

The Dynamic Interrelationships of Actin and Vinculin in Cultured Cells

Joseph Schlessinger and Benjamin Geiger

Department of Chemical Immunology, The Weizmann Institute of Science, Rehovot, Israel

The dynamic state of cytoskeletal proteins actin and vinculin was studied in living cells using microinjection of fluorescently-labeled proteins combined with fluorescence photobleaching recovery (FPR). It is shown that both proteins maintain a dynamic equilibrium between their diffusible pools in the cytoplasm and their "organized" cytoskeletal fraction. These interrelationships could be simulated in model systems consisting of isolated substrate attached membranes. It was demonstrated that fluorophore bound vinculin was incorporated into the exposed focal contacts and that this binding was largely actin independent. These results are in line with the hypothesis that local contacts induce binding of vinculin to the endofacial surface of the membranes and that this region serves as a nucleation center for the assembly of actin bundles.

Key words: focal contacts, cytoskeleton, microinjection, mobility

INTRODUCTION

It is now generally accepted that many of the mechanical properties of the plasma membrane are determined and controlled by meshworks of cytoskeletal filaments which are associated with the cytoplasmic faces of the membrane. Of the various classes of cytoskeletal meshworks the actin-containing microfilaments appear to be most tightly attached to the membrane in specific sites. One of the common areas for membrane-microfilament association are regions in which the cell forms local contacts either with its neighbours, with pericellular connective tissue or with artificial substrates provided under culture conditions. Besides actin, these contact regions, denoted "adhaerens junctions," contain α -actinin and, in closer proximity to the membrane, vinculin [Geiger, 1979; Burridge and Feramisco, 1980; Geiger et al, 1980, 1981]. In these areas vinculin shows an apparent association with the termini of actin bundles. These data and related ultrastructural and biochemical studies suggested the possibility that vinculin is involved in the linkage of actin to the cell membrane in these junctions. Our hypothesis was that immobilization of certain "membrane receptors" in the area of contact induces the transition of vinculin from a soluble state in the cytoplasm to the membrane. It was also assumed that subsequently the membrane-

associated vinculin serves as a nucleation site for the assembly of actin bundles (for details see Geiger [1981, 1982]; Geiger et al [1983]).

The model outlined above implies that both membrane components and cytoskeletal elements associated with such cell contacts maintain a dynamic equilibrium between their "free" and "anchored" forms and that the association of vinculin with the membrane is largely actin independent. These aspects were directly investigated, as will be described below.

THE DYNAMIC PROPERTIES OF CYTOSKELETAL STRUCTURE ASSOCIATED WITH ADHAERENS JUNCTIONS IN LIVING CELLS: MICROINJECTION COMBINED WITH FLUORESCENCE PHOTBLEACHING RECOVERY MEASUREMENTS

A new approach for the investigation of the distribution and dynamic properties of cytoskeletal elements involved the microinjection of fluorescently labeled cytoskeletal elements into living cells. We have recently used this technique to introduce fluorescently labeled actin, α -actinin, vinculin, and MAP₂ into living cultured cells [Kreis et al, 1982; Geiger et al, 1983]. Image intensification microscopy was used to follow the fate and localization of the injected proteins in the cells and fluorescence photobleaching recovery (FPR) measurements were used to determine the mobility of the cytoskeletal elements within the various cellular domains. To explore the dynamics of the various cytoskeletal proteins attached to focal contacts of cultured cells, we, in collaboration with T. Kreis, have injected actin, α -actinin, and vinculin into chicken gizzard fibroblasts. Shortly after their microinjection, actin and α -actinin were detected in stress fibers, focal contacts, leading lamella and interfibrillary space (see Kreis et al [1982]). Vinculin was localized only in focal contacts and within the interfibrillary space.

The mobility of the various fluorescently labeled proteins determined by the FPR method is presented in Table I. Control proteins, such as rhodamine-labeled

TABLE I. Mobility of Rhodamine-Labeled Actin, α -Actinin, and Vinculin in Microinjected Chicken Gizzard Fibroblasts as Measured by Fluorescence Photobleaching Recovery

Protein	Cellular domain	Diffusion coefficient $D(\text{cm}^2/\text{s}) \times 10^{-9}$	Mobile fraction	Half-time for recovery of fluorescence ($\tau_{1/2}$) into the "immobile" areas
Actin	Focal contact or stress fibers	3.1 ± 1.1 (30) ^a	18 ± 7	4.1 ± 2.8 (23)
α -Actinin	Focal contact	2.8 ± 1.0 (10)	40 ± 10	2.7 ± 1.2 (6)
Vinculin	Focal contact	3.5 ± 1.2 (20)	43 ± 8	2.1 ± 0.9 (15)
Actin	Interfibrillary	3.2 ± 1.2 (35)	65 ± 13	ND ^b
α -Actinin	Interfibrillary	2.5 ± 1.4 (12)	76 ± 5	ND
Vinculin	Interfibrillary	2.9 ± 1.1 (30)	~ 80	ND
Bovine serum albumin	Border area	6.2 ± 0.9 (14)	> 90	—
	Perinuclear	6.3 ± 0.9 (7)	> 90	—
Goat immunoglobulin	Border area	6.0 ± 1.3 (15)	> 90	—
	Perinuclear	6.6 ± 0.7 (12)	> 90	—

^aNo. of determinations.

^bNot determined.

bovine serum albumin or IgG, were mobile with $D = 6 \times 10^{-9}$ cm²/s. Their fractional recovery (mobile fraction) is higher than 90%. The mobilities of actin, α -actinin, and vinculin as well as their fractional recoveries were significantly lower. Actin in interfibrillary regions had mobile fraction of about 70%, and in focal contacts and stress fibers the mobile fraction was smaller than 20%. Similarly, significant proportion of vinculin and α -actinin molecules (mostly those in focal contacts regions) did not diffuse freely (~40% recovery, while the cytoplasmic protein was mostly mobile [~80%]). In spite of the apparent immobility of the cytoskeletal elements within the stress fibers and focal contacts, it was possible to measure a slow rate of fluorescence recovery into these regions after short photobleaching. The rate of the slow recovery into either stress fibers or focal contacts was independent of the size of the bleaching spot, in contrast to the fast recovery of the diffusible proteins, which was dependent on the square of the beam radius. Extensive measurements with rhodamine-actin in focal contacts or stress fibers indicated that the half time ($\tau_{1/2}$) of the slow recovery was within the range of 1–11 min (with an average of 4.1 min, see Table I). The structural and molecular basis for this wide variability is not yet clear. Vinculin and α -actinin appeared to be more uniform in their dynamic behavior and had slow recovery with $\tau_{1/2}$ of about 2–3 min. Additional experiments indicated that the slow recovery represented an exchange of components between the soluble cytoplasmic pool and the “cytoskeletal” pool, rather than rearrangement of components within the cytoskeleton [Kreis et al, 1982].

DYNAMIC PROPERTIES OF MEMBRANE CONSTITUENTS IN AREAS OF CELL-SUBSTRATE CONTACT

The FPR measurements approach was also used by us, in collaboration with Z. Avnur, to determine the lateral mobilities of membrane proteins and lipids within cell-substrate focal contacts. In order to perform such experiments on single substrate-associated membranes, we have developed a technique for the isolation of these membranes without affecting their substrate contacts (for details see Avnur and Geiger [1981]). In brief, cells were incubated for 2 min in buffer A (50 mM MES buffer, 5 mM MgCl₂, 3 mM EGTA 1 mM ZnCl₂, pH 6.0), then washed and sheared with a stream of phosphate-buffered saline. Analysis of these preparations with interference reflection optics or scanning electron microscopy (SEM) indicated that most of the cells were opened up, leaving attached their ventral filaments associated with them [Avnur and Geiger, 1981].

Fluorescent labeling of cells, prior to the preparation of ventral membranes, was performed using either a lipid probe WW 591 (5-[3- γ -sodium sulphopropyl-6,7-benzo-2-(3H)-benzoxazolylidene-butinylidene]-1,3-dibutyl-2-thiobarbituric acid, Hillman and Schlessinger [1982]) or an amino group-reactive, nonpenetrating fluorophore lissamine-rhodamine B sulfonyl chloride (RB 200 SC). With respect to the latter, it was verified that it does not significantly label membrane lipids or cytoplasmic proteins. The substrate-attached membranes, labeled with either WW591 or with RB 200 SC, retained their focal contacts and enabled us to measure the lateral diffusion of the lipid probe and of the rhodamine-labeled proteins in the attached and free membrane domains by FPR. The values obtained for the diffusion coefficients in the two domains indicated that the lateral diffusion of both lipids and proteins in focal contacts was reduced by a factor of 1.5–2 as compared to those measured in unattached regions (see Geiger et al [1982]). The relative retardation of lateral mobility in contact areas was similar for both WW591 and the rhodamine-labeled proteins.

An important difference between the lipid probe and the labeled proteins was the extent of fluorescence recovery after photobleaching. The lipid probe exhibited essentially complete recovery, while over 50% of the protein label was essentially immobile ($D < 3 \times 10^{-12} \text{ cm}^2/\text{s}$). This indicates that protein molecules in focal contacts are heterogeneous with respect to their dynamics and fall into at least two categories: those proteins that are free to diffuse into and away from the contact areas and others which are essentially immobile. Such organization of focal contacts allows for rather rapid exchange of components and addition or loss of contact receptors which may be important for the regulation of focal contact formation, maintenance, and reversal.

BINDING OF FLUORESCENTLY LABELED VINCULIN TO SUBSTRATE-ATTACHED MEMBRANES

The model outlined above suggested that new binding sites for vinculin are formed as a consequence of attachment to the substrate and that after the binding of vinculin to these sites it organizes actin into bundles. To study the binding of vinculin to the membrane we have prepared opened-up substrate-bound membrane fragments and incubated them with fluorescently labeled vinculin. Observation through the fluorescence microscope indicated that the labeled vinculin was associated specifically with focal contacts. Chick vinculin could bind to focal contacts in cells of a large variety of species including chicken, human, bovine, mouse, rat and rat-kangaroo. The addition of unlabeled vinculin in excess (up to tenfold) brought about only partial inhibition of binding, suggesting that vinculin might undergo self-aggregation in the focal contact.

In a series of studies carried out in collaboration with V. Small and Z. Avnur [Avnur et al, 1983], it was shown that the binding of vinculin to the membrane was largely actin-independent. Substrate-attached membranes were incubated with fragmin in the presence of Ca^{+2} to remove actin from their cytoplasmic faces and then stained for actin and vinculin or "decorated" with rhodamine-labeled vinculin. The results indicated that while actin and other associated proteins were removed by this treatment, the endogenous vinculin was not affected. Similarly, the removal of actin from the membranes did not impair their capacity to bind exogenous soluble vinculin.

CONCLUSIONS

The studies described briefly here suggest that the different microfilament-associated components of focal contacts maintain a dynamic steady-state equilibrium between their free and anchored forms. Moreover, the plasma membrane in contact areas do not contain macroscopic barriers for diffusion and therefore both lipids and proteins (at least those which are not "anchored") can diffuse through the contact area. We have also shown that vinculin has the unique property that its association with focal contacts is largely actin-independent, in contrast to most of the other microfilament-associated proteins (α -actinin, filamin, myosin, tropomyosin) whose primary association appears to be with actin. These results support the major features of the proposed dynamic model for adherens junctions formation, and possibly for the biogenesis of adherens junctions in general.

ACKNOWLEDGMENTS

Most of the studies described in this article were performed in collaboration with Thomas Kreis, Zafira Avnur, and Victor Small. We would like to thank these colleagues for most enjoyable collaboration and illuminating discussions. These studies were supported by grants from the Muscular Dystrophy Association (B.G.) and Israel-U.S. Binational Foundation (J.S.).

REFERENCES

- Avnur, Z., and Geiger, B. (1981): Substrate-attached membranes of cultured cells: Isolation and characterization of ventral cell membranes and the associated cytoskeleton. *J. Mol. Biol.* 153: 361-379.
- Avnur, Z., Small, J.V., and Geiger, B. (1983): Actin independent association of vinculin with the cytoplasmic aspect of the plasma membrane in cell contact areas. *J. Cell Biol.* (in press).
- Burridge, K., and Feramisco, J.R. (1980): Microinjection and localization of a 130K protein in living fibroblasts: A relationship to actin and fibronectin. *Cell* 19:587-595.
- 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. (1981): Transmembrane linkage and cell attachment. In: "International Cell Biology 1980-1981." Berlin: Springer-Verlag, pp. 761-773.
- Geiger, B. (1982): The involvement of vinculin in contact-induced cytoskeletal interactions. *Cold Spring Harbor Symp. Quant. Biol.* 46:671-682.
- Geiger, B., Tokuyasu, K.T., Dutton, A.H., and Singer, S.J. (1980): Vinculin, an intracellular protein localized at specialized sites where microfilament bundles terminate at cell membranes. *Proc. Natl. Acad. Sci. USA* 77:4127-4131.
- Geiger, B., Dutton, A.H., Tokuyasu, K.T., and Singer, S.J. (1981): Immunoelectron microscopic studies of membrane-microfilament interactions. The distribution of α -actinin, tropomyosin and vinculin in intestinal epithelial brush border and in chicken gizzard smooth muscle cells. *J. Cell Biol.* 91:614-628.
- Geiger, B., Avnur, Z., and Schlessinger, J. (1982): Restricted mobility of membrane constituents in cell-substrate focal contacts of chicken fibroblasts. *J. Cell Biol.* 93:495-500.
- Geiger, B., Avnur, Z., Kreis, T.E., and Schlessinger, J. (1983): The dynamics of cytoskeletal organization in areas of cell contact. Invited review in Shay, J.W. (ed): "Cell and Muscle Motility, Vol. 5." (In preparation).
- Hillman, G., and Schlessinger, J. (1982): The lateral diffusion of epidermal growth factor complexed to its surface receptor does not account for the thermal sensitivity of patch formation and endocytosis. *Biochemistry* 21:1667-1672.
- Kreis, T.E., Geiger, B., and Schlessinger, J. (1982): The mobility of microinjected rhodamine-actin within living chicken gizzard cells determined by fluorescence photobleaching recovery measurements. *Cell* 9:835-845.