Transformation-dependent increase in endogenous cytochalasin-like activity in chicken embryo fibroblasts infected by Rous sarcoma virus

(actin polymerization/neoplastic transformation/oncogene product/oncogenic virus/cytochalasin B)

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ABSTRACT Transformation of chicken embryo fibroblasts by infection with Rous sarcoma virus has been shown to cause disruption of actin filament organization as seen with fluorescence staining techniques. This study is an attempt to use quantitative biochemical techniques to compare actin-related parameters in normal and transformed cells. Normal cells and cells infected with a temperature-sensitive mutant virus (NY68) and grown at the restrictive temperature of 41.5°C have normal bundles of actin filaments, or F-actin; these cells also have about the same number of high-affinity cytochalasin binding sites at the ends of F-actin (≈ 5 pmol of sites per mg of cellular protein; K_d , 20 nM). In contrast, infected cells grown at the permissive temperature of 37°C have a more diffuse pattern of actin filaments, and the number of cytochalasin binding sites in these transformed cells was below the level of detection. DNase I inhibition assays showed that the percent of unpolymerized actin, or G-actin, in cell extracts was not significantly different between normal and transformed cells ($\approx 50\%$). In assays of cell extracts for endogenous cytochalasin-like activity on actin filaments (i.e., retardation of filament assembly at the fast-growing end, inhibition of cytochalasin binding to actin "nuclei," and decrease of low-shear viscosity of solutions of actin filaments), infected cells at 37°C showed a higher level of activity per mg of protein than did uninfected cells or infected cells at 41.5°C. These results suggest that the increase in endogenous cytochalasin-like activity in transformed cells may relate to the decrease in measurable cytochalasin binding sites and the abnormal distribution of actin filaments previously seen by fluorescence staining techniques.

Normal fibroblasts in culture have sheaths of actin filament bundles (also called actin cables or stress fibers) that often run the length of the cell (1) as shown by immunofluorescence studies. In contrast, virally transformed cells lack this ordered type of actin structure (2-5); the diffuse fluorescence staining in these cells shows cytoplasmic actin to be disorganized. Experiments with temperature-sensitive mutants of tumor viruses demonstrated the disappearance of bundles of actin filaments or filamentous actin (F-actin), in virus-infected cells at the permissive but not at the restrictive temperature, which would indicate that a functional product of the transforming gene of the virus (e.g., the *src* gene of Rous sarcoma virus) is producing this effect. Furthermore, studies of these virus mutants in combination with inhibitors of protein synthesis suggest that the viral gene product does not act on the actin cytoskeleton through newly synthesized proteins (6).

Much progress in understanding the molecular actions of proteins encoded by transforming genes of tumor viruses has occurred over the past several years. In the case of the Rous

sarcoma virus, the product of the *src* gene (protein pp60^{src}) appears to be a protein kinase that catalyzes phosphorylation of tyrosine residues of many cellular proteins (7, 8). However, little molecular information exists at present concerning the linkage of this kinase activity to the disorganization of the actin cytoskeleton in the transformed cell.

This study is an attempt to use quantitative biochemical techniques to examine several actin-related parameters in normal and transformed chicken embryo fibroblasts (CEF) infected with a temperature-sensitive mutant of Rous sarcoma virus (NY68RSV). Our results suggest that the transformation-dependent changes in actin organization may relate to an altered activity of the cellular proteins that affect actin assembly in a manner similar to that of the cytochalasins, the fungal alkaloid metabolites that have potent activity on actin-related cytoskeletal and motile functions.

MATERIALS AND METHODS

Materials. Triton X-100, ATP (grade II), phenylmethylsulfonyl fluoride and 2-mercaptoethanol were obtained from Sigma. DNase and calf thymus DNA were supplied by Millipore (Bedford, MA). Unlabeled cytochalasin B (CB) and cytochalasin D (CD) were purchased from Aldrich. [³H]CB was obtained from New England Nuclear. The Sephacryl S-200 was a Pharmacia product.

Cell Culture. Fibroblasts were isolated from chicken embryo as described by Rubin (9) and grown in Ham's F10 medium (GIBCO or MA Bioproducts) supplemented with 10% trypsin phosphate broth (GIBCO or Scott Laboratories, Richmond, CA)/10% heat-inactivated calf serum (GIBCO or Colorado Serum, Denver, CO)/0.5% beef embryo extract (GIBCO)/1× penicillin/streptomycin/fungizone mixture (GIBCO or MA Bioproducts). All experiments were done on secondary or tertiary cell cultures.

Virus Stocks. Our initial stocks of wild-type (Schmidt-Ruppin B) and the temperature-sensitive mutant of Rous sarcoma virus (NY68, originally from the laboratory of H. Hanafusa, Rockefeller University) were generously provided by K. Yamada of the National Cancer Institute. We prepared our own stocks by the following procedure. One milliliter of virus was added to a 100-mm dish of freshly isolated CEF. The cells were then grown at 37°C and subcultured twice. Growth medium of confluent tertiary cultures was replaced with fresh growth medium containing 1% (vol/vol) dimethyl sulfoxide, and the cells were incubated at 37°C for 2 hr. The resulting virus-containing medium was collected, centrifuged to re-

Abbreviations: CEF, chicken embryo fibroblasts; G-actin, monomeric actin; F-actin, filamentous actin; CB, cytochalasin B; CD, cytochalasin D; PBS, Dulbecco's phosphate-buffered saline; NY68-RSV, temperature-sensitive mutant of Rous sarcoma virus.

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move cells, and frozen in 1.5-ml aliquots at -70°C . To obtain infected cells, we added 1.5 ml of virus stock to each 100-mm dish of freshly isolated CEF. The cells were grown at 37°C and split at a 1:3 ratio. Secondary and tertiary cell cultures grown at the nonpermissive temperature (41.5°C) were used only after growing for at least 24 hr.

Binding of [^3H]CB to Cultured Cells. Cells used for CB binding experiments were plated on 60-mm dishes and grown to confluence. After the plated cells had been rinsed three times with L15 medium (GIBCO), one ml of L15 containing [^3H]CB with or without $20\ \mu\text{M}$ CD was added to each plate. The cells were incubated at 37°C for 15 min, and the medium was removed for scintillation counting to determine the concentration of free [^3H]CB. The cell layer was dissolved in 1 M NaOH and then neutralized with acetic acid. Aliquots of this solution were used for determining protein content and bound [^3H]CB by scintillation counting. Specific binding is defined as binding that can be eliminated by the presence of $20\ \mu\text{M}$ CD in the assay medium.

Preparation of Cell Extracts. Extracts of CEF were prepared by modification of the method of McClain *et al.* (6). Confluent cells were rinsed twice with phosphate-buffered saline (PBS; pH 7.4) and gently scraped into fresh buffer. After centrifugation at 1000 rpm in an International Equipment HN-S centrifuge for 10 min, the supernatant fraction was aspirated, and the cell pellet was quickly rinsed with ice-cold buffer A (0.2 mM CaCl_2 /0.5 mM 2-mercaptoethanol/0.2 mM ATP/0.2 mM phenylmethylsulfonyl fluoride/5 mM Tris-HCl, pH 8.0 at 22°C). All subsequent procedures were done on ice or at 4°C . The cell pellet was resuspended in buffer A with a loose-fitting (B-type) Dounce homogenizer and then sonicated for 20 sec with a Branson Sonifier fitted with a microprobe at the lowest power setting and 50% duty cycle. The homogenate was centrifuged at 62,000 rpm for 35 min in a Beckman type 65 rotor. The supernatant fraction to which was added 100 units/ml of aprotinin was designated as the cell extract.

Actin Preparations. Actin was prepared from acetone powder of rabbit skeletal muscle by the method of Spudich and Watt (10) and further purified over a Sephacryl S-200 column. The peak fractions were pooled, and actin concentration was measured by absorption at 290 nm (E , $0.637\ \mu\text{l}/\mu\text{g}\cdot\text{cm}$). Stable actin "nuclei" in the form of spectrin-band 4.1-actin complex were extracted from erythrocyte membranes at low ionic strength and used without further purification (11).

DNase I Assay. The methods of Blikstad *et al.* (12) and Carlsson *et al.* (13) were used to measure monomeric actin (G-actin) by the inhibition of DNase. DNA was dissolved in 0.1 M Tris-HCl, pH 7.5/2.5 mM MnCl_2 . The substitution of MnCl_2 for CaCl_2 and MgCl_2 resulted in slightly higher DNase activity and gave a more linear response.

Other Procedures. Protein concentrations of extracts and cell lysates were determined with the method of Hartree (14). Binding of CB to erythrocyte spectrin-band 4.1-actin complex was measured with the isoelectric precipitation method (15). Actin filament assembly was monitored with an Ostwald-type viscometer (11). Low-shear viscosity of filamentous actin (F-actin) was determined with the falling-ball method described by MacLean-Fletcher and Pollard (16). Measurements were made at an angle of 60° , and glycerol standards were used to compute apparent viscosity of the samples.

RESULTS AND DISCUSSION

Binding of CB to Fibroblasts. CB, a fungal metabolite that affects many different forms of cytoskeletal and motile functions in nonmuscle cells (17), can bind with high affinity to the fast-growing end (i.e., the "barbed end" after deco-

ration with heavy meromyosin) of actin filaments (18, 19). Because the affinity of this compound is much higher for F-actin than for G-actin (19), we have devised a cytochalasin binding assay as a way to study the state of polymerization of F-actin in intact cells. For instance, when human platelets are activated by thrombin or other agents, an increase in actin filaments—i.e., F-actin—can be observed with electron microscopy (20), and a shift of actin in cell lysate from the monomeric to the polymeric state can be measured with the DNase assay for G-actin and a sedimentation assay for

FIG. 1. Binding of CB to CEF. Confluent monolayers of fibroblasts grown at 37°C (●) and NY68RSV-infected fibroblasts grown at 37°C (■) or 41.5°C (○) were assayed for CB binding. Specific binding, B, defined as the amount of [^3H]CB binding that is eliminated by excess unlabeled CD in the medium, was measured at various concentrations of free CB, F. (Top) B measured at lower ranges of F. (Middle) B measured at higher ranges of F. (Bottom) Scatchard plot analysis of the data of Top.

F-actin (21). These changes are accompanied by an increase in high-affinity cytochalasin binding sites in the stimulated platelets (22). Similarly, we found that when Chinese hamster ovary (CHO) cells in suspension spread on a substrate, the appearance of actin filament bundles is also accompanied by an increase in high-affinity cytochalasin binding sites in the cells (23).

In the present study, we compared the binding of [3 H]CB to normal and transformed fibroblasts. To distinguish cytochalasin binding sites associated with actin filament ends from those located on sugar transport proteins (24), we defined actin-related sites as those that bind both cytochalasin B and D with saturation kinetics (25). In all binding experiments, the [3 H]CB concentrations ($<0.8 \mu\text{M}$) were below the threshold that produces observable change in the cellular F-actin organization as revealed by staining with fluorescently labeled phalloidin (data not shown).

[3 H]CB binding that could be eliminated with excess CD was significantly higher for cells at the normal state (i.e., uninfected cells and NY68RSV-infected cells at the restrictive temperature of 41°C) than for cells at the transformed state (i.e., NY68RSV-infected cells at the permissive temperature of 37°C) (Fig. 1 *Top*). This difference diminished and finally disappeared when increasingly higher [3 H]CB concentrations were used (Fig. 1 *Middle*). Scatchard plot analysis (26) of the data obtained at the lower cytochalasin concentrations shows that the high-affinity portion of the binding curve of normal cells was not detectable in transformed cells (Fig. 1 *Bottom*). This difference is highly reproducible (see Table 1). These results appear opposite to those of Salter and Weber (27), who found an increased level of cytochalasin binding to transformed cells; those investigators, however, measured total [3 H]CB binding, which includes glucose transport-related sites as well as actin-related sites. When we measured the amount of [3 H]CB binding that is eliminated with excess amounts of unlabeled CB, rather than with CD, we also found increased binding to the transformed cells (data not shown).

Measurement of G-Actin Content in Cell Lysates. That actin filaments may undergo depolymerization when the cells go from the normal to the transformed state is one obvious interpretation for our CB binding results. This would decrease the number of filament ends that bind cytochalasin to a level below the sensitivity of the cytochalasin binding assay. We, therefore, measured the level of G-actin in lysates of the normal and transformed cells, using the DNase inhibition assay, and discovered no significant difference in G-actin levels between the normal and transformed cells (Table 2). In these experiments, though, there is an interval of ≈ 2 min from the time of cell lysis until the initiation of the DNase assay. As any differences in G-actin levels existing in the two cell types could have disappeared in that interval, we took DNase measurements at different time intervals after cell lysis. We did find a slow increase in G-actin levels over time, but this effect was reduced to a negligible level by using

Table 2. G-actin levels in lysates of chicken embryo fibroblasts

Cell type	Temperature, $^\circ\text{C}$	G-actin, % of total actin	
		Salt buffer	Sucrose buffer
Normal	37	48 ± 2	31 ± 2
NY68-infected	37	51 ± 3	
NY68-infected	41.5	47 ± 3	
Wild type			
RSV-infected	37		33 ± 3

Confluent monolayers of cells were scraped into prelysis buffer and placed into test tubes. Triton X-100 was immediately added, and the mixtures were agitated with a Vortex mixer. Aliquots were then assayed for inhibition of DNase activity. Values are expressed as the average $\% \pm \text{SEM}$ of total actin measured as G-actin in four different experiments. The salt buffer represents conditions according to Carlsson *et al.* (13), and the sucrose buffer represents conditions according to Blikstad and Carlsson (28).

buffer conditions that are designed to minimize filament depolymerization *in vitro* (28). Although changes in the absolute value of G-actin level occurred (depending on the experimental conditions), the *relative* values obtained for normal and transformed cells remained nearly the same in all of these experiments.

Studies on Cytochalasin-Like Activity in Cell Extracts. The absence of a detectable level of high-affinity cytochalasin binding sites in transformed cells without appreciable depolymerization of actin filaments may be explained by an increased activity of proteins that interfere with cytochalasin interaction with filament ends (29–31). These proteins can affect actin assembly by high-affinity binding to the barbed end of actin filaments and nuclei in a cytochalasin-like manner (29–32). We therefore compared the cytochalasin-like activity of extracts of normal and transformed cells as measured by several different assay methods.

In the first experimental approach, the effect of extracts on apparent viscosity of F-actin was measured with a low-shear falling-ball viscometer. Although the theoretical basis of this assay remains unclear, cytochalasins and proteins that bind the ends of F-actin lower its “low-shear” viscosity in a concentration-dependent manner (33). The extract of cells in the transformed state was more effective than extracts of cells in the untransformed state in lowering the viscosity of F-actin (Fig. 2). Growing uninfected cells or cells infected with wild-type Rous sarcoma virus at 41.5°C did not appreciably change the specific activity of the extracts, indicating that the difference seen in Fig. 2 was not simply a result of the shift in temperature used to culture the NY68RSV-infected cells.

To further substantiate cytochalasin-like activity, we examined the combined effects of the cell extracts and CB. The fibroblast extract/cytochalasin mixtures had a dose-dependent as well as additive effect upon the low-shear viscosity of F-actin (see Table 3).

A more specific assay for cytochalasin-like activity is the

Table 1. Summary of results obtained by Scatchard analysis of binding of CB to fibroblasts

Cell type	$K_d (\times 10^{-8} \text{ M})$		Binding sites, pmol/mg protein	
	High affinity	Low affinity	High affinity	Low affinity
Normal	1.8 ± 0.4 (6)	25 ± 7	5.0 ± 0.9	42 ± 11
NY68-infected (41.5°C)	2.0 ± 0.5 (3)	27 ± 7	5.0 ± 0.6	39 ± 7
NY68-infected (37°C)	ND (4)	20 ± 4 (4)	ND	36 ± 7

Binding data were obtained as described in Fig. 1. Results were normalized per mg protein. Corrections for interactions between high-affinity and low-affinity binding curves were not made. ND, not determined. Values are given as averages $\pm \text{SEM}$; numbers in parentheses indicate the number of experiments.

FIG. 2. Effect of fibroblast extract on the low-shear viscosity of F-actin. Extracts of fibroblasts grown at 37°C (●), or of NY68RSV-infected fibroblasts grown at 37°C (■) or 41.5°C (○) were added to G-actin in buffer A on ice. After KCl (to 0.1 M) and MgCl₂ (to 2 mM) had been added to initiate actin polymerization, the samples were immediately drawn into capillary tubes and then incubated at 30°C for 30 min. The apparent viscosity of the samples was measured with the falling ball method. cP, centipoise (1 P = 0.1 Pa-sec).

measurement of G-actin addition to the barbed end of growing actin filaments and nuclei; cytochalasin inhibits this addition. In this assay technique, a low-salt medium (0.4 mM MgCl₂) minimizes filament elongation at the pointed end, so cytochalasin-sensitive elongation of exogenously added actin nuclei at the barbed end can be monitored with an Ostwald viscometer (11). The extract of transformed cells inhibited actin filament assembly more than did the extracts of the untransformed cells (Fig. 3), results consistent with the conclusion from measurements with the falling ball viscometers—extracts of transformed cells have a higher cytochalasin-like activity per mg of protein.

Our laboratory group has previously found that proteins with cytochalasin-like activity against actin assembly interfere with the high-affinity binding of cytochalasin to actin nuclei in a dose-dependent manner (29, 31). The extract of transformed cells inhibited cytochalasin binding to actin nuclei more than the extracts of the cells at the nontrans-

Table 3. Combined effects of CB and fibroblast extract on the low-shear viscosity of actin

CB, μ M	Extract, μ g protein	Apparent viscosity, cP
0	0	250
	2.5	147
	5.0	116
	10.0	66
0.2	0	165
	2.5	103
	5.0	80
	10.0	50
0.4	0	130
	2.5	83
	5.0	68
	10.0	30
0.8	0	90
	2.5	65
	5.0	39
1.0	0	78

Assays were performed as in Fig. 2, except that the samples contained the indicated concentrations of CB in addition to various amounts of extract of NY68RSV-infected fibroblasts grown at 37°C. cP, centipoise.

FIG. 3. Effect of fibroblast extract on "nucleated" assembly of F-actin. All samples contained G-actin (0.3 mg) and stable actin nuclei in the form of erythrocyte spectrin-band 4.1-actin complex (25 μ g of protein) in 0.6 ml of buffer A. Some of the samples also contained extract (5 μ g of protein) of fibroblasts grown at 37°C (●), or NY68RSV-infected fibroblasts grown at 37°C (■) or 41.5°C (○); no extract was added to the control sample (▲). At time zero MgCl₂ was added (to 0.4 mM) to the samples, and assembly of F-actin was monitored viscometrically at 25°C. Under these conditions no significant assembly of the actin occurred without stable actin nuclei in the assay medium.

formed state (see Fig. 4). This result may explain the decrease in high-affinity cytochalasin binding sites in the transformed cells to a level below the sensitivity of the binding assay (Fig. 1 Bottom).

Conclusions. Extracts of transformed cells have a significantly higher level of cytochalasin-like activity than extracts of nontransformed cells. Although the molecules responsible for this activity are yet unidentified, we can speculate that they belong to a class of actin binding proteins known to interact with the barbed end of F-actin *in vitro* (for a recent review, see ref. 34). Of particular interest are gelsolin (35) and actin capping proteins (36, 37), which have been isolated from vertebrate cells and tissues. Whether one or more of these proteins participate in the observed increase in activity from

FIG. 4. Effect of fibroblast extract on binding of CB to erythrocyte spectrin-band 4.1-actin complex. Binding of [³H]CB to the complex (25 μ g of protein) was measured in the absence or presence of various amounts of fibroblast extract grown at 37°C (●), or of NY68RSV-infected fibroblasts grown at 37°C (■) or 41.5°C (○). Binding was expressed as a ratio of bound CB and concentration of free CB.

transformed-cell extracts awaits studies using specific antibodies to these proteins. The difference in activity between the extracts could reflect an increase in specific activity and/or the number of copies of actin-modulating proteins in the transformed cells. This conjecture is supported by the report of Fuchtbauer *et al.* (36) that microinjection of such a protein into normal cells produces transformation-like rearrangements of the actin cytoskeleton. Alternatively, the differences described in our study may result from increased extractability of these proteins from membranous or cytoskeletal structures.

These experiments on cell extracts, together with cytochalasin-binding studies on whole cells, show that transformation of chicken embryo fibroblast by Rous sarcoma virus is accompanied by significant changes in a number of actin-related parameters that can be quantitatively measured with biochemical methods. How these changes are related to the disorganization of the actin cytoskeleton and whether these methods can be developed into useful diagnostic assays for neoplastic transformation are questions that encourage further investigations.

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