

Actin-independent Association of Vinculin with the Cytoplasmic Aspect of the Plasma Membrane in Cell-Contact Areas

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ABSTRACT We investigated the mode of association of vinculin with areas of contact between the termini of microfilament bundles and the cell membrane in sites of focal contact with the substrate by selective removal of actin from these areas. Opened-up substrate-attached membranes of chick fibroblasts as well as detergent-permeabilized cells were treated with fragmin from *Physarum* in the presence of Ca^{+2} . This treatment removed actin filaments from the cytoplasmic faces of the membranes, along with several actin-associated proteins (α -actinin, tropomyosin, myosin, and filamin). Vinculin distribution was not affected by this treatment. Moreover, rhodamine- or fluorescein-conjugated vinculin, when added to these preparations, became specifically associated with the focal contacts regardless of whether the latter were pretreated with fragmin or not. We conclude that the association of vinculin with focal contacts is largely actin-independent. We discuss the implications of these findings in the molecular mechanisms of microfilament membrane association in areas of cell contact.

It is now generally accepted that cytoskeletal filaments are associated with the plasma membrane in areas of cell contact (1, 4, 9, 16, 17, 29, 33). In particular, it has been shown that bundles of actin-containing microfilaments are attached to the cytoplasmic faces of the membrane in cell-substrate focal contacts of cultured cells and related *adherens* type intercellular junctions (1, 8, 29, 31, 32). Besides actin, which is a major constituent of microfilaments, at least two actin-related proteins are found close to or in the area of microfilament-membrane association in contact regions. α -Actinin is bound to actin bundles along their length and, in particular, near their membrane-attached termini (14, 24, 25, 27), and vinculin, a 130,000-dalton protein (6, 9), is exclusively associated with the cytoplasmic aspect of contact areas closer to the membrane than α -actinin (9–11, 14, 15, 30). On the basis of the electron microscopic and immunofluorescence observations, as well as of in vitro studies on vinculin interaction with actin (7, 21–23, 26, 35), Geiger has proposed that vinculin might be involved in the transmembrane induction of actin-bundle formation in intercellular *adherens* junctions (10–11). Geiger proposed that local changes in the cell membrane in contact regions (i.e., lateral aggregation of certain receptors in the cell membrane) induce an association of diffusible cytoplasmic vinculin with the membrane in that region and that this association may be

a prerequisite for a local actin bundle formation (11). This model implies that the primary association of vinculin with the contact areas is at least partially or transiently actin-independent. To test this directly, we selectively removed actin from permeabilized cells and isolated substrate attached membranes and determined the effect of this treatment on the distribution of endogenous vinculin as well as on the affinity of exogenously added vinculin for the membrane attachment sites.

MATERIALS AND METHODS

Chick fibroblasts from embryonic gizzard or heart were cultured on glass coverslips (9) or silver, hexagonal electron microscope grids (28), as previously described. Ventral membranes were prepared by the ZnCl_2 method (2), including short incubation with 1 mM ZnCl_2 in buffer containing 50 mM MES (*N*-morpholinoethansulfonic acid; Serva, Heidelberg, W. Germany), 5 mM MgCl_2 , 3 mM EGTA, pH 6.0, followed by shearing with a stream of phosphate-buffered saline (PBS). The substrate-attached opened-up membranes retained their focal contacts, actin bundles, and vinculin, as described in detail previously (2). Alternatively, membrane fragments were prepared by shearing with a stream of PBS without a previous ZnCl_2 treatment (3). Permeabilization of cells were performed by incubating cells with 50 mM MES buffer, 3 mM EGTA, 5 mM MgCl_2 , 0.5% Triton X-100, pH 6.0 for 2 min. Fragmin from *Physarum* (18–20) was kindly provided by Dr. H. Hinssen (University of Bonn, Germany).

Membrane fragments and permeabilized cells were incubated with different concentrations of fragmin in fragmin buffer (50 mM MES buffer, 0.1 mM CaCl_2 , 0.5 mM DTT, 2 mM MgCl_2 pH 6.3, or with the same buffer without fragmin).

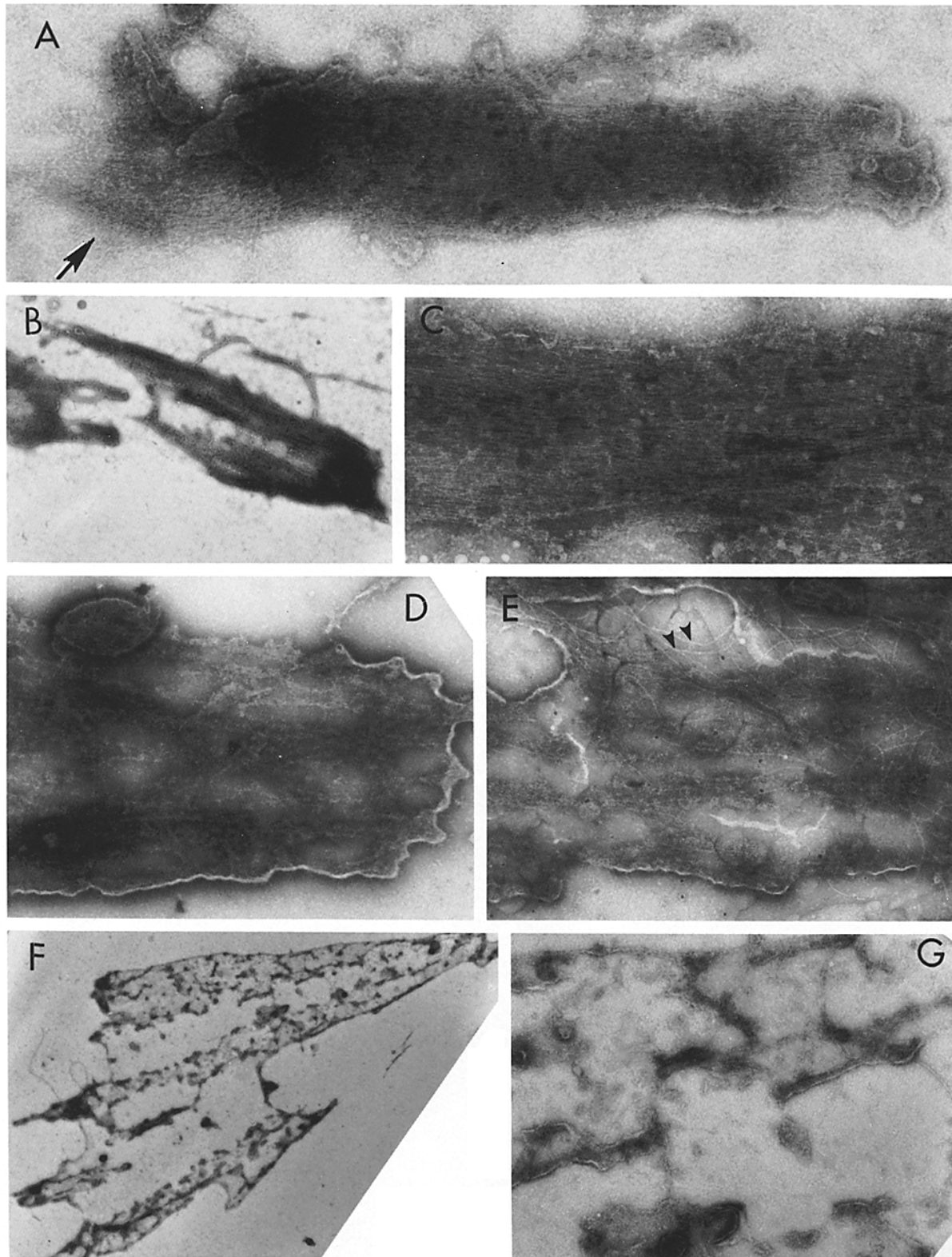


FIGURE 1 (A–C) control preparations of ventral membranes. (A) attached membranes on silver grid, fixed directly after flushing. Note fraying of actin filaments at left end (arrow), indicating severing site of stress fiber. (B and C) Ventral membranes after 5-min incubation in fragmin buffer, without fragmin. (A and C) $\times 62,500$. (B) $\times 10,000$. (D–G) Focal contacts after incubation with fragmin (0.4 mg/ml) for different time periods. (D) 2 min. (E) 5 min. (F and G) 10 min. Note rapid loss of actin filaments as well as the presence, in some cases, of intermediate filaments (arrowheads) in the posterior region of the focal contacts. (D, E, and G) $\times 40,000$. (F) $\times 8,000$. Negative staining was done in 1% aqueous sodium silicotungstate (A–E) or 1% aqueous uranyl acetate (F and G).

Fixation of membranes for electron microscopy was carried out with 1% glutaraldehyde in cytoskeleton buffer (see reference 28), followed by negative staining in cold uranyl acetate or sodium silicotungstate. Ventral membranes on glass coverslips were fixed with 3% paraformaldehyde and fluorescently labeled as described (9). For double-labeling experiments, the specific antibodies against α -actinin (13) or vinculin (9) were mixed with fluorescein-conjugated phalloidin which specifically stains actin filaments (34, 36). Subsequently, the samples were rinsed and incubated with rhodamine-labeled goat anti-rabbit immunoglobulin. Double immunofluorescent labeling for both actin and vinculin was performed with rabbit antibodies to chick muscle actin and guinea-pig antibodies to vinculin, followed by rhodamine-conjugated goat anti-rabbit IgG and fluorescein-labeled goat anti guinea pig IgG (2, 14).

We prepared fluorescently labeled vinculin by mixing pure chicken-gizzard vinculin with either lissamine-rhodamine B sulfonylechloride (RB200SC) (5) in 0.2 M sodium carbonate buffer, pH 8.5, at a molar ratio of 1 to 10 or with dichlorotriazinyl amino fluorescein (DTAF) at a similar molar ratio. The labeled proteins were isolated by gel filtration on Sephadex G-50 columns, equilibrated with 50 mM MES buffer, pH 6.0, and either used immediately or quickly frozen in liquid nitrogen in small aliquots. Rhodamine-labeled bovine serum albumin (BSA) and fluorescein-labeled goat immunoglobulin were prepared by a similar procedure. α -Actinin was labeled as described (12). "Decoration" experiments with the fluorescently labeled proteins were performed by incubating unfixed membranes or opened-up cells with 30 μ g/ml of the labeled proteins for 5 min. Subsequently, the specimens were rinsed with PBS and fixed with 3% paraformaldehyde.

Fluorescence microscopy was carried out with a Zeiss Photomicroscope III equipped with epiluminator and selective filters for fluorescein and rhodamine. Cells were photographed on Kodak Tri-X film. Exposure times for control samples were identical to those of the corresponding experimental figure.

RESULTS

Selective Removal of Cellular Actin by Fragmin Does Not Alter Vinculin Distribution

Substrate-attached ventral membranes prepared by the $ZnCl_2$ -method (2) were associated with cytoskeletal filaments, mostly microfilaments. Fig. 1 A-C shows electron micrographs of negatively stained ventral membranes of chick fibroblasts which were cultured directly on the electron microscope grids.

For the selective removal of actin from the ventral membranes, we used fragmin from *Physarum*. This protein induces the fragmentation of F-actin in the presence of micromolar concentrations of Ca^{+2} as described in detail by Hinssen (19, 20) and by Hasegawa et al. (18). Addition of fragmin in concentrations between 20 and 800 μ g/ml to the ventral membranes resulted in a rapid disappearance of actin filaments from their cytoplasmic faces. Partial removal of actin could be detected even after short exposure (20 s) to 40 μ g/ml fragmin and was essentially complete after \sim 5 min of treatment. The effect of fragmin on the membrane-bound filaments, as detected by electron microscopy, is shown in Fig. 1 D-G. The dense arrays of membrane-associated microfilaments disappeared and only sparsely distributed intermediate filaments

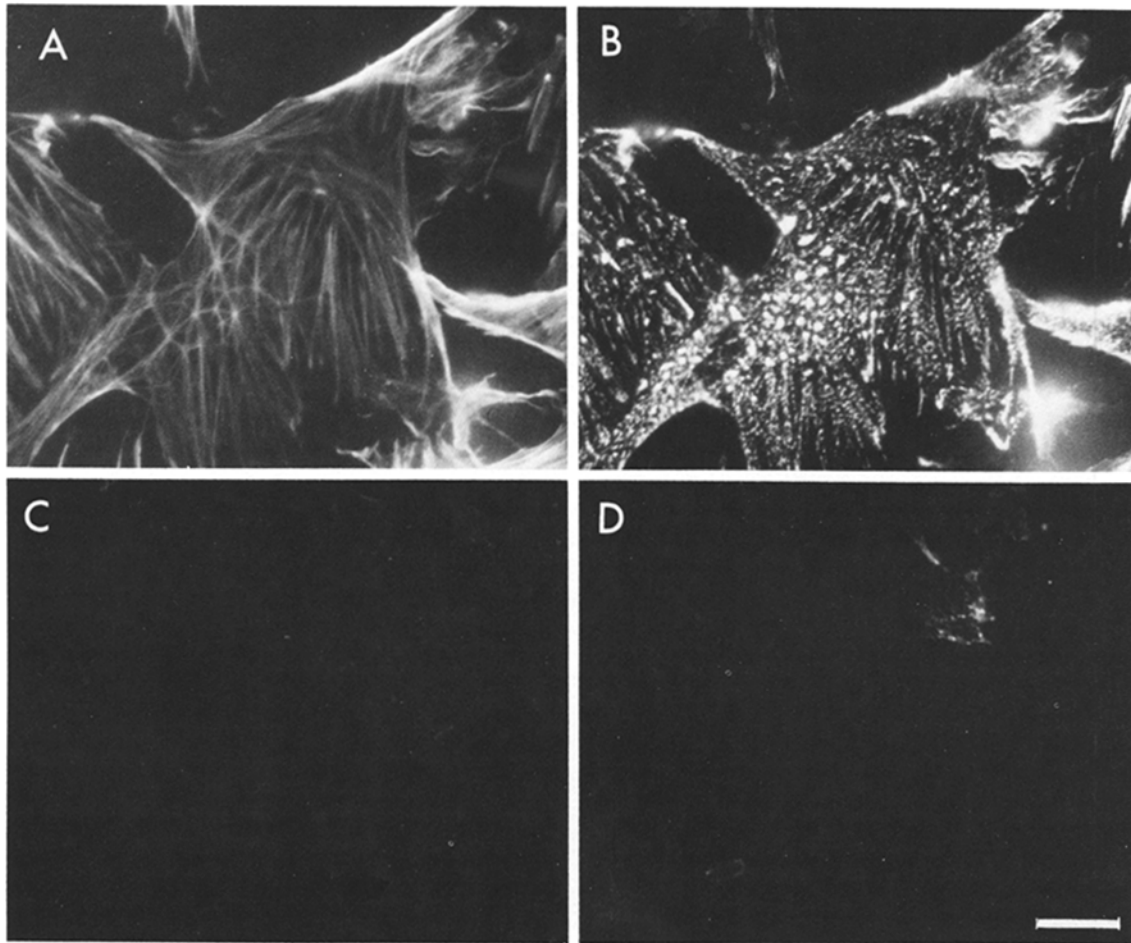


FIGURE 2 Double fluorescent labeling of ventral membranes for actin with fluorescein-phalloidin (A and C) and for α -actinin (B and D). The membranes were incubated for 3 min in fragmin buffer (see text) without fragmin (A and B) or with 0.4 mg/ml fragmin (C and D), then fixed and labeled. Notice the punctate distribution of α -actinin along actin bundles in the untreated membrane, and the simultaneous removal of both proteins after incubation with fragmin. Bar, 10 μ m. (A-D) \times 980.

could sometimes be seen associated with the ventral membranes (Fig. 1 *D–G*, arrowheads).

Fluorescent labeling of the membranes with fluorescein-phalloidin (Figs. 2 *A* and 3 *A*) or with actin antibodies (Fig. 4 *B*) revealed well-organized membrane-bound bundles. Immunolabeling of the same membrane for α -actinin indicated that this protein was distributed periodically along the actin bundles and was enriched near their termini (Fig. 2 *B*). Immunolabeling of the membranes for vinculin (Fig. 3 *B*) revealed an exclusive association of this protein with the termini of actin bundles (see matching arrows in Fig. 3 *A* and *B*). Examination of the same membranes with interference-reflection optics (Fig. 3 *C*) showed the coincidence between the vinculin-rich plaques and cell-substrate focal contacts. Fluorescent labeling with either actin antibodies or fluorescent phalloidin following fragmin treatment of these membranes (Figs. 2 *C* and 3 *D*) confirmed that essentially all the detectable membrane-bound actin was

removed. The same fragmin-treated membranes were also labeled for α -actinin and vinculin. As shown in Fig. 2 *D*, the membrane-associated α -actinin was removed together with actin. Vinculin, on the other hand (Fig. 3 *E*), remained associated with the cytoplasmic aspect of the focal contacts (interference reflection in Fig. 3 *F*) long after actin was visibly removed (>10 min of treatment). It should be pointed out that even after short treatments with fragmin (40 $\mu\text{g}/\text{ml}$, 30 s) actin was extracted from the termini of the bundles near the focal contacts. This excluded the possibility that this area is less accessible to fragmin. After prolonged incubations with fragmin (>5 min) we noticed some decrease in the intensity of vinculin immunolabeling as well as nonuniform staining of the focal contacts. This, however, occurred long after actin was apparently removed and was accompanied by progressive detachment of the entire ventral membrane from the substrate. It could also be correlated with the presence of fragmented

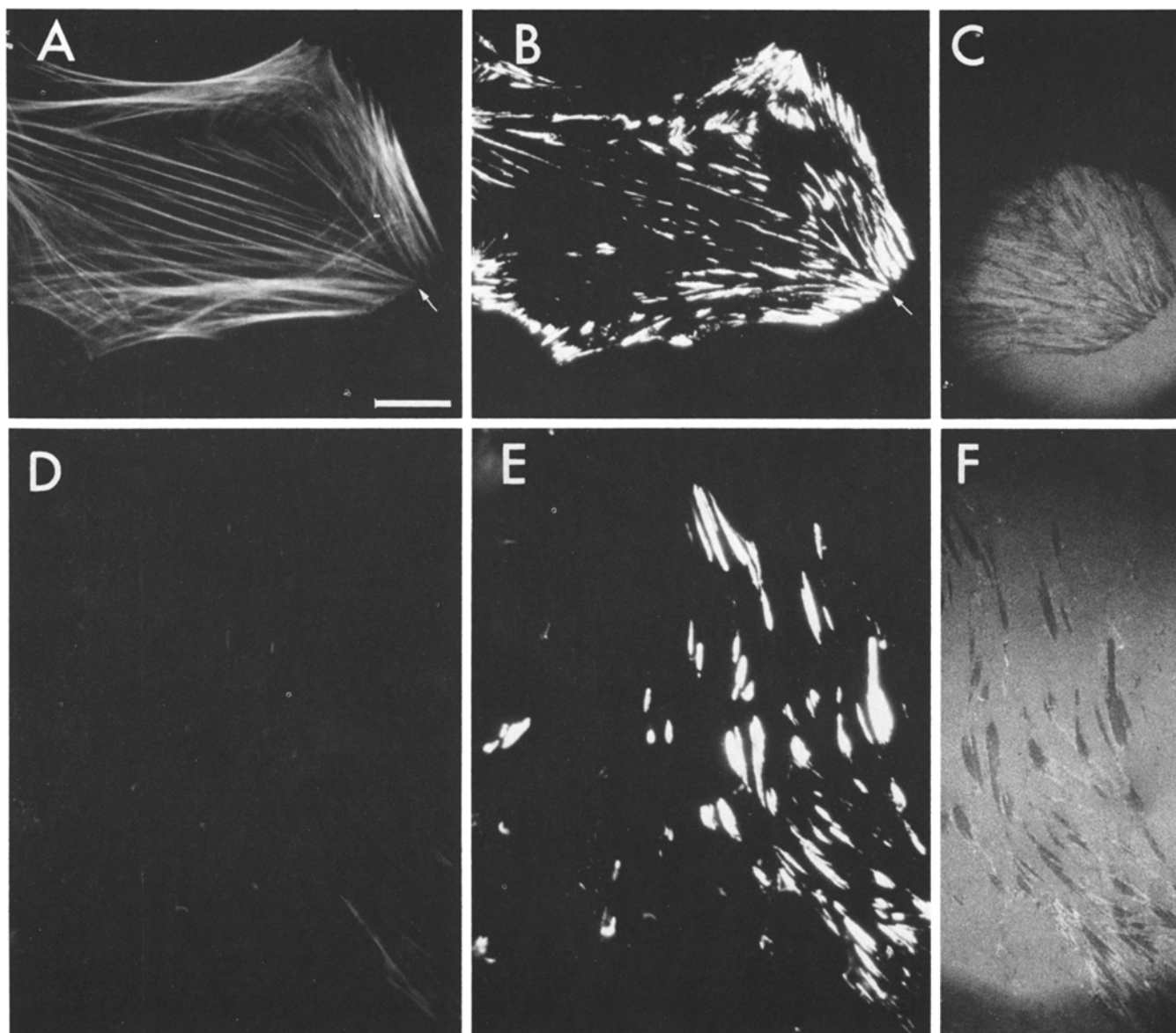


FIGURE 3 Double fluorescent labeling of ventral membranes for actin with fluorescein-phalloidin (*A* and *D*), vinculin (*B* and *E*, respectively), and the corresponding interference reflection images (*C* and *F*). The membranes were incubated for 3 min in buffer without fragmin (*A–C*) or with 0.4 mg/ml fragmin (*D–F*). Notice the localization of vinculin near the ends of actin bundles at focal contact areas (arrows). After the fragmin treatment, actin is removed essentially completely (*D*) while vinculin labeling and the pattern of focal contacts are apparently normal. Bar, 10 μm . (*A–F*) $\times 980$.

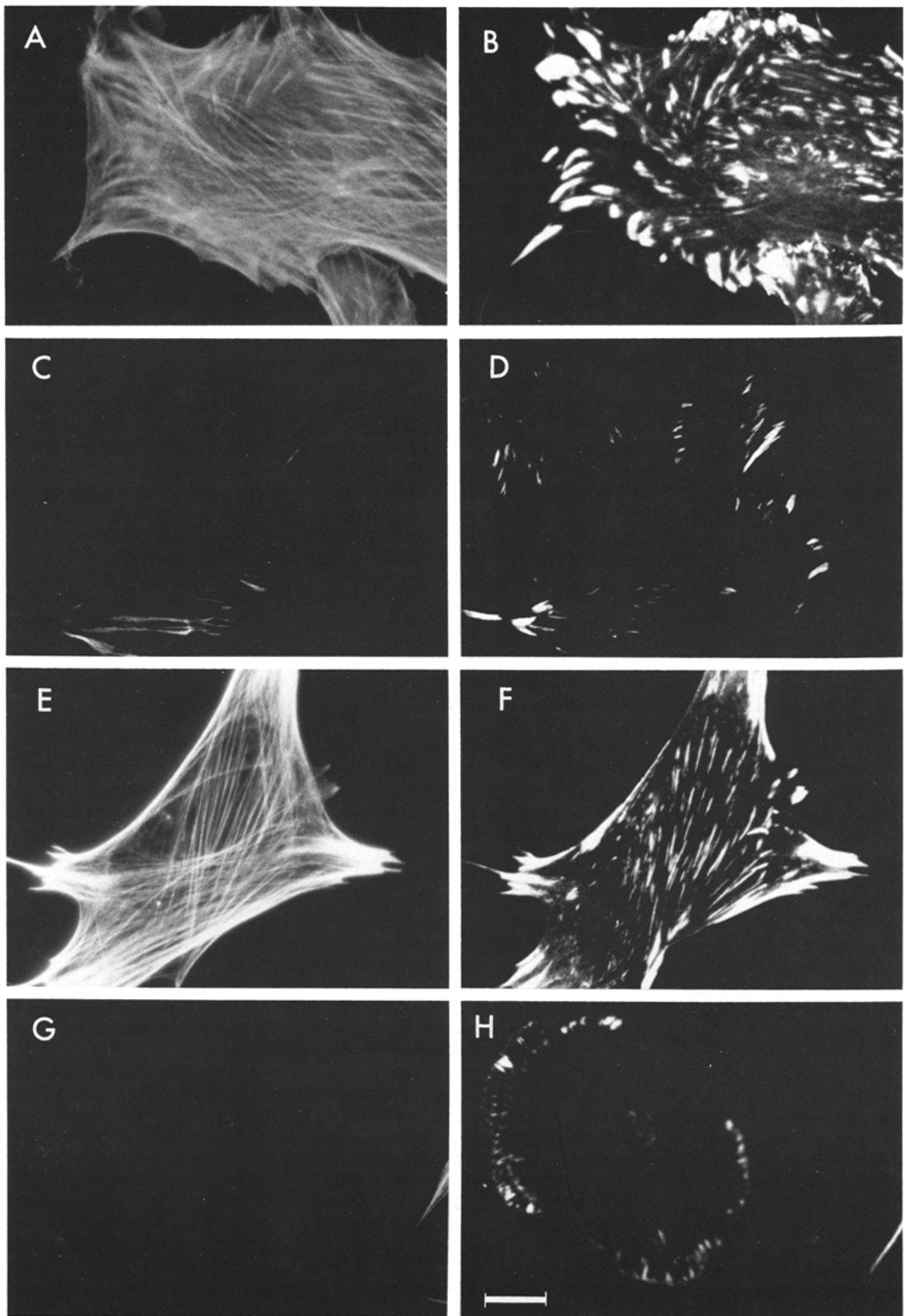


FIGURE 4 The effect of fragmin treatment on actin and vinculin distribution in Triton X-100-permeabilized cells (A-D) or buffer-sheared membranes without ZnCl_2 pretreatment (E-H). The different preparations were incubated with $40 \mu\text{g/ml}$ fragmin (C, D, G, and H) or with fragmin buffer without fragmin (A, B, E, and F) for 12 min, then fixed and double-labeled for actin and vinculin using rhodamine-phalloidin in conjunction with anti-vinculin and fluorescein-goat anti-rabbit IgG. Notice the relationships between vinculin and actin in the untreated sample, the presence of residual vinculin on fragmin-treated focal contacts and the apparent absence of actin labeling in these sites. Bar, $10 \mu\text{m}$. (A-H) $\times 980$.

membranes seen by electron microscopy. These results indicate that the endogenous vinculin in "mature" focal contacts does not require the presence of actin for its binding to that region. On the other hand, α -actinin, which is also enriched near focal contacts, was highly actin dependent (fragmin sensitive), in its distribution both along the bundles and near their termini. It should be added that several additional actin-associated proteins, including tropomyosin, myosin and filamin, were readily removed by fragmin treatment (not shown).

The yield of ventral membranes purified by the $ZnCl_2$ method is relatively high, and they display remarkable structural preservation. However, we had to consider the possibility that vinculin might have been "immobilized" and rendered fragmin insensitive due to the $ZnCl_2$ treatment.

We therefore tested the differential effect of fragmin on actin and vinculin in Triton X-100-treated cells (Fig. 4A-D) and in substrate-bound membranes prepared by mechanical shearing of cultured chick-gizzard cells without $ZnCl_2$ treatment (Fig. 4E-H).

The results indicated that regardless of the procedure used for exposure of the endofacial surfaces of focal contacts, it was possible to remove most of the visible actin from them without affecting significantly vinculin distribution. It should, however, be pointed out that the rate of actin removal in detergent-permeabilized cells was relatively slow and 10 min or more were needed for a satisfactory removal of actin.

Binding of Fluorescently Labeled Vinculin to the Cytoplasmic Surfaces of Focal Contacts

Fluorescent derivatives of vinculin were prepared by reacting the pure protein with either DTAF or RB200SC. When added to Triton X-100-permeabilized cells, the fluorescent vinculin became readily associated with areas of focal contacts. This "decoration" was obtained with diverse cell types, including chick-gizzard fibroblasts (Fig. 5A), human epithelial line CCL₆ (Fig. 5B), and rat Kangaroo PtK₂ cells (Fig. 5C, the inset shows the corresponding interference-reflection image). In PtK₂, the fluorescent vinculin was found also in regions of cell-cell-contact as indicated by arrows in Fig. 5C. Irrelevant proteins (BSA or goat immunoglobulin) did not bind to these sites (see below). Moreover, fixation of the detergent-treated cells with formaldehyde prior to decoration abolished completely the capacity of the permeabilized cells to bind exogenously added vinculin.

Specific binding of fluorescently labeled vinculin was also obtained with isolated substrate-attached membranes prepared by shearing with buffer, with or without previous $ZnCl_2$ treatment. In both preparations specific association of the labeled protein was obtained as demonstrated in Fig. 6A and C. Comparison of the fluorescence patterns obtained with rhodamine-labeled ventral membranes of chick-gizzard fibroblasts with the corresponding interference-reflection images (Fig. 6B) indicated that the protein was associated predominantly with focal contacts. In that area the decorating vinculin was apparently associated with the termini of actin filament bundles as shown in the paired photographs Fig. 6C and D and Fig. 7A and B.

To rule out the possibility that the fluorescently labeled vinculin is particularly "sticky" or that isolated focal contacts tend to bind exogenously added proteins nonspecifically, we used several control proteins instead of vinculin. Rhodamine-labeled BSA or fluorescein-goat immunoglobulin, when added to ventral membranes (Fig. 6E and F, respectively), did not

bind to any specific cellular structure. When incubation was carried out with another rhodamine-labeled cytoskeletal protein, α -actinin, specific labeling was not restricted to focal contacts but was found in spotted distribution along the stress fibers (Fig. 6G and reference 12).

An interesting observation was that the decoration with rhodamine-vinculin was not inhibited efficiently by excess of unlabeled vinculin. Unlike our previous experience with α -actinin (12), the addition of 10-fold excess of the unlabeled protein caused only limited reduction in the decoration of focal

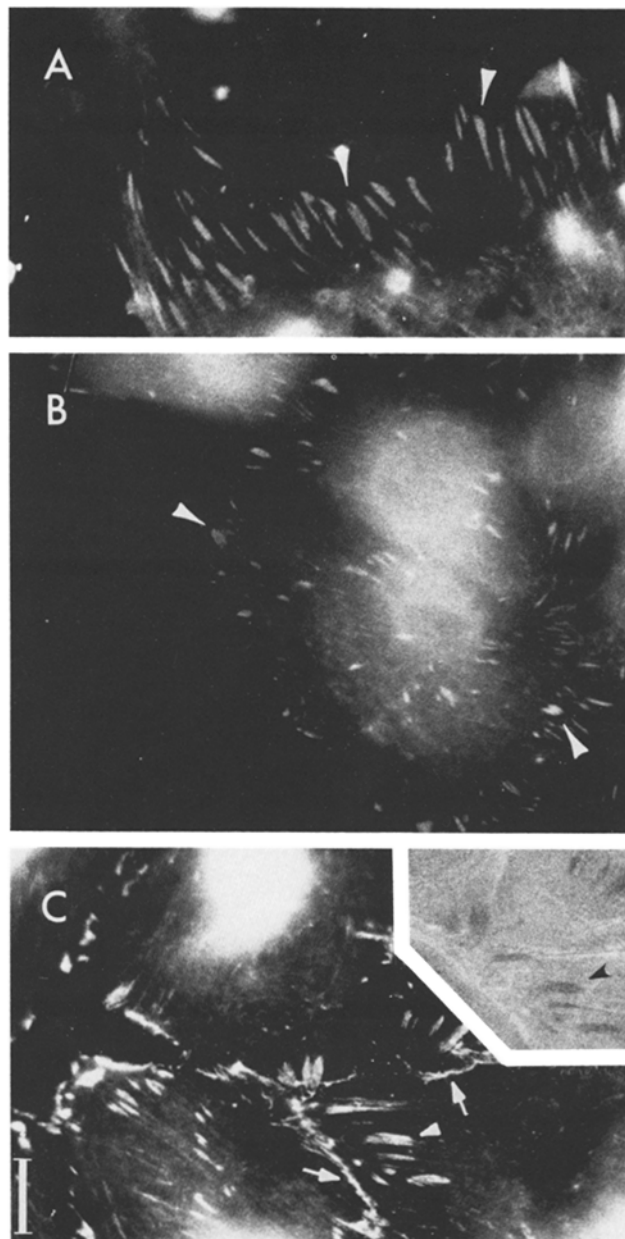


FIGURE 5 Decoration of Triton X-100-permeabilized cells with fluorescein-conjugated vinculin. The cells were permeabilized, then incubated with 30 μ g/ml of the labeled vinculin, rinsed, and fixed with paraformaldehyde. The cells used were chick-gizzard fibroblasts (A), human epithelial line CCL₆ (B), and rat kangaroo PtK₂ (C). The inset in C is of the residual interference reflection pattern of focal contacts in the same field. It is apparent that vinculin binds not only to cell-substrate focal contacts but also to areas of cell-cell contact (arrow, C). Arrowheads point to few cell-substrate focal contacts. Notice the specific association of the labeled vinculin with focal contacts in the three cell types. Bar, 10 μ m. (A-C) \times 980.

contacts and, even after addition of 20- to 30-fold excess of vinculin, the inhibition was only partial. The significance of these, apparently nonsaturation kinetics of binding will be discussed below.

Incorporation of Rhodamine-Vinculin into Focal Contacts Is Largely Actin-independent

As mentioned above, the rhodamine-labeled vinculin was bound to ventral membranes at or near the termini of the associated actin filament bundles (Fig. 7*A and B*). To determine whether the presence of organized actin bundles was necessary for the incorporation of the exogenously added vinculin, we pretreated the ventral membranes used for decoration with fragmin to remove the actin bundles. As shown in Fig. 7, the removal of actin from ventral membranes did not affect their capacity to bind fluorescently labeled vinculin.

DISCUSSION

Studies on the molecular mechanism of membrane-microfilament interaction directed much interest recently to the protein vinculin. This protein was specifically localized at or near the cytoplasmic aspects of cell contact areas including focal contacts with tissue culture substrate, dense plaques of smooth muscle, *zonula-* and *fascia-adherens* of polarized epithelium and cardiac muscle, etc. (9-11, 13-15, 30). Immunoelectron microscopic localization of vinculin in the various tissues indicated that the protein was found in the vicinity of the junctional membrane, closer than other actin-associated proteins, including α -actinin and tropomyosin (14, 30). Double fluorescent labeling of the same cells for actin and vinculin during early stages of cell spreading indicated that vinculin was associated with distinct focal contacts before significant actin bundles could be detected in these areas (10). These and

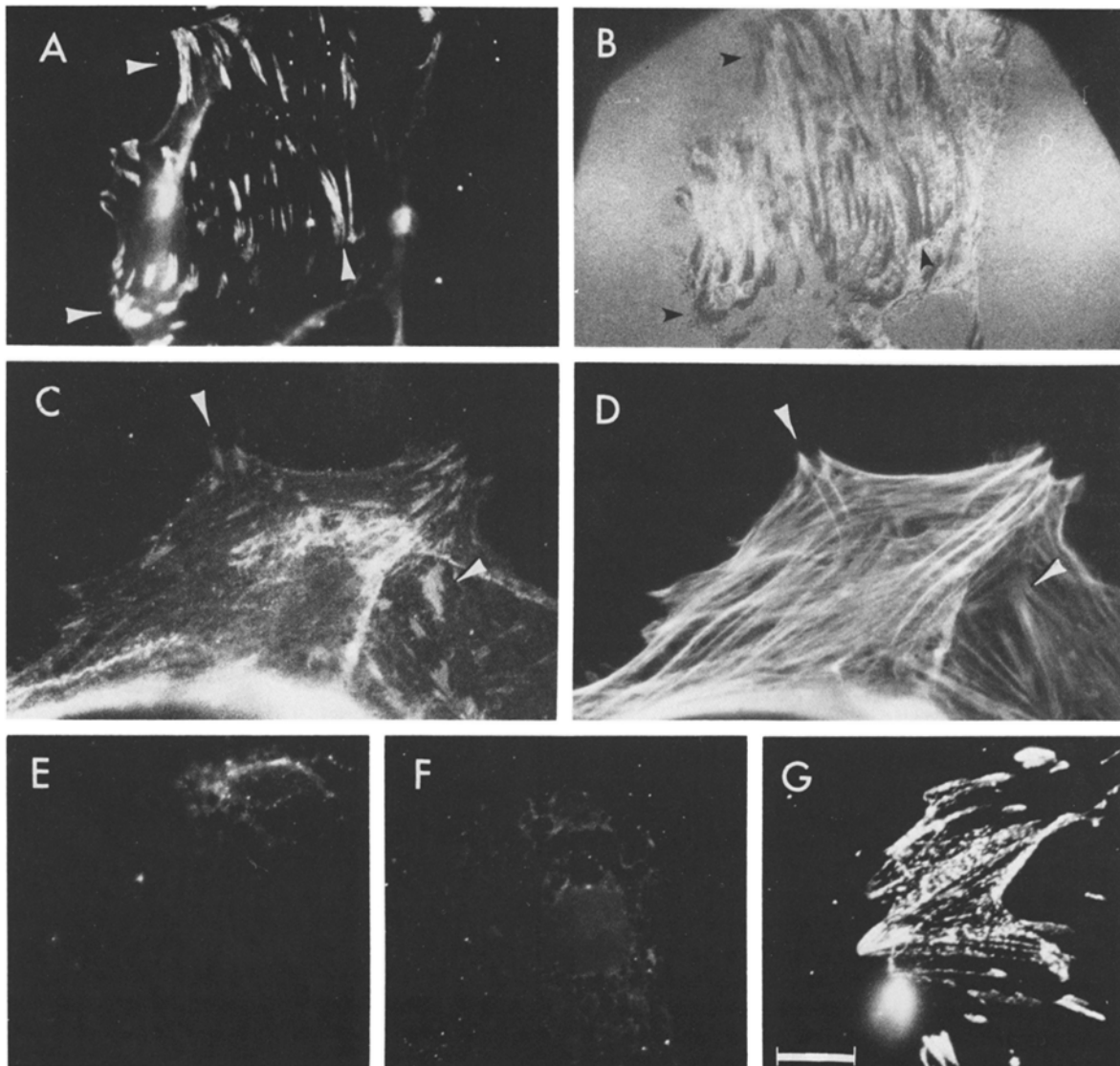


FIGURE 6 (A and C) Decoration with rhodamine-vinculin of ventral membranes prepared with (A) or without (C) $ZnCl_2$ treatment. The interference reflection pattern of the membrane "decorated" in A is shown in B. The distribution of actin rhodamine-phalloidine labeling in the buffer-sheared cells in C is shown in D. (E-G) Control experiments in which ventral membranes prepared by the $ZnCl_2$ method were incubated with rhodamine BSA (E), fluorescein-goat IgG (F), or rhodamine-labeled α -actinin (G). Notice the specific association of rhodamine-vinculin with focal contacts and actin bundle termini (arrowheads in A-D point to the same locations) and the absence of labeling with fluorescently labeled BSA and IgG (the exposure time in E and F was approximately double the regular exposure of the same specimens after vinculin decoration); α -actinin was bound both near focal contacts and, in a dotted pattern, along the residual stress fibers. (A-G) $\times 980$.

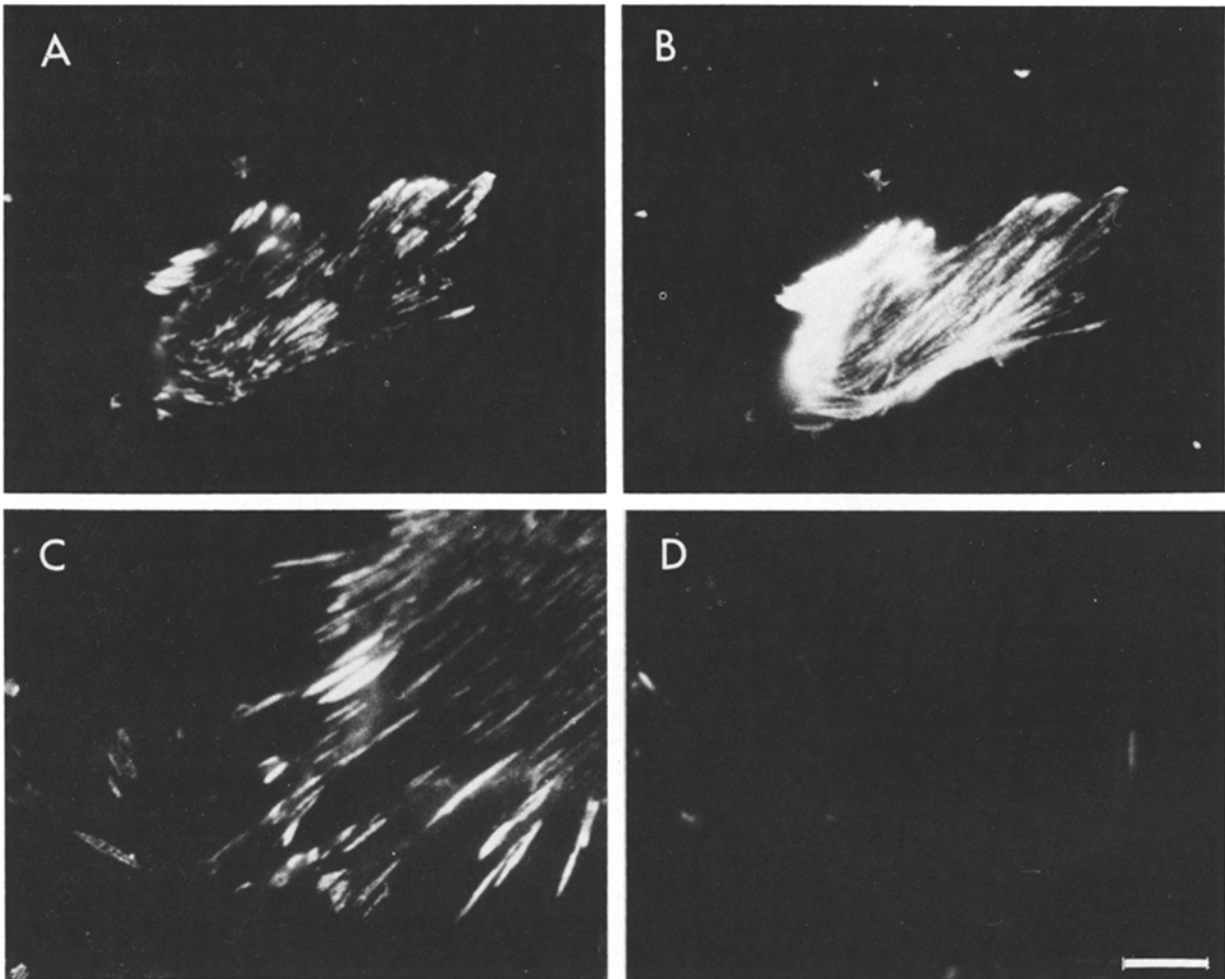


FIGURE 7 Decoration of ventral membranes with rhodamine-labeled vinculin (A and C). The membranes were incubated for 3 min with fragmin buffer (A and B) or with 40 $\mu\text{g}/\text{ml}$ fragmin (C and D), then incubated with rhodamine-vinculin (30 $\mu\text{g}/\text{ml}$ in MES buffer pH 6.0) for 10 min, then rinsed, fixed, and immunolabeled indirectly for actin using fluoresceinated antibodies. Notice that rhodamine-vinculin in both preparations binds to elongated plaques which could be identified as cell-substrate focal contacts by interference reflection microscopy (not shown). The binding of vinculin to these areas was not affected by the apparent removal of actin (D) from the substrate attached membranes. Bar, 10 μm . (A-D) $\times 980$.

additional observations led us to propose that vinculin binds to the cell membrane in newly formed contact regions and subsequently induces the formation of actin bundle in those sites (11). This hypothetical model gained some corroboration from the direct demonstration that vinculin interacts with actin *in vitro* and induces its bundling (22, 23, 26, 35). The model, however, predicted that vinculin can also bind to the junctional membrane in an actin-independent fashion.

In the present study, we showed that extensive removal of actin from isolated ventral membranes and permeabilized cells does not significantly alter the distribution of endogenous vinculin nor does it affect the binding to focal contacts of exogenously added vinculin. These findings may represent the interrelationships between the membrane, vinculin, and actin in living cells only if the method used for exposure of focal contacts does not affect these interrelationships. Therefore, we employed several methodological approaches for opening up the cells using ZnCl_2 pretreatment, detergent permeabilization, or simple shearing with buffer. We found that, in all systems tested, the interaction of vinculin with focal contacts was essentially actin-independent, though the ZnCl_2 -ventral membranes displayed superior overall structural preservation and a

high yield. In Tritonized cells the rate of actin severing was slower, and in the case of buffer-sheared samples the yield was relatively low with many resealed membrane fragments the cytoplasmic faces of which were not exposed. Nevertheless, in all cases actin removal had little or no apparent effect on vinculin. We cannot exclude the possibility that very small amounts of actin, below the levels detectable by fluorescent phalloidin or by antibodies (both of which were used in the present study), are still present in focal contacts after fragmin treatment. Nevertheless, high sensitivity to fragmin of the terminal areas of the stress fibers and the dramatic increase in the molar ratio of vinculin-to-actin render such explanation unlikely.

An additional relevant aspect involves the decoration experiments. We verified the specificity of the process using several controls including irrelevant fluorescently-labeled proteins which gave no specific labeling and rhodamine-labeled α -actinin which binds also to stress fibers. Moreover, the same rhodamine-vinculin used here, when microinjected into living cells, was readily incorporated into focal contacts (T. E. Kreis, J. Schlessinger, and B. Geiger, unpublished results; see also reference 6). Interestingly, unlabeled vinculin in excess was not

an efficient inhibitor of the decoration. The reasons for this phenomenon are not clear and two possibilities which may be mentioned here are the possible presence of a large number of vinculin-binding sites in focal contacts or a local self aggregation of vinculin in that area.

The largely actin-independent binding of vinculin as shown here, together with *in vitro* results on the actin-bundling activity of vinculin (22, 23, 26, 35), strongly supports the suggestion that vinculin binds to contact regions before actin becomes associated with them and that from this site it acts as a nucleation center for the assembly of microfilament arrays.

It still remains to be determined what is the nature of the vinculin-binding constituents in focal contacts. Are these integral membrane components? Are they yet additional peripheral proteins or is it vinculin itself which may undergo homotypic aggregation in the contact area? Answers to these questions may be very helpful for understanding the transmembrane signaling in areas of cell-substrate contact and related intercellular junctions.

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