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Assay for early cytoplasmic effects of the *src* gene product of Rous sarcoma virus

(microfilaments/microinjection/growth control/surface modulating assembly/actin immunofluorescence)

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ABSTRACT **When microinjected into normal fibroblasts, cytoplasmic extracts of cells transformed by Rous sarcoma virus caused dissolution of microfilament bundles. This activity was not found in extracts of normal cells. The maximum effect was seen within 30 min of injection, and the activity could still be measured after a 10-fold dilution of the cytoplasmic extracts (14 mg/ml original protein concentration). The activity was trypsin sensitive and was destroyed by boiling, but was not RNase sensitive. Protein synthesis was not required for the disruption of actin-containing stress fibers by the injected activity. Microinjected cytoplasts prepared from normal 3T3 cells also showed dissolution of microfilament bundles, indicating that the cell nucleus was not required for expression of activity. Extracts made from fibroblasts transformed by Rous sarcoma virus having a temperature-sensitive mutation in the *src* gene were also temperature sensitive in the microinjection assay. Thus, the activity of extracts from cells infected with *src* mutant virus, but not from cells infected with wild-type virus, was destroyed either by *in vitro* incubation of the extract at the nonpermissive temperature before injection or by incubation of recipient cells at the nonpermissive temperature after injection.**

We conclude that the microinjection assay can detect a cytoplasmic activity coded for by the *src* gene of Rous sarcoma virus and that an early direct or indirect target of the *src* gene product is the cytoskeleton and cell motility system. This result is discussed in relation to the hypothesis that submembranous arrays of microfilaments, microtubules, and their associated proteins interact with cell surface receptors to form a surface modulating assembly that functions as a key regulator of cell growth.

Rous sarcoma viruses (RSVs) can oncogenically transform both avian and mammalian cells. The various coordinated changes in growth control, cell shape, and metabolism that occur upon transformation can be ascribed to the effects of a single gene (*src*) in the viral genome (1). Knowledge of the site and mechanism of action of the protein product of the *src* gene would be particularly valuable for our understanding of growth control in both normal and transformed cells. The recent availability of antisera to the *src* gene product (2) should facilitate investigation of structural aspects of this problem. So far, however, there has been no assay system available to detect the presence in cell extracts of any of the activities of the *src* gene product. In this paper, we describe such an assay, which has been designed to detect early cytoplasmic effects of the product in normal cells.

We have hypothesized that the *src* gene product alters interactions of cytoskeletal and cell motility proteins, either directly or indirectly (3, 4). In addition, we have suggested that submembranous arrays of cytoskeletal elements such as microtubules, microfilaments, and their associated proteins interact with membrane proteins to form a surface modulating assembly or SMA (5, 6). Components of this assembly appear to control the mobility of cell surface receptors (6, 7) as well as regulate signals from the cell surface that are concerned with the stimulation and inhibition of growth (6, 8, 9). Comparisons of the morphology of microfilaments and microtubules in normal and RSV-transformed cells demonstrated that these SMA components and the behavior of surface proteins were altered in transformed cells (3). Additional perturbation experiments (4) on cells infected with temperature-sensitive mutants of RSV suggested that the *src* gene product acted in the cytoplasm to affect components of the SMA.

These observations raised the possibility that the *src* gene product might induce similar changes in normal uninfected cells which could then serve to assay for its presence. The assay described here consists of the microinjection of cellular extracts from transformed cells into normal cells, followed by examination of actin-containing structures in these cells by immunofluorescence microscopy. We have found that the injection of extracts of RSV-transformed fibroblasts into normal 3T3 fibroblasts and enucleated 3T3 cytoplasts cause the dissolution of normal actin stress fibers in the recipient cells. Of particular significance for the specificity of this assay was the finding that the activity of extracts obtained from cells transformed with viruses having temperature-sensitive mutations in the *src* gene was also temperature-sensitive *in vitro*.

MATERIALS AND METHODS

Mouse 3T3 fibroblasts (American Type Culture Collection, Bethesda, MD) were grown on glass coverslips (18 × 18 mm) in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and gentamicin at 20 μg/ml. Chick embryo fibroblasts (CEF) were used as secondary cultures in Scherer's medium containing 10% tryptose phosphate broth, 5% calf serum, and gentamicin at 20 μg/ml. Freshly plated secondary chick fibroblasts were infected with the Prague strain of RSV subgroup A (Pr-A) or with a Pr-A strain having a temperature-sensitive mutation in the *src* gene (tsLA24-A, kindly provided by John Wyke, Imperial Cancer Research Fund Laboratories, London, England).

For injection of cytoplasts, 3T3 fibroblasts were enucleated by using cytochalasin B (Aldrich, Metuchen, NJ). Cells on plastic coverslips were incubated 30 min at 37° in medium with cytochalasin B at 10 μg/ml. The coverslips were inverted and fit into a centrifuge tube and centrifuged at 15,000 × *g* for 30 min. The cells were then washed four times at 30-min intervals to allow recovery from the drug before being injected.

Extracts from transformed CEF or from normal uninfected

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Abbreviations: RSV, Rous sarcoma virus; SMA, surface modulating assembly; CEF, chick embryo fibroblasts; P_i/NaCl, phosphate-buffered saline.

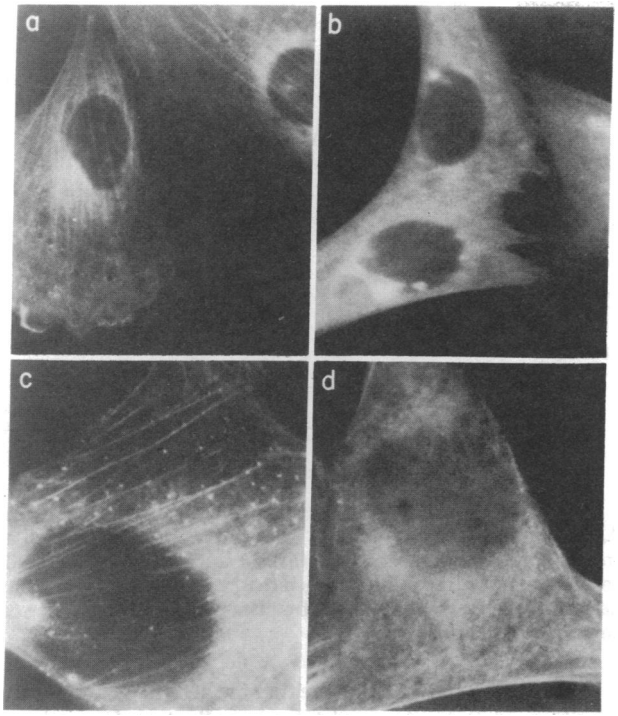

FIG. 1. 3T3 fibroblasts fluorescently stained with antibodies to actin 50 min after being microinjected with extracts of CEF. Cells in *a* and *c* were injected with an extract of normal cells; cells in *b* and *d* received extracts of Pr-A-transformed cells. The cells in *a* and *c* show normal stress fiber morphology; the cells in *b* and *d* show diffuse staining with anti-actin antibody. (*a* and *b*, ×650; *c* and *d*, ×1400.)

CEF were made after the cultures became confluent (3–5 days). Cells were harvested with a rubber policeman and washed twice in ice-cold phosphate-buffered saline (P_i/NaCl). A protease inhibitor, Trasylol (FBA Pharmaceuticals, New York), was added to the cell pellet after the second wash. The cell pellet was then homogenized in a tight-fitting Dounce homogenizer (20 strokes at 4°) and sonicated for 15 sec in a 24° water bath. The disrupted cells were spun at 100,000 × *g* for one hour at 4°, and the supernatant was retained for microinjection. All extracts and solutions were filtered through a 0.22-μm Millipore filter (Bedford, MA) before injection. Protein determinations were made using the Bio-Rad protein assay (Richmond, CA).

For trypsin treatment, an extract of Pr-A-CEF was prepared in the absence of Trasylol. A 200-μl sample of the extract was incubated with 0.28 μg of trypsin (40 units/mg, three times recrystallized; Worthington Biochemicals, Freehold, NJ) for 30 min at room temperature, then 7 μl of Trasylol was added to neutralize the trypsin. As a control, trypsin and Trasylol were added simultaneously to an extract and left at room temperature 30 min. This latter treatment had no effect on activity.

Ribonuclease A on polyacrylamide beads (Sigma, St. Louis, MO) was washed three times in 0.14 M KCl, and 0.5 mg was incubated with 200 μl of Pr-A–CEF extract for 30 min at room temperature. Under these conditions, none of the RNase was released from the beads, and a control sample of yeast RNA was completely digested.

The microinjection procedure was modified from that described by Diacumakos (10). Microinjection needles were made on a DeFonbrune microforge, loaded from the rear with extract, and connected to a micrometer-controlled syringe filled with silicone oil. The needle was mounted on a Leitz micromanipulator. Sparsely populated fibroblasts within a marked area on an inverted coverslip were injected while being visualized in a fixed-stage Zeiss microscope with ×40 phase optics. During the injection procedure, the cells were in Hanks' balanced salt solution containing 10% fetal calf serum and 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes).

Table 1. Effects on the morphology of stress fibers after microinjection of cell extracts into normal cells

Injected material	% diffusely stained cells*
None	10 (280)
Extract of Pr-A-transformed CEF	61 (322)
Extract of normal CEF	16 (37)
KCl, 0.14 M	7 (75)

* Numbers in parentheses refer to total number of cells injected.

Antisera directed against chicken gizzard actin were prepared according to the method of Groeschel-Stewart *et al.* (11). Fibroblasts to be stained were fixed in 3.7% formaldehyde in P_i/NaCl for 20 min at room temperature. The coverslips were then rinsed in P_i/NaCl, incubated with 0.1 M glycine in P_i/NaCl for 5 min to quench the formaldehyde, and rinsed again with P_i/NaCl. To allow the antibody access to intracellular proteins, the cells were made permeable by treating with 0.2% Triton X-100 in P_i/NaCl for 3 min. After washing with P_i/NaCl (three times, 5 min each), the cells were reacted for 20 min at room temperature with the Ig fraction from the antiserum (2.5 mg/ml), washed with P_i/NaCl, and then incubated with fluorescein-conjugated goat anti-rabbit Ig for 20 min. After washing in P_i/NaCl, the coverslips were inverted onto glass slides containing a drop of glycerol and the morphology of cellular microfilaments was assessed by immunofluorescence microscopy (3).

RESULTS

Because cytoskeletal networks are altered in cells as a result of oncogenic transformation (1, 3), it was of particular interest to see whether a cytoplasmic extract of transformed cells could induce such alterations when microinjected into the cytoplasm of normal cells. We used the immunofluorescent staining pattern with anti-actin antibodies in mouse 3T3 fibroblasts as an indicator of possible alterations. The normal staining pattern with anti-actin, generally consisting of parallel arrays of stress fibers comprised of bundles of filamentous actin, is shown in Fig. 1 *a* and *c*. In Fig. 1 *b* and *d* are shown 3T3 cells that had been microinjected with a cytoplasmic extract of CEF infected with wild-type Pr-A RSV. The staining is generally diffuse, although a few residual fibers were occasionally noted. Approximately 10% of the injected and noninjected cells exhibited a staining pattern intermediate between diffuse and nondiffuse. It is not known whether this pattern resulted from cell heterogeneity, cell cycle variation, or inconsistencies in the microinjection technique.

The quantitation of the stress fiber dissolution in the cell population is presented in Table 1. About 60% of 3T3 cells injected with extracts of RSV-transformed CEF showed the diffuse staining pattern 50 min after being microinjected. An extract made from rat kidney cells transformed by the Schmidt-Ruppin D strain of RSV also exhibited this activity. Extracts of normal CEF or solutions of 0.14 M KCl injected into 3T3 cells did not have this activity: the frequency of cells with diffuse actin staining patterns after these injections was close to the frequency of diffusely stained cells in uninjected populations. The effect of extracts from transformed cells on microfilament

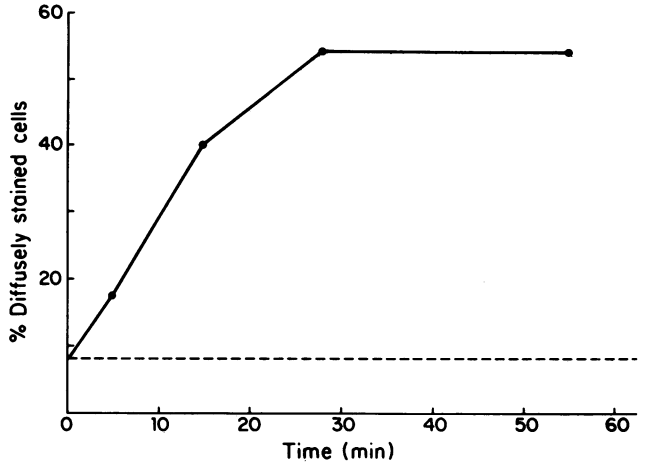

FIG. 2. Appearance of diffusely staining cells as a function of time after microinjection of a Pr-A–CEF extract. Cells on coverslips were microinjected at room temperature and returned to 37° at time 0. At various times (abscissa), the coverslips were fixed and processed for immunofluorescence microscopy with antibodies to actin. The horizontal broken line represents the frequency of diffusely staining cells in uninjected populations.

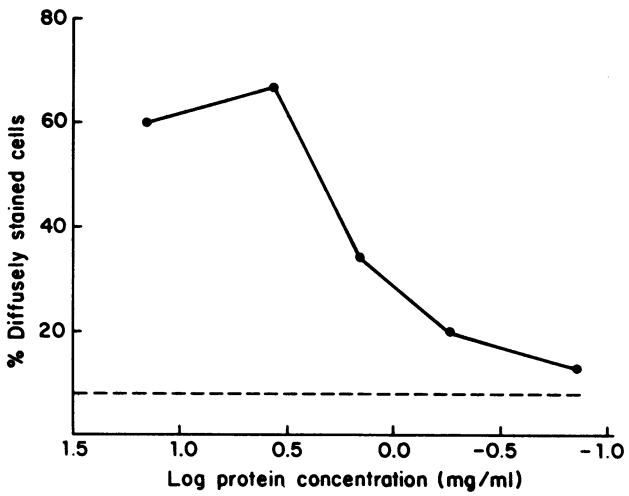

FIG. 3. Effect of dilution on the activity of Pr-A–CEF extract. A Pr-A–CEF extract (14 mg/ml) was diluted with 0.14 M KCl and injected into 3T3 cells. All cells were fixed and stained with antibodies to actin 50 min after being returned to 37° after injection. The horizontal broken line represents the frequency of diffusely staining cells in uninjected populations.

morphology was also seen when CEF were used as recipient cells. The 3T3 cells were easier to maintain and to inject, however, and therefore they were used routinely as recipient cells in most of the experiments.

Analysis of the data by the χ^2 test showed that the percent of cells diffusely stained after injection of transformed cell extracts is significantly different ($P = 0.005$) from that obtained after injection of normal cell extracts. In this analysis, cells that were intermediate in their staining pattern were assigned to the category of staining pattern that was the more conservative for estimates of significance. In other words, cells injected with transformed cell extracts that exhibited an intermediate staining pattern were assigned to the nondiffusely stained category; cells injected with normal cell extracts that exhibited an intermediate pattern were assigned to the diffusely stained category. Such an assignment would tend to minimize the differences observed before and after injection; even with this convention, significant differences were readily apparent.

The effect of the extract from transformed cells on normal actin morphology was rapid, and a plateau in the frequency of diffusely staining cells was reached within 30 min after the microinjection (Fig. 2). In view of these results, 50-min incubations were routinely used in further experiments. In Fig. 3 is shown the effect on the activity of diluting the extract. Extracts were still active at protein concentrations of 1.4 mg/ml. Accordingly, most of the experiments were done using extracts diluted to approximately 3 mg/ml in 0.14 M KCl, the lowest concentration of extract that exhibited maximal activity.

These data indicate that extracts of RSV-transformed cells differ from those of normal cells in having an activity that disrupts actin-containing structures. Although this activity is an eventual result of the expression of the *src* gene product of RSV, it could be due either to direct action of microinjected *src* gene product or to action by some substance (either a small molecule or a macromolecule) induced secondarily by the *src* gene product. To decide among these possibilities, we made use of a mutant of RSV, tsLA24, derived from the Pr-A strain of virus, that expressed a temperature-sensitive *src* gene product. Cells infected with these viruses are transformed at the permissive temperature, 37°, but are phenotypically normal at the restrictive temperature, 41°, despite the fact that viral replication can proceed normally at that temperature.

In Table 2 is shown evidence that the activity is temperature sensitive in extracts made from cells transformed with tsLA24-A. Temperature sensitivity was not observed with active extracts from wild-type RSV-transformed cells. Moreover, the temperature sensitivity was seen both when the recipient cells were incubated at the restrictive temperature and when the extract itself was heated *in vitro* and the recipient cells were incubated at the permissive temperature (37°). These results suggest strongly that the observed changes in actin morphology resulted from the direct or indirect action of the *src* gene product on actin stress fibers.

The activity was trypsin sensitive but not RNase sensitive (Table 3), confirming that the microinjection of actin-disrupting activity was due to the presence of a protein rather than to a small molecule in the extract or a protein synthesized from injected mRNA. Furthermore, the activity was still observed when the recipient cells were previously incubated with puromycin or cycloheximide, suggesting that ongoing protein synthesis is not required for dissolution of the actin cables.

Several additional lines of evidence suggested that the assayed activity was independent of the cell nucleus. With whole cells

Table 2. Temperature sensitivity of the activity of transformed cell extracts

Extract	Incubation temperature of recipient cells, °C	% diffusely stained cells*
Pr-A-transformed CEF		
Extracted in cold	37	61 (322)
	41	71 (255)
Extracted in cold and then treated 45 min at 41°	37	60 (20)
tsLA24A-transformed CEF		
Extracted in cold	37	63 (171)
	41	26 (101)
Extracted in cold and then treated 45 min at 41°	37	26 (42)

* Numbers in parentheses refer to total number of cells injected.

Table 3. Alteration of activity by various treatments of Pr-A-transformed CEF extracts and of recipient 3T3 cells

Modification of assay	% diffusely stained cells*
Treatment of cell extract	
None	58 (52)
Trypsin	7 (87)
Trypsin + Trasylol (control)	54 (76)
RNase, insolubilized on polyacrylamide beads	60 (20)
100°, 5 min	48 (50)
100°, 10 min	20 (25)
100°, 20 min	20 (55)
Treatment of recipient cells	
None	54 (26)
Puromycin, 0.1 mM	64 (59)
Cycloheximide, 0.1 mM	58 (70)

* Numbers in parentheses refer to total number of cells injected.

as recipients, extracts were injected into the cytoplasmic region adjacent to the nucleus. Microinjection of anti-actin Ig followed by a 50-min incubation at 37°, fixation of the cell, and staining with fluorescent anti-Ig showed that the injected material had completely permeated the cytoplasm. The nucleus was not stained, however, suggesting that large macromolecules do not enter the nucleus with this procedure over the time we observed them.

As direct evidence that the *src* gene product does not act in this assay at the level of gene expression, we observed the effects of injected Pr-A–CEF extracts on enucleated cells. The data in Table 4 show that enucleated 3T3 fibroblasts responded to injection of extracts containing *src* gene product with dissolution of stress fibers. The frequency of diffusely staining enucleated cells was approximately the same as for whole cells: cytoplasts injected with Pr-A–CEF extracts were 79% diffusely stained and uninjected cells were 20% diffusely stained.

DISCUSSION

This paper describes an assay for the *src* gene product based on its ability to cause the dissolution of bundles of microfilaments in normal cells within half an hour after microinjection. The activity was present in extracts from three different types of RSV-transformed cells but not in extracts of normal CEF. In view of its sensitivity to proteolysis and boiling, we suggest that the activity is due to the presence of a protein in the extract. The dissolution of actin was not due to injected RNA that was translated into protein inasmuch as the activity was not sensitive to prior RNase treatment of the extract. Furthermore, the activity was expressed in cells whose protein synthesis had been inhibited by puromycin or cycloheximide.

Most important, however, was the finding that the activity of extracts made from cells transformed with viruses having temperature-sensitive mutations in the *src* gene was temperature sensitive *in vitro* (Table 2). Activity in extracts of cells infected with tsLA24 RSV was lost when the extract was heated at the nonpermissive temperature (41°), whereas an extract from wild-type Pr-A-infected cells was not affected by such a treatment. Temperature sensitivity of activity from tsLA24 RSV-infected cells was also observed when the tsLA24 extract was injected into recipient cells incubated at 41°.

Table 4. Effect of injection of Pr-A–CEF extract on actin structures in enucleated 3T3 fibroblasts

Cells	Injected	% diffusely stained
Enucleated 3T3 fibroblasts	+	79
	−	20
Normal 3T3 fibroblasts	+	55
	−	11

Under optimal conditions, only 75% of the injected cells exhibited diffuse staining with anti-actin antibody, the average being about 60%. The nature of the unresponsive cells is unclear. Some of these cells were probably not injected with enough of the *src*-containing extract, because the volume of injection is difficult to control. Moreover, it is known that population heterogeneity exists in the morphology of cytoskeletal components even in completely transformed populations (3, 4). Whether this heterogeneity reflects a stable characteristic of a few cells or a cell-cycle-dependent characteristic of all cells is not known. The proportion (approximately 10%) of normal cells that exhibited diffuse staining might well be accounted for by cells in the G_2 and M phases of the cell cycle.

These observations on the effects of the *src* gene product on microfilament morphology do not discriminate whether the protein acts directly or indirectly to exert its effect on the surface modulating assembly (SMA). For example, the *src* gene product may bind directly to components of the SMA or it may compete with the binding of another normal SMA component. Alternatively, the *src* gene product could be an enzyme, producing an effector that alters the SMA. If this were the case, the product produced by the enzyme would have to be short lived; otherwise, injected extracts of transformed cells that contained this product would have induced the cytoskeletal alterations independently of the activity of the *src* gene product. The *src* gene could also code for an enzyme that covalently modifies components of the SMA, for example by phosphorylation, a possible regulatory mechanism for some contractile proteins (12).

In any event, it is now clear that the *src* gene product activity is ascribable to a protein that can act in the cytoplasm, and having as an early target components of the SMA. The *src* gene product can act upon these components independently of protein synthesis and of alterations in normal gene expression. More directly, it was possible to show that enucleated cells responded to the *src* gene product, confirming earlier observations (13) and our earlier more indirect experiments (3, 4). The present experiments still do not indicate, however, whether the alterations in the SMA are the causes of the alteration seen in growth control of transformed cells. Obviously, the *src* gene product could have independent effects on several parallel nuclear and cytoplasmic systems, including the SMA. Alternatively, the observed changes in microfilament organization might be the only direct effects of the *src* gene product and at least some of the other cellular changes of transformation might result from these primary changes in the SMA. This would be consistent with the hypothesis that the SMA is a regulator of cell growth and with earlier observations (4) concerning the effects of alterations in the SMA on the expression of *src*-induced transformation. The combined use of antibodies to *src* gene products (2) and of various microinjection assays designed to reveal effects on SMA structures and on cell growth should help decide among the various possibilities.

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