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Decrease in Membrane-Associated Actin of Fibroblasts after Transformation by Rous Sarcoma Virus

(viral transformation/cytoplasmic actin/temperature-sensitive virus)

GARY WICKUS*, ERIC GRUENSTEIN†, PHILLIPS W. ROBBINS, AND ALEXANDER RICH

Biology Department, Massachusetts Institute of Technology, Cambridge, Mass. 02139

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ABSTRACT **The actin content of membranes prepared from cultured chick embryo fibroblasts has been measured on polyacrylamide gels. The actin was identified by tryptic peptide mapping. After transformation of the cells by Rous sarcoma virus, the amount of actin associated with the membranes is decreased by 30–50%. This result is not due to infection *per se*, since infection by a temperature-sensitive strain of the virus decreases membrane-associated actin only at the permissive temperature. A shift from the nonpermissive (41°) to the permissive (36°) temperature results in an increase in the percentage of total cellular protein synthesis devoted to actin production, so that the decrease in membrane-associated actin appears to be a selective displacement from the membrane rather than a general decrease in total cellular actin.**

Alterations in the plasma membranes of cells have been associated with transition of the cell from the normal to the transformed state. Transformation is associated with changes in cell shape (1), membrane transport (2), agglutination by plant lectins (3), and contact inhibition of growth (4, 5). A number of recent studies have reported either increases or decreases in individual proteins identified as bands on polyacrylamide gels of plasma membranes of transformed as compared to normal cells (6–14). While such studies characterizing the difference between these membrane constituents are clearly of very great interest, it is often difficult to assign specific functional significance to these altered polypeptide patterns. However, we have focused our attention on the characterization of a single band in polyacrylamide gels arising from membranes of normal and transformed cells which we have identified as actin. Here we report that virus-induced cell transformation is associated with a decrease in membrane actin content, even though the total actin content of the cell is unchanged.

Actin is known to be present in a wide variety of nonmuscle eukaryotic cells (reviewed recently in ref. 15). Actin-containing microfilaments are seen close to the plasma membrane in the electron microscope and microfilaments have been shown to contact the plasma membranes of acanthamoeba (16) and intestinal microvillae (17). Recently, actin has been chemically identified through its tryptic peptide map in plasma membrane preparations of 3T3 mouse fibroblasts and HeLa cells (18) and antibodies to fibroblast actin have been shown to bind close to the inner surface of plasma membranes in several cell types (19). Weber *et al.* have reported that tumor transformed cells show a modified pattern of antibody-labeled actin (20). The close association between plasma membranes and this protein which is capable of functioning in a contractile role, together with the well-known alterations in cellular morphology and movement after transformation suggested to us that there may be functionally important changes in the membrane actin content following infection of cells with a transforming virus.

METHODS AND MATERIALS

Chick embryo fibroblasts were grown from 11 day embryos (SPAFAS, Norwich, Conn.) and infected with Rous sarcoma virus as previously described (10). Secondary cultures were prepared by trypsinizing the primary cultures and passing them to fresh plates at a density of 5×10^6 cells per 100 mm petri dish. Unless otherwise indicated, secondary cultures were maintained at the permissive temperature (36°) for an additional 40 hr with medium changes every 12–18 hr until full morphological transformation occurred. Half the secondary cultures were then changed to the nonpermissive temperature (41°). After an additional 16 hr of culture, the cultures incubated at 41° showed identical morphology to contact-inhibited normal chick embryo fibroblasts or TS-68 infected chick embryo fibroblasts maintained at 41°. The cultures which were maintained at 36° remained morphologically transformed.

For pulse-labeling experiments in which cells were shifted down from 41° to 36°, uninfected or infected chick embryo fibroblast cultures were pulse-labeled with [^{35}S]methionine (7.5 μCi/ml) in Eagle's minimum essential medium prepared without methionine, but supplemented with 2% tryptose phosphate broth and 4% calf serum. In shift-down experiments, cells were cultured at the higher temperature at all stages prior to shifting to the lower temperature. Cells labeled with a ^{14}C-labeled amino acid mixture for actin peptide mapping were prepared by replacing the normal growth medium with Eagle's minimal essential medium minus amino acids and containing trace quantities of 15 amino acids that were uniformly labeled with ^{14}C (0.5 mCi/ml). Incubation was continued in this medium until the uptake of radioactivity from the medium began to plateau at around 6 hr. At this time the cells were washed and harvested. In all other cases, cells were labeled 56 hr after plating of the secondary cultures by the addition of fresh medium containing 4 μCi/ml of L-[^{35}S]methionine. After 18 hr, the cultures were collected by scraping with a rubber policeman.

Plasma membrane vesicles were prepared as described by Perdue *et al.* (21) with minor modifications. Plasma membrane sheets were prepared by the method of Brunette and Till (22) in a two-phase system. This procedure was modified

* Current address: Gundersen Clinic, Ltd., 1836 South Ave., La Crosse, Wisc. 54601.
† Current address: Department of Biochemistry, University of Cincinnati, College of Medicine, 231 Bethesda Avenue, Cincinnati, Ohio 45267.

slightly as suggested by Sheinin (12) by treating the cells for 1 min with 0.1% trypsin solution in citrate-saline buffer (0.016 M Na citrate, 0.01 M KCl, pH 7.6) while the cells were still attached to the surface of the petri dish. In all cases 1 mM phenylmethylsulfonylfluoride was included in the homogenizing solution to inhibit proteolysis.

Plasma membranes and total cell homogenates were analyzed by dissolution at 100° in 1% sodium dodecyl sulfate, 1% 2-mercaptoethanol, 10% glycerol, and 0.05 M Tris·HCl, pH 6.8. Suitable samples containing 50 μg of protein were subjected to electrophoresis on polyacrylamide slab gels or on disc gels in which case 100 μg of protein were applied. In both cases the gel system used was that described by Laemmli (23). After electrophoresis the slab gels were dried and the position of the labeled proteins determined by autoradiography. Three independent methods were used to quantitate actin. (*i*) The slab gel autoradiogram was scanned and the area under the actin peak was integrated and divided by the total radioactivity applied to the gel column. (*ii*) Membrane proteins analyzed by disc gel electrophoresis were stained with Coomassie Brilliant Blue, scanned at 550 nm, and the area under the actin peak compared to the area under the total scan. (*iii*) Membranes were analyzed on disc gels, and the gels were cut transversely into 1 mm thick slices and the radioactivity in each slice measured. Percent of radioactivity as actin was determined by dividing the radioactivity in the actin peak by the total radioactivity in all slices. For purposes of comparison among different experiments, the value obtained for nontransformed cells was normalized to 100 and all other values compared to this.

Tryptic peptide maps of the presumptive actin bands from polyacrylamide disc gels were prepared as previously described (18). The band which migrated in the position of pure actin was cut out and the protein eluted in the presence of 0.5 mg of added purified chicken muscle actin. The protein was precipitated with trichloroacetic acid, digested with trypsin, and analyzed on thin-layer cellulose plates by chromatography and electrophoresis (24). Ninhydrin staining material represents carrier muscle actin while autoradiographic spots represent presumptive actin arising from chick embryo fibroblast membrane.

Tissue culture materials were obtained from Grand Island Biological Supply Co. [^{35}S]methionine, (7.5 μCi/ml) and ^{14}C-labeled amino-acid mixture, were obtained from New England Nuclear Co., phenylmethylsulfonylfluoride from Sigma Co., and thin-layer cellulose plates from Brinkmann. All other compounds were commercially available reagent grade. The Rous sarcoma virus and its temperature-sensitive mutant, TS-68, were the generous gift of Dr. H. Hanafusa.

RESULTS

The initial observation on which the subsequent experiments are based is shown in Fig. 1A. Membranes prepared by the method of Perdue (21) from TS-68 infected chick embryo fibroblasts that were labeled for 18 hr with [^{35}S]methionine showed a 30% decrease in labeled actin content when grown at the permissive (36°) relative to the nonpermissive (41°) temperature. The infected cells are transformed at 36°, but not at 41°. Rous sarcoma virus (TS-68) production is equivalent at both temperatures. Since membranes prepared by this procedure form vesicles (21), our first concern was to confirm these results using membranes prepared by the Brunette and Till procedure (22) in which rolled sheets of membranes predominate. In this case (Fig. 1B), the decrease of labeled protein as actin was even more pronounced, with transformed cell membranes having only 50% as much as their untransformed counterparts. These data raised several questions. (*1*) Is the gel band we are measuring which co-electrophoreses with authentic muscle actin really actin? (*2*) Is our method of quantitation of membrane actin valid? (*3*) Is the effect due to temperature rather than transformation? (*4*) Are we simply seeing a change in total cellular actin synthesis which is reflected in the membrane actin content, or is there a subcellular redistribution of actin associated with transformation?

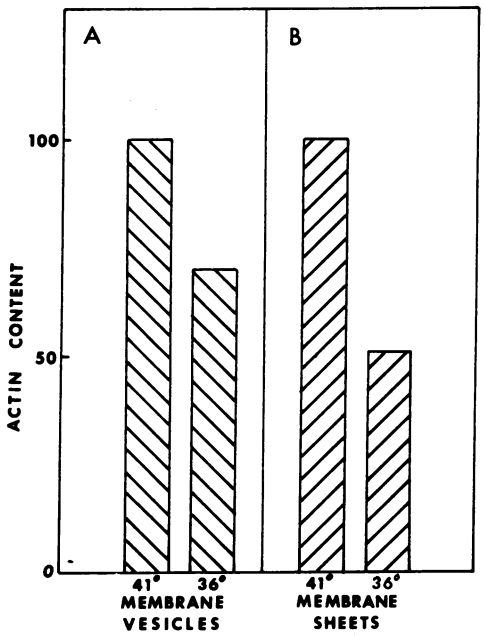

FIG. 1. Incorporation of [^{35}S]methionine into the membrane-associated actin of TS-68 Rous sarcoma virus infected chick embryo fibroblasts at the permissive (36°) and nonpermissive (41°) temperatures. Cells were infected and labeled as described in *Methods and Materials*. Plasma membranes were then prepared by the method of Perdue (A) and of Brunette and Till (B). Normalized actin contents of transformed and untransformed cells were determined as described in *Methods and Materials*.

In order to unambiguously identify the gel band, we compared its tryptic peptide map to that of authentic actin. Membranes were prepared from TS-68 infected chick embryo fibroblast cells labeled with a mixture of ^{14}C-labeled amino acids and analyzed on sodium dodecyl sulfate polyacrylamide disc gels. Labeled protein from the gel band co-electrophoresing with actin was eluted, mixed with excess unlabeled muscle actin, digested with trypsin and mapped in two dimensions by chromatography and electrophoresis. The resultant map obtained from cells grown at 41° is seen in Fig. 2. Similar results (not shown) were obtained from cells grown at 36°. Thirty-two major ninhydrin spots have autoradiographic spots associated with them, whereas, there are nine without apparent associated radioactivity. Eighteen autoradiographic spots are present without associated ninhydrin-sensitive material. Based on many similar experiments in which the maps of identical proteins and of proteins known to be different are compared (18), we conclude from the extensive coincidence of ninhydrin and radioactive peptides seen here that actin is the major protein component of the presumptive actin gel band. There are probably one or more

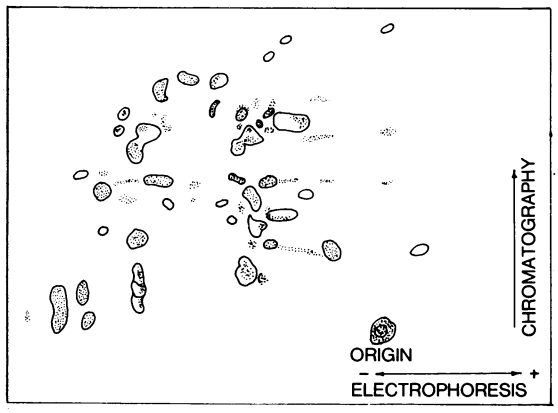

FIG. 2. Tryptic peptide map of the radioactively labeled (^{14}C-labeled amino-acid mixture) presumptive actin band from gels of TS-68 infected chick embryo fibroblast membranes (*stippled areas*) compared to ninhydrin stained carrier chick muscle actin peptides (*solid outlines*).

minor contaminating proteins, but these are present to a similar extent in both transformed and untransformed cells and cannot account for a 30–50% difference in band intensity.

Fig. 3A shows the relative labeled actin content of plasma membrane vesicles from normal, uninfected chick embryo fibroblasts with wild-type virus transformed cells, both grown at 39°. Associated with transformation there is a 30% decrease in membrane actin labeled with [^{35}S]methionine. The shift in temperature from 36° to 41° of Fig. 1 thus does not appear to be necessary to obtain the decrease in actin. Fig. 3B and 3C show results of the same experiment calculated (see *Methods and Materials*) from Coomassie Blue stained disc gels and from labeled sliced disc gels, respectively. The

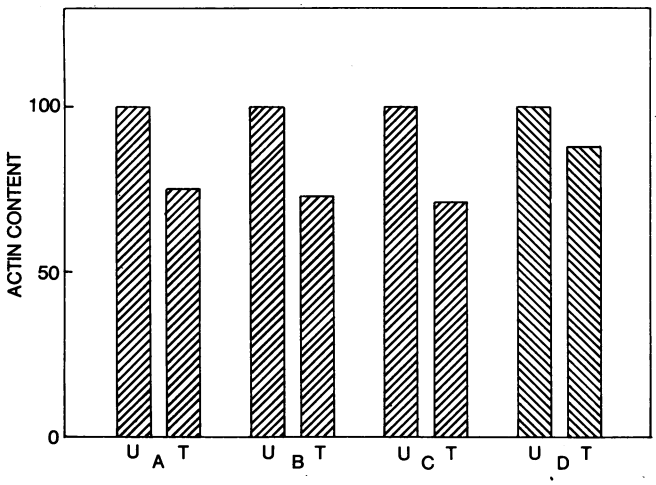

FIG. 3. Actin content of normal and wild-type (SR-RSV) transformed chick embryo fibroblast membranes and whole cells. Membranes were prepared by the method of Perdue from uninfected (U) and transformed (T) cells grown and labeled with [^{35}S]methionine as described in *Methods and Materials*. (A) Membrane proteins were resolved by sodium dodecyl sulfate slab gel electrophoresis and autoradiograms prepared. (B) Membrane proteins were analyzed by sodium dodecyl sulfate disc gel electrophoresis, stained with Coomassie Brilliant Blue and scanned at 550 nm. (C) Membranes were analyzed on sodium dodecyl sulfate disc gels as in (B), but were not stained. Gels were cut into 1 mm thick slices and the radioactivity of each slice was measured. Methods for quantitation and normalization of actin content are described in *Methods and Materials*. (D) Actin content of whole cells measured as in (A) above.

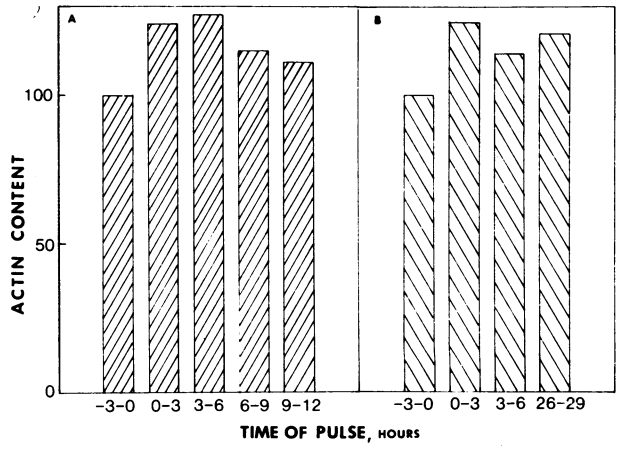

FIG. 4. Incorporation of [^{35}S]methionine into the actin of whole cells infected with TS-68 (panel A) or uninfected (panel B) during 3 hr pulses after a temperature shift-down from 41° to 36°. Quantitation and normalization of actin content are described in *Methods*.

results are not significantly different from the slab gel autoradiographic scans of Fig. 3A. The differences observed between normal and transformed cells are, therefore, probably not due to artifacts in the method of quantification. The data of Fig. 3D are the same as those of 3A except that total cell homogenates rather than membrane vesicles have been analyzed. There is a small decrease in total cellular actin in transformed cells, but not enough to account for the much larger decrease in membrane-associated actin.

In order to see whether a shift in temperature could account for the change in membrane-associated actin at any period during the transition from the normal to transformed state, cells were pulsed for 3 hr periods after a temperature shift-down. The results for both uninfected and TS-68 infected cells are shown in Fig. 4. At all times following the shift from 41° to 36°, there is an increase in the percentage of total protein synthesis devoted to actin for both uninfected and TS-68 infected cells. We conclude that a temperature shift cannot account for the decrease in membrane-associated actin. Indeed, under at least some circumstances the temperature shift may partially mask the true magnitude of the intracellular actin redistribution.

The reason for the apparent discrepancy between these data and those of Fig. 3D in which total actin content decreases is not entirely clear. However, it may be related to the fact that the short pulses of [^{35}S]methionine in Fig. 4 should reflect primarily the synthesis of new actin, whereas, longer labeling in Fig. 3D will also take into account the rate of degradation (25). Transformed cells may have increased proteolytic activity (26, 27) and the data would be consistent with a simultaneous increase in both synthesis and degradation of actin.

DISCUSSION

Membrane-associated actin is decreased after transformation. This decrease is not related to viral infection *per se*, to temperature effects, or to a concomitant decrease in cellular actin synthesis. We conclude that, in association with transformation, there is a redistribution of cellular actin away from the plasma membrane. However, it should be pointed out that this interpretation assumes no gross changes in the isolation and recovery of plasma membranes associated with

transformation. Two different isolation techniques were used, but it is likely that the recovery is less than 100%.

What is the significance of these results in relation to cell functions? Actin may serve in more than one role in nonmuscle cells. In addition to promoting movement both within the cell and of the plasma membrane, actin may participate in the regulation of membrane structural rigidity. There is some evidence to support this concept. Microfilaments, presumably composed at least partially of actin, are commonly observed in two general morphologies. One form is designated sheath, α-, or stress filaments, and the other lattice or network filaments (27). Sheath filaments are found in bundles parallel and immediately subjacent to the plasma membrane generally in regions where the membrane is immobile and refractile. Wessells, Spooner, and Luduena (28) have suggested that microfilaments in this morphological arrangement may be primarily involved in maintenance of structural rigidity. Lattice filaments are also often seen near and sometimes extending into the plasma membrane, especially in regions of active membrane movement such as the anterior ruffled edges of fibroblasts. In further contrast to sheath filaments, lattice filaments do not appear to be associated with the membrane along the entire course of the filament, but rather at discrete points similar to the actin filaments found hanging from the plasma membrane of acanthamoeba (16). It is possible that we may be isolating primarily sheath-type actin associated with the fibroblast membranes, as suggested by the lack of effect of cytochalasin B on the actin content of 3T3 fibroblast membranes (18) and the similar refractory nature of sheath filaments (but not lattice filaments) to structural modification by cytochalasin B (27).

The observation that actin is lost from membranes isolated from transformed as compared to normal fibroblasts is consistent with a loss of structural parameters associated with the maintenance of membrane rigidity. As a consequence, the continued mobility of transformed cells beyond confluence could be viewed as a release from inhibition of movement rather than as an excess of motility promoting factors. In agreement with this concept, there is a decrease in the number of visible α-filaments (sheath filaments) in the anterior regions of transformed 3T3 cells compared to normal and revertant 3T3 lines (29). It has been suggested that there is probably a close relationship between decreased α-filaments and loss of contact inhibition of movement in transformed cells. Our data further suggest that this morphological change is specifically associated with a diminished association between actin and the plasma membrane. Transformed cells may have a decrease in the amount of polymerized actin or a decreased number of actin binding sites.

There is circumstantial evidence linking transformation with altered cell mobility. Treatment of subconfluent L-929 fibroblast cultures with dibutyryl-adenosine 3′:5′-cyclic monophosphate (cyclic AMP) causes a marked decrease in cell mobility relative to untreated cells (30). This effect is rapid and reversible, suggesting that neither RNA nor protein synthesis is directly involved. Cyclic AMP increases in normal cells at confluence, but not in transformed cells (31). The phenomena of transformation, cell motility, and contact inhibition may thus be related through the effects of cyclic AMP on microfilaments and their interaction with the cell membrane. In addition, any role played by actin in contractile processes is likely to involve myosin as well. It should be noted that the myosin content of a transformed rat kidney cell line is decreased to one-half that of the normal cell (32). However, further work will be needed to clarify the role of actin and myosin changes in transformed cells.

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