Transformation-sensitive Isoactin in Passaged Chick Embryo Fibroblasts Transformed by Rous Sarcoma Virus

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ABSTRACT Transformation by Rous sarcoma virus (RSV) has been reported to block the expression of differentiated cell products in chicken cells. The expression of these proteins may or may not be suppressed when temperature-sensitive mutants are shifted from the nonpermissive to the permissive temperature. A general characteristic of cellular transformation is the disruption of the microfilament system. In passaged chick embryo fibroblast cultures (CEF), this system is principally composed of isomeric forms of actin designated α, β, and γ by their isoelectric focusing and when subjected to SDS-PAGE behavior. We present evidence that an α-actin in CEF cultures, identified by its electrofocusing behavior, retention in the cytoskeleton, and DNase I binding properties, is selectively and dramatically reduced in amount upon transformation by RSV. Little or no reduction is observed in the β- and γ-isoactins. The reduction of α-actin is shown to be reversible and transformation related by use of a temperature-sensitive mutant, tsNY68. The decrease in this transformation-sensitive isoactin is apparently due to a decrease in synthesis, though other possibilities are discussed. A specific decrease in a particular isoactin after transformation may give insight into the mechanism by which the microfilaments are normally maintained.

In vitro transformation by Rous sarcoma virus (RSV) in a variety of cell types leads to specific changes in the expression of differentiated cell products (3, 13, 31, 39). For instance, fibronectin, a cell surface protein involved with adhesion, is decreased after transformation (21). Another event closely linked to RSV transformation is the disruption of the cytoskeleton (11), particularly the microfilament complex (4, 32, 47). The microfilaments, a major constituent of the cytoskeleton, are composed primarily of actin (18, 44). In normal cells, microfilaments exist in a dynamic state and undergo rapid assembly and disassembly (19). After transformation, their rapid disruption may involve alterations in the microfilament-associated proteins, actin(s), or both. As examples of the former, α- and β-tropomyosin (26) are markedly decreased (20), while vinculin, a phosphoprotein that appears to be involved in actin filament bundling and termination (7, 17, 23), may be phosphorylated by the transforming gene product of RSV (pp60<sup>src</sup>) (9). As an example of the latter, the expression of variant actins in some transformed cell lines have been reported (5, 28).

Actin is a highly conserved component of all eukaryotic cells and is a major protein in muscle as well as nonmuscle cells (25, 33). Multiple forms of this protein exist (45), and by high resolution, two-dimensional gel electrophoresis three forms: α, β, and γ can be resolved (16, 36, 48). Each of these forms has a tissue-specific isomer that cannot be resolved by isoelectric focusing. The α-actins are the most acidic of the three isoelectric focusing variants, and unique isomers are found in skeletal and cardiac muscle (45, 46) and vascular smooth muscle cells (14, 15). Passaged chick embryo fibroblasts (CEF) contain three isoelectric forms: α, β, and γ of actin (36, 43). Recently, the α-form in CEF has been shown to be the vascular smooth muscle isoactin (46).

We report here that the transformation of CEF by RSV leads to a specific decrease in the level of this α-isoactin. The lowered level of this isomer appears to result from a decrease in synthesis rather than an increase in degradation. This alteration parallels the decrease in the tropomyosins (20). Experiments with a temperature-sensitive mutant of RSV show that the decrease of this isomer is reversible and correlates with the appearance of the transformed phenotype.

MATERIALS AND METHODS

Cells and Virus: CEF were derived from serial passage of cells prepared by trypsinization of eviscerated 10-d chick embryos. Cells used for the studies described here ranged from fourth to tenth passage, and results were not dependent on passage number. The cells were grown on plastic dishes or glass coverslips using either Richter's IMEMZO (Associated Biomedical Systems, Inc.,
Buffalo, NY) or H-21 (Gibco Laboratories, Grand Island, NY) medium supplemented with 5% calf serum, 10% tryptose phosphate broth, penicillin (100 u/ml), and streptomycin (100 #g/ml).

After the third passage, cells were infected with either Schmidt-Ruppin strain A or a temperature-sensitive mutant (tsNY68) of RSV. Transformation occurred within 4-6 d after infection and was monitored by changes in morphology as well as the appearance of biochemical markers of transformation, such as specific alterations in cellular phosphoproteins (35, 50) and pp60v~ kinase activity (9).

Labeling of Cells and Two-dimensional Gel Electrophoresis: Cell cultures were metabolically labeled with [35S]methionine usually in methionine-free Dulbecco's minimum essential medium. Labeling times were variable depending on the experiment and, if longer than 4 h, 10% of the normal amount of unlabeled methionine was included. For some experiments, cells were grown on glass coverslips (22-mm diam). Labeled cells were scraped directly into buffer comprised 2% SDS, 5 mM Tris-Cl (pH 8.0), 5% mercaptoethanol, and 10 mM EDTA, then heated to 93°C for 3 min. If the cells had been grown on coverslips, 20 #l of this lysis buffer was used; if cells were grown on 35-mm dishes, 50 #l of buffer was used. Preparation for two-dimensional gel electrophoresis followed the procedure of Ames and Nikaido (1), and electrophoresis was done essentially as described by O'Farrell (30) except for the following modifications: the pH gradient was formed with a combination of 0.67% 2-I-l ampholytes and 1.33% 4-6 ampholytes (Serva Heidelberg, Federal Republic of Germany), isoelectric focusing was done for 12 h at 300 V followed by 4 h at 800 V, and SDS-PAGE was done on 10% acrylamide gels.

Determination of Relative Amounts of Each Isoactin: Estimation of the amount of each isoactin variant on the Coomassie Blue-stained two-dimensional gel generally followed the method of Quitschke and Schechter (34). Briefly, the regions of the dried slab gels containing the isoactins were enlarged by photography, and the individual isomers were scanned as described on a Joyce-Loebl Chromoscan. The relative ratios of each variant on the gel were determined. Consequently, variations in Coomassie Blue staining between gels was not of great significance. Each spot was subjected to two or more scans, with little or no alteration in the results.

The quantities of each isomer were also determined by pulse experiments. Lysates were obtained from cells pulsed with [35S]methionine (50-100 #Ci/ml) for 4 to 24 h. The gels were stained with Coomassie Blue (12), permeated with 1 M sodium salicylate (8), and dried. Autoradiography was carried out at -70°C using either Cronex or Kodak XAR x-ray film. Quantitation of spots was accomplished by excision from salicylated gels and counting in a Beckman LSC, using a toluene-based scintillant.

Preparation of Cytoskeletons: Cells were incubated with 10 #Ci/ml of [35S]methionine in medium containing 10% of the normal methionine concentration. Labeling was carried out for 18 h. To prepare cytoskeletons, the monolayer was washed with phosphate-buffered saline and extracted with 5% Triton X-100 (6) and 0.6 M KCl (41). The attached cytoskeletons were washed twice with this solution, dissolved in 2% SDS-containing buffer, and analyzed by two-dimensional gel electrophoresis.

DNase I Sepharose Purification of Actin: The purification procedure used was a modification of that of Lazarides and Linberg (27). Briefly, normal chick fibroblasts from two 100-mm plates were disrupted by nitrogen cavitation in 1 ml of 150 mM NaCl, 50 mM Tris-Cl, 1 mM CaCl, pH 7.4 and centrifuged at 130,000 g for 30 min. The supernate was diluted 1:1 in 0.3 M sodium acetate, 1 mM CaCl, and 30% glycerol. DNase I conjugated to Sepharose was equilibrated in this latter buffer, and 100 #l of this was added to the clarified cell lysate. After a 30-min incubation at 4°C, the beads were collected by centrifugation and washed four times in ice-cold buffer followed by four washes in ice-cold buffer containing 0.75 M guanidine HCl. Actin was extracted from the beads by adding 150 #l of a solution comprising 2% SDS, 5 mM Tris-Cl, 10 mM EDTA, 5% mercaptoethanol, and heating to 95°C for 3 min. The sample was then analyzed by twodimensional electrophoresis.

Purification of a-Actin: a-Actin was a gift of Dr. Gary Miller and was purified from rat skeletal muscle by the method of Spudich and Watt (42).

RESULTS

When normal chick embryo fibroblast cultures were analyzed by two-dimensional gel electrophoresis, a highly reproducible pattern of three spots was seen at mol. wt. 43,000 and near pl 5.5 (Fig. 1 A, B). This complex was a major species in whole cell lysates and appears to represent three isolectric variants of chick actin (36, 43). These results were independent of passage number (through 10 passages).

The steady-state levels of the three putative isoactins in untransformed cells were determined by two independent methods. Densitometric scanning of Coomassie Blue-stained gels (34) indicated that the spot corresponding to a-actin constituted ~55% of total actin, while the spots that focused as a- and y-actins constituted 23 and 22%, respectively, (Fig. 1 A). When chick fibroblasts transformed by RSV were compared with sister cultures of normal cells, a dramatic reduction in the level of the most acidic species of this complex was noted (Fig. 1 B). This protein was still detectable in these transformed cells but now constituted <5% of the total actin by densitometric scanning. The levels of a- and y-isoactins were unchanged. An attempt was also made to determine the relative abundance of the three putative isoactins by quantitation of [35S]methionine

FIGURE 1 Comparison of isoactins in normal and virus-transformed CEF. Normal (sixth passage) chick fibroblasts or a sister culture that had been previously infected with RSV (Schmidt-Ruppin strain A) were lysed directly into SDS-containing buffer and analyzed by two-dimensional gel electrophoresis. Gels were stained with Coomassie Blue (12) and dried. A shows the pattern obtained from normal cells, while B shows that obtained from transformed cells. A pattern similar to that in B was seen with a variety of strains of nondefective RSV. Inserts are enlargements of the putative isoactin complex from A and B, respectively, showing the transformation-specific decrease in the level of the most acidic species. No proteins appear in the regions covered by the inserts. Numerals on the left-hand side of A and B refer to molecular weights.
incorporation. This study supported a four- to fivefold decrease in \(\alpha\)-isoactin after transformation but gave somewhat different results for the fraction of total actin as \(\alpha\)-isomer. For example, in a 24-h pulse with \([\text{35S}]\)methionine in normal CEF, we obtained values for the \(\alpha\)-species ranging from 4.5 to 6% of total actin, while transformed cell values ranged from 0.9 to 1.5%. Once again, no difference in the quantity of \(\beta\)- or \(\gamma\)-isomer present was noted. It is not clear why the difference exists between these data and those obtained by quantitation of Coomassie Blue staining. Nonetheless, with both methods, transformed CEF display a level of the putative \(\alpha\)-isoactin that is four- to fivefold less than is found in the normal CEF. Concomitant reductions were also noted in both \(\alpha\)- and \(\beta\)-tropomyosin as previously reported (20).

A specific reduction in the level of this \(\alpha\)-like isoactin protein, independent of passage number, time in culture, and cell density, was always found to accompany transformation by RSV, but we considered that this might represent a cell selection phenomenon. In other words, an infection of passaged chick fibroblast cultures with the transforming virus might lead to enhanced proliferation of a specific subset of cells that normally contain low levels of this putative isoactin. To examine this possibility, we conducted studies with a temperature-sensitive mutant of RSV, tsNY68. Cells infected with this viral mutant are normal at the nonpermissive growth temperature of 41°C, but are transformed when grown at 35°C (24). Temperature shifts in these temperature-sensitive infected cells lead to rapid transformation or phenotypic reversion. tsNY68-infected cells were grown at 35°C on 22-mm coverslips. At varying times, these coverslips were shifted to 41°C, normal morphology appearing within 4–6 h after temperature shift. These cultures were then pulsed with \([\text{35S}]\)methionine for 2 h and the labeling of the putative isoactins was examined by two-dimensional gel analysis. tsNY68-transformed CEF that were not shifted (Fig. 2A) show a low level of incorporation of label into the most acidic species, while cells that had been shifted to the nonpermissive temperature 22 h before the pulse (Fig. 2C) show a level of incorporation into this protein similar to that of uninfected cells. The increase in labeling of this putative isoactin is detectable at 4 h (Fig. 2B) postshift, which coincides with the onset of normal morphology (24) and is concomitant with or precedes an increase in \(\alpha\)- and \(\beta\)-tropomyosin synthesis. Conversely, when tsNY68-infected cultures maintained at 41°C for up to 72 h and phenotypically normal were shifted to 36°C for 24 h, the rate of \([\text{35S}]\)methionine incorporation into this protein was equivalent to that seen in wild-type transformed cells (data not shown). Neither a specific temperature dependence of label incorporation into this species nor a difference in apparent half-life was noted in either normal or wild-type transformed chick fibroblasts (data not shown).

To confirm that all three proteins did represent the isoactins present in passaged CEF, two approaches were used. Cytoskeletons were prepared by extraction of intact cells with nonionic detergent and high salt. The cytoskeletal residue is deficient in most cellular proteins and enriched in certain structural proteins of intermediate filaments (vimentin in these cells) and microfilaments (the isoactins) (41). Tubulin, on the other hand, is solubilized by this procedure (2). Fig. 3A demonstrates the pattern of the intact cells while Fig. 3B represents the proteins of the cytoskeleton that remain after nonionic detergent extraction. The retention of this 43,000-dalton complex in the cytoskeletal preparation along with vimentin and fibronectin is consistent with its representing actin. The relative abundance of each isoactin in the cytoskeleton is similar to that of the whole cell lysate, suggesting that all three forms share similar solubility behavior.

To further confirm that the complex represented authentic actins, we purified actin from chick fibroblasts by affinity binding to DNase I (27). Fig. 4A demonstrates that the putative...
The presence of the transformation-sensitive protein in affinity-purified actin, as well as its location with respect to the other isoactins after isoelectric focusing and SDS-PAGE, suggests that this is an α-isomer of actin. α-Isoactins from skeletal, cardiac, and vascular smooth muscle co-migrate in two-dimen-

isoactin complex is present in the 130,000-g supernate of cells disrupted by nitrogen cavitation. Vimentin and fibronectin have been largely removed by centrifugation. After purification on DNase I, the putative actin complex is the only species detectable and all three species are present in the same relative abundance as in intact cells (Fig. 4B). This confirms that this complex is authentic actin and strongly suggests that each species present corresponds to at least one isoactin.
sional gel electrophoresis. Confirmation of this species as an α-like isoactin was given by its comigration with purified skeletal muscle α-actin (Fig. 5).

DISCUSSION

The presence of α-actin in secondary cultures of CEF has been documented (36, 43), although some other nonmuscle cells apparently do not contain this isoactin (16). A recent publication indicates that the α-isomer present in passaged CEF is the smooth muscle isoactin (46). CEF also have been shown to contain both vinculin and α- and β-tropomyosin (17, 26) in the microfilament complex. A recent study demonstrated that both viral and chemical transformations lead to marked reductions in α- and particularly β-tropomyosin in avian fibroblast cultures (20). Vinculin, thought to interact with actin, is greatly enriched in phosphotyrosine content after RSV transformation, although not reduced in amount (40). Both of these alterations have been suggested to play a role in the disruption of the cytoskeleton after transformation.

We found that RSV transformation of CEF cultures always leads to a dramatic and selective decrease in the steady-state levels of a cytoskeletal protein identified as an α-isomer of actin. No alterations in the levels of β- or γ-actin were observed. The total amount of actin appears reduced (also noted by others [49]) as a result of the decrease in the more acidic isomer. The reduction of α-actin in RSV-CEF is apparently due to a decreased synthesis rather than an increase in degradation. This is supported by two pieces of evidence. First, pulse-chase studies revealed no differences in apparent half-life of the α-like isoactin between wild-type and uninfected cells. Second, transformed cells that were pulse-labeled with [35S]methionine for brief periods showed very little incorporation of label into α-like isoactin, relative to normal cells (data not shown).

Two methods were used to quantitate the relative abundance of the isoactins resolved from whole cell lysates. Direct densitometric scanning indicated that α-isoactin comprised >20% of the total actin, while incorporation of metabolic label gave a significantly lower value. Vanderckhove and Weber (46), using a novel technique, reported a value of 10% for the α-isoactin fraction of total actin in passaged chick fibroblasts. This suggests that densitometry may give an artificially high result. Irrespective of this point, both methods demonstrated a dramatic four- to fivefold reduction of α-isoactin after transformation.

Our results, as with all studies using embryonic cell cultures, could be explained on the basis of mixed cell types in our culture. The presence of an α-actin that is vascular smooth muscle specific in passaged CEF (46) suggests that either these fibroblasts express this actin or the cultures normally contain smooth muscle cells as a contaminant (14, 15). The fact that the amount of α-isoactin present is independent of passage number suggests that this isomer is not due to contaminating smooth muscle cells, since, with multiple passages, one might expect to select either for or against a smooth muscle contaminant, leading to a decreased or increased amount of the α-isomer. But even if smooth muscle cells are the source of the α-isoactin, the studies conducted with the temperature-sensitive mutant tsNY68 clearly demonstrate that the level of α-actin is linked to transformation in a reversible manner. We conclude that transformation is specifically controlling the expression of α-isoactin rather than eliminating a subset of cells.

It has been shown previously that the morphologic changes that accompany transformation of tsNY68-infected chick fi-

![Figure 5](image-url)
require up to 12 h and are among the last parameters of transformation to occur. It is not clear at this point whether the decrease in an α-actin-like isomer after transformation plays a direct role in these cytoskeletal changes or whether the decrease arises secondary to transformation-induced alterations in the cytoskeleton. Our observation that there is an apparent increase in synthesis of this isoactin within 2–4 h after shift of tsNY68-infected cells to the nonpermissive temperature is consistent with the notion that this isomer is essential for stabilization of normal microfilament morphology in these cells. It is also possible that the decrease in the α-actin is secondary to morphological changes. One could speculate, for instance, that disruption of microfilaments by another mechanism leads to the selective release of the acidic isoactin in a soluble (G-actin) form. This could conceivably cause a specific decrease in synthesis by a negative-feedback mechanism such as that noted for tubulin (2).

Our findings are consistent with a specific decrease in synthesis of the α-like isoactin species in transformed cells, but other mechanisms are being considered. A recent study (37) found that β- and γ-actins are released into the medium of differentiating myoblasts, while α-actin is specifically retained. However, our experiments have not shown a preferential release of any isoactin species from transformed cells into the media (data not shown). The possibility that alteration(s) in other mechanisms are being considered. A recent study (37) for tubulin (2).

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