified by interference-reflection microscopy. However, since our previous results indicated that such a pattern may result, at least partly, from reduced accessibility for the antibodies, we have investigated this phenomenon further using a rhodamine-labeled BSA carpet. The results (Figure 6B) pointed to a reduced fluorescence in elongated areas under the spreading cells. The margins of these areas were, however, more diffuse than those found with the fluorescent fibronectin, and the accumulation of fibrillary materials typical for fibronectin was not observed.

Discussion

Extensive efforts have been invested in recent years in an attempt to characterize the mechanisms involved in the process of cell adhesion. Two major experimental approaches have been employed in such studies: first, anatomical characterization of those areas in the membrane directly associated with intercellular contacts or with sites of adhesion to extracellular matrix or tissue-culture substratum (Izzard and Lochner, 1976; Abercrombie and Dunn, 1975; Heath and Dunn, 1978); and second, search for extracellular components that promote cell attachment and spreading. Such materials may either be produced by the cells or may be supplied to them exogenously (Grinnell 1978; Vaheri and Mosher, 1978; Yamada and Olden, 1978).

The attachment of cultured fibroblasts to the substratum as identified by electron microscopy and interference-reflection optics occurs at limited areas over the ventral cell-surface-denoted focal contacts or focal adhesion plaques. In these restricted areas the cell membrane was found to run parallel to the surface of the substratum, leaving a gap of only 100–150 Å between them (Izzard and Lochner, 1980). In addition, broader areas of less firm attachment were also detected by interference-reflection microscopy while the rest of the ventral cell surface remained loosely attached to the surface. The focal adhesion plaques are not only the sites of closest contact with the substratum but also are characterized by the association of their cytoplasmic faces with the termini of actin-containing microfilament bundles. This was originally found using electron microscopy (Abercrombie et al., 1971; Heaysman and Pegrum, 1973) and later by a combination of interference-reflection microscopy and immunofluorescent labeling for actin and its associated proteins (Heath and Dunn, 1978; Geiger, 1979; Wehland et al., 1979). Recently it was demonstrated that a newly described smooth muscle protein, denoted by us vinculin, is specifically concentrated in the sites of microfilament–membrane contacts at the focal adhesion plaques (Geiger, 1979; Geiger et al., 1980; Feramisco and Burridge, 1980). In the studies described here the simultaneous immunolabeling for vinculin and fibronectin on the same cell was found to be very useful for the determination of the spatial relationships between fibronectin and the focal contacts.

The attachment- and spreading-promoting properties of fibronectin, together with its pattern of distribution on the cell membrane and the apparent transmembrane association with actin-containing microfilament bundles, raised the possibility that fibronectin directly mediates the contact between the cell and the substratum at the focal adhesions. However, the results presented here indicate that the relationships between fibronectin and focal contacts are considerably more complex.

The results of double immunolabeling for fibronectin and vinculin on the same cells together with interference-reflection microscopy (Figure 1) pointed to the absence of labeling from focal contact, in an agreement with the recent data of Badley et al. (1980), Birchmeier et al. (1980), Fox et al. (1980) and Grinnell (1980). However, a major difficulty in the interpretation of these results is the possibility that the absence of labeling of fibronectin inside the focal contact results merely from reduced accessibility for the antibodies rather than from its actual absence from these areas. The studies performed with the uniform fibronectin carpet provided us with means to look into that problem. When we fixed and tritonized cells growing on a rhodamine-conjugated fibronectin carpet and then immunolabeled them indirectly for fibronectin using fluoresceinated antibodies (Figures 3I and 3J), there were many areas from which the labeling was missing (arrowheads in Figure 3J), although the fibronectin of the carpet in those areas was still intact. The same areas (not shown) contained vinculin and appeared dark in interference-reflection microscopy, indicating that there is indeed a reduced penetration of antibodies to focal contacts. Thus an absence of immunolabeling in that specialized region, as demonstrated also by Badley et al. (1980) and by Birchmeier et al. (1980), should not be taken as an indication for actual absence of fibronectin. Several approaches were designed to circumvent the accessibility problem. One of them made use of a brief detergent treatment before fixation. Immunolabeling for fibronectin of cultures growing on rhodamine-fibronectin carpet indicated that absence of labeling was restricted only to those areas from which the rh-CIG was removed (Figures 3G and 3H). Chen and Singer (1980) immunolabeled ultrathin frozen sections of chick fibroblasts simultaneously for fibronectin and for Con A-binding proteins at the cell surface. Their results indicated that fibronectin labeling was indeed reduced or missing from focal contacts which were extensively labeled for Con A-binding sites.

In a second set of experiments we have determined the spatial distributions of exogenous fibronectin- and vinculin-containing focal contacts in cells growing on a uniform carpet of fibronectin (human CIG). This set of experiments was performed on two types of fibronectin carpets; the first was of native fibronectin and the second used rhodamine-labeled fibronectin. The experiments performed with fluorescent fibronectin layer enabled us to determine its distribution directly and thus avoid the necessity for immunolabeling and the possible artifacts which may result from uneven penetration of the antibodies. The experiments performed with unmodified fibronectin, on the other hand, are believed to represent more reliably the interaction of cells with the native protein. Nevertheless, both the native and rhodamine-conjugated protein promoted attachment and spreading of a variety of cells including chicken gizzard fibroblasts, 3T3, L cells and human foreskin fibroblasts.

The removal of substrate-attached fibronectin was progressive and by 6 hr of incubation most of the protein was removed. The lateral displacement of fibronectin and the formation of fibronectin cables outside the cells occur most probably via a dynamic membrane process in sites of cell–substrate contact. However, the correlation between the fibronectin-free areas and vinculin-containing focal contacts was quite complex as may be appreciated from Figure 5. Many areas from which fibronectin was partially or extensively removed coincided with focal contacts. This was observed most commonly in early stages of spreading. Nevertheless, the removal of fibronectin was by no means a prerequisite for the formation of focal contacts as determined by interference-reflection microscopy and vinculin immunolabeling, since many of the focal contacts were located on an intact or only partially perturbed fibronectin carpet (Figures 5A–5C). Similarly, many of the fibronectin-free areas were not related to focal contacts and were often located outside the cell borders (see, for example, Figures 5E and 5F). This observation is related most likely to the dynamic nature of the spreading process and to cell locomotion. This extensive removal of fibronectin by moving cells may also be related to the formation of phagokinetic tracks on coloidal gold, as observed by Albrecht-Buehler (1977). Finally, there were also areas in which accumulation of fibronectin was directly related to vinculin-rich areas (Figures 5E and 5F, arrays). Usually the vinculin organization in these sites was "fibrillary" rather than focal. This finding may be related to the previously described transmembrane association of fibronectin with actin (Heggeness et al., 1978; Hynes and Destree, 1978; Singer, 1979) and to the recent demonstration of spatial relationships between fibronectin and microinjected vinculin (Feramisco and Burridge, 1980). We have no direct data concerning the temporal sequence of events related to contact formation and fibronectin removal, though our data support the view that fibronectin promotes cell contact, but once the contact is established, fibronectin may be removed from the substrate at that area.

The removal of substrate-attached proteins seems to be a general phenomenon, since it was observed also with cells growing on a BSA layer. BSA did not promote attachment and spreading as did fibronectin, but the distribution of the rhodamine-labeled BSA was also altered under the spreading cells. Unlike fibronectin, however, there was no apparent accumulation of the labeled BSA, certainly not in a fibrillary form.

In addition to the lateral displacement of extracellular fibronectin, we also observed progressive endocytosis of this protein by the cells (Figure 5). Cells in early stages of spreading usually displayed such endocytosis in cell regions close to areas from which fibronectin was also laterally displaced, and thus the two processes could occur simultaneously in the same area. We have tried to determine whether the endocytosis of fibronectin is related to clathrin-coated vesicles by labeling cells with anticlathrin antibodies. This comparison was usually difficult to do due to the large number and high density of both fibronectin- and clathrin-containing structures. In some cells, however, some coincidence was found suggesting partial or transient association (Figures 5G and 5H).

The phenomena described here, namely the spatial relationships between cell–substrate contacts and reorganization of the matrix protein fibronectin may be mutually related. The removal of substrate-attached fibronectin by both lateral displacement and by endocytosis is a contact-related process. Nevertheless, these alterations in fibronectin distribution, together with de novo production and secretion of this protein by the cells may, in turn, further affect cell attachment and locomotion. These cell–matrix relationships may thus be of great importance for the normal development and motility of cultured cells.

Experimental Procedures

Purification of Human Plasma Fibronectin

Fibronectin (cold insoluble globulin) was purified from human plasma using two cycles of gelatin-Sepharose column chromatography (Ruoslahti et al., 1978; Orly and Sato, 1979) and was apparently homogeneous as determined by polyacrylamide gel electrophoresis (gradient slab gels of 6–15% acrylamide) in the presence of SDS (Studier, 1973). Phenylmethylene sulfonyl fluoride (PMSF) 0.2 mM was included throughout the purification and storage to minimize proteolysis. The purified fibronectin was stored in 2 M urea at 4°C and dialyzed against PBS before use. Human fibronectin and similarly purified bovine fibronectin were dialyzed exhaustively against 0.1 M potassium phosphate buffer to remove the urea and coupled to glutaraldehyde-activated Ultrogel AcA 22 (LKB, France) to be used as an immunoabsorbent (Ternynck and Avrameas, 1976).

Immunochemical Reagents

Antibodies to human plasma CIG were raised in rabbits and in guinea pigs by intradermal injection of 400 μg of the antigen in complete Freund's adjuvant (Difco). The rabbits were also injected directly into the poplitheal lymph nodes. The animals were boosted 3 and 4 weeks after the primary immunization and were bled weekly starting 7 days later. Antibody titer was estimated by double immunodiffusion in 1% agarose gels and by immunofluorescent labeling of cultured chicken cells. Purified antibodies to human CIG were prepared by affinity chromatography on Ultrogel-immobilized human CIG and stored at 4°C in the presence of 10 mM NaN_3. The limited crossreactivity of the antihuman fibronectin with bovine plasma fibronectin was eliminated by adsorption of the antibodies on a bovine CIG column.

Rabbit antibodies to vinculin as well as to BSA and goat antibodies to rabbit and guinea pig IgGs were prepared essentially as described earlier (Geiger, 1979). Pure antibodies to clathrin were provided by D. Louvard, EMBL Heidelberg. Monospecificity of all antibody preparations was verified by immunoprecipitation as previously described (Geiger, 1979). Conjugation of antibodies to rhodamine-lissamine sulfonyl chloride (Research Organics Inc.) was performed according to the method of Brandtzaeg (1973). Conjugation of dichlorotriazinyl amino fluorescein (DTAF, Research Organics Inc.) to antibodies was carried out for 1 hr at pH 9.0, using a molar ratio of 10:1 between DTAF and IgG. The fluorophore-conjugated antibodies were chromatographed on DEAE-cellulose (Brandtzaeg, 1973) to isolate the antibody fraction that contain 3–4 fluorophores per IgG molecule.

Microscopy

Fluorescence microscopy was performed with a Zeiss Photoscope III equipped with filter sets for double (fluorescein–rhodamine) immunofluorescence (Geiger and Singer, 1979).

Interference-reflection microscopy was performed in either Photoscope III as previously described (Geiger, 1979) or in a similar fashion, with a Zeiss Universal microscope equipped with epiilluminator.

Cells and Tissue Culture

Cultures of embryonic chicken gizzard cells were prepared and maintained in culture in DME/F-12 (1:1) media in the presence of 10% fetal calf serum. For immunofluorescence experiments, cells were grown on 18 or 22 mm square glass coverslips. Fibronectin or BSA carpets were prepared by incubating the coverslips for 30–60 min at 25°C with fibronectin (15 μg/ml in phosphate buffered saline [PBS]), or with BSA (500 μg/ml in PBS), and then extensively rinsed. To prepare rhodamine-labeled fibronectin or BSA carpets, precoated coverslips were incubated for 5 min with 1 ml of 0.2 M sodium carbonate buffer (pH 9.0) containing 6 μg of rhodamine–lissamine sulfonyl chloride at room temperature. Conjugation of the protein in solution with rhodamine prior to preparation of the carpet was not effective since the subsequent association with the glass was considerably reduced. Both untreated and rhodamine-labeled fibronectin exhibited a similar capability to bind to a gelatin column and to promote cell attachment and spreading of suspended chicken gizzard cells in serum-free medium. Fixation and indirect immunofluorescent labeling were carried out as previously described (Geiger and Singer, 1979).

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References

Abercrombie, M. and Dunn, G. A. (1975). Adhesions of fibroblasts to substratum during contact inhibition, observed by interference-reflection microscopy. Exp. Cell. Res. *92*, 57–62.

Abercrombie, M., Heaysman, J. E. M. and Pegrum, S. M. (1971). The locomotion of fibroblasts in culture. IV. Electron microscopy of the leading lamella. Exp. Cell. Res. *67*, 359–367.

Albrecht-Buehler, G. (1977). Phagokinetic tracks of 3T3 cells: parallel between the orientation of track segment and of cellular structures which contain actin and tubulin. Cell *12*, 333–339.

Ali, I. U., Mautner, V., Lanza, R. and Hynes, R. O. (1977). Restoration of normal morphology: adhesion and cytoskeleton in transformed cells by addition of a transformation-sensitive surface protein. Cell *11*, 115–126.

Badley, R. A., Woods, A., Smith, C. G. and Rees, D. A. (1980). Actomyosin relationships with surface features in fibroblast adhesion. Exp. Cell. Res. *126*, 263–272.

Birchmeier, C., Kreis, T. E., Eppenberger, H. M., Winterhalter, K. H. and Birchmeier, W. (1980). Corrugated attachment membrane in WI-38 fibroblasts: alternating fibronectin fibers and actin-containing focal contacts. Proc. Nat. Acad. Sci. USA. *77*, 4108–4112.

Brandtzaeg, P. (1973). Conjugates of immunoglobulin G with different fluorophores: I. Characterization by anionic exchange chromatography. Scand. J. Immunol. *2*, 273–290.

Chen, W.-T. and Singer, S. J. (1980). Fibronectin is not present in the focal adhesions formed between normal cultured fibroblasts and their substrate. Proc. Nat. Acad. Sci. USA *77*, 7318–7322.

Curtis, A. S. G. (1964). The mechanism of adhesion of cells to glass: a study by interference-reflection microscopy. J. Cell. Biol. *20*, 199–215.

Feramisco, J. and Burridge, K. (1980). Microinjection and localization of a 130K protein in living fibroblasts: a relationship to actin and fibronectin. Cell, *19*, 587–595.

Fox, C. H., Cottler-Fox, M. H. and Yamada, K. M. (1980). The distribution of fibronectin in attachment sites of chick fibroblasts. Exp. Cell Res. *130*, 477–481.

Geiger, B. (1979). A 130K protein from chicken gizzard: its localization at the termini of microfilament bundles in cultured chicken cells. Cell, *18*, 193–205.

Geiger, B. and Singer, S. J. (1979). The participation of α-actinin in the capping of cell membrane components. Cell, *16*, 213–222.

Geiger, B., Dutton, A. H., Tokuyasu, K. T. and Singer, S. J. (1980). The intracellular distributions of three cytoskeletal proteins in intestinal epithelial and gizzard smooth muscle cells as determined by double immuno-electron microscopy. J. Cell Biol. *83*, 2916a.

Grinnell, F. (1978). Cell adhesiveness and extracellular substrata. Int. Rev. Cytol. *53*, 65–144.

Grinnell, F. (1980). Visualization of cell substratum adhesion plaques by antibody exclusion. Cell Biol. Int. Rep. *4*, 1031-1036.

Grinnell, F. and Feld, M. K. (1979). Initial adhesion of human fibroblasts in serum-free medium: possible role of secreted fibronectin. Cell *17*, 117–129.

Grinnell, F. and Hays, D. G. (1978). Cell adhesion and spreading factor. Exp. Cell. Res. *115*, 221–229.

Heath, J. P. and Dunn, G. A. (1978). Cell to substratum contacts of chick fibroblasts and their relation to the microfilament system. A correlated interference reflection and high-voltage electron microscopic study. J. Cell Sci. *29*, 197–212.

Heaysman, J. E. M. and Pegrum, S. M. (1973). Early contacts between fibroblasts. An ultrastructural study. Exp. Cell. Res. *78*, 71–78.

Heggeness, M. H., Ash, J. F. and Singer, S. J. (1978). Transmembrane linkage of fibronectin to intracellular actin-containing filaments in culture human fibroblasts. Ann. N.Y. Acad. Sci. *312*, 414–417.

Höök, M., Rubin, K., Oldberg, A., Obrink, B. and Vaheri, A. (1977). Cold insoluble globulin mediates the adhesion of rat liver cell to plastic petri dishes. Biochem. Biophys. Res. Commun. *79*, 726–733.

Hynes, R. O. (1973). Alteration of cell-surface proteins by viral transformation and proteolysis. Proc. Nat. Acad. Sci. *70*, 3170–3174.

Hynes, R. O. and Destree, A. T. (1978). Relationships between fibronectin (LETS protein) and actin. Cell *15*, 875–886.

Izzard, C. S. and Lochner, L. R. (1976). Cell-to-substrate contacts in living fibroblasts: an interference reflexion study with an evaluation of the technique. J. Cell. Sci. *21*, 129–159.

Izzard, C. S. and Lochner, L. R. (1980). Formation of cell-to-substrate contacts during fibroblast motility: an interference-reflexion study. J. Cell. Sci. *42*, 81–116.

Morrison, P., Edsall, R. and Miller, S. G. (1948). Preparation and properties of serum and plasma proteins. XVIII. Separation of purified fibrinogen from fraction of human plasma. Am. J. Chem. Soc. *70*, 3103–3108.

Orly, J. and Sato, G. (1979). Fibronectin mediates cytokinesis and growth of rat follicular cells in serum-free medium. Cell *17*, 295–305.

Pearlstein, E. (1976). Plasma membrane glycoprotein which mediates adhesion of fibroblasts to collagen. Nature *262*, 497–500.

Ruoslahti, E. and Vaheri, A. (1975). Interaction of soluble fibroblast surface antigen with fibrinogen and fibrin. Identity with cold insoluble globulin of human plasma. J. Exp. Med. *141*, 497–501.

Ruoslahti, E., Vuento, M. and Engvall, E. (1978). Interaction of fibronectin with antibodies and collagen in radioimmuno assay. Biochim. Biophys. Acta. *534*, 210–218.

Singer, I. 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.

Studier, F. W. (1973). Analysis of bacteriophage T7 early RNAs and proteins on slab gel. J. Mol. Biol. *79*, 237–248.

Ternynck, T. and Avrameas, S. (1976). Polymerization and immobilization of proteins using ethylchloroformate and glutaraldehyde. Scand. J. Immunol. Suppl. *3*, 29–35.

Vaheri, A. and Ruoslahti, E. (1974). Disappearance of a major cell-type specific surface glycoprotein (SF) after transformation of fibroblasts by Rous Sarcoma Virus. Int. J. Cancer *13*, 579–586.

Vaheri, A. and Mosher, D. (1978). High molecular weight, cell surface-associated glycoprotein (fibronectin) lost in malignant transformation. Biochim. Biophys. Acta *516*, 1–25.

Wehland, J., Osborn, M. and Weber, K. (1979). Cell-to-substratum contacts in living cells. A direct correlation between interference-reflexion and indirect immunofluorescence microscopy using antibodies against actin and α-actinin. J. Cell. Sci. *37*, 257–273.

Yamada, K. M. and Olden, K. (1978). Fibronectins-adhesive glycoproteins of cell surface and blood. Nature *275*, 179–184.

Yamada, K. M., Yamada, S. S. and Pastan, I. (1976). Cell surface protein partially restores morphology, adhesiveness and contact inhibition of movement of transformed fibroblasts. Proc. Nat. Acad. Sci. USA. *73*, 1217–1221.

Yamada, K. M., Olden, K. and Pastan, I. (1978). Transformation-sensitive cell surface protein: isolation, characterization and role in cellular morphology and adhesion. Ann. N.Y. Acad. Sci. *312*, 256–277.