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Cell Biology

Microinjection of fluorescently labeled α-actinin into living fibroblasts

(cytoskeleton/α-actinin)

JAMES R. FERAMISCO

Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724

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ABSTRACT **α-Actinin from chicken gizzard labeled with tetramethylrhodamine isothiocyanate has been incorporated into living fibroblast cells by microinjection. Fluorescent labeling of α-actinin was carried out such that the conjugated protein was functional *in vitro* as shown by its ability to bind to F-actin. Within 1–2 hr after injection, diffuse fluorescence was observed throughout the cytoplasm and only faint fluorescence was apparently associated with the stress fibers. During the ensuing 2–15 hr, however, most of the fluorescence was seen as periodicities along the stress fibers and as foci of the microfilament polygonal networks. This distribution of α-actinin in the living cells was strikingly similar to that found by indirect immunofluorescence localization of endogenous α-actinin in fixed samples of the same cell type. Control studies in which heat-treated (100°C, 2 min) fluorescent α-actinin or tetramethylrhodamine isothiocyanate alone was injected into the cells indicated that the stress fiber and polygonal network labeling was specific for "native" fluorescently labeled α-actinin. These results suggest that the dynamic properties of proteins and structures in cultured mammalian cells can be studied with the use of microinjection and fluorescence microscopic techniques.**

During the past several years, much information has been obtained in the identification and localization of contractile and cytoskeletal proteins in nonmuscle cells (for review, see ref 1). For example, by electron microscopy and immunofluorescence microscopy, the presence of actin filaments (e.g., refs 2 and 3), myosin (4, 5), tropomyosin (6), α-actinin (7), and filamin (8) in the stress fibers of nonmuscle cells has been demonstrated. Though it is not yet proven that the stress fibers of nonmuscle cells function as contractile organelles, the presence of contractile proteins in the stress fibers, the alternating "sarcomeric" arrangement of myosin and α-actinin along the fibers (9), and the observation that stress fibers contract in response to ionic conditions that induce muscle contraction (10) suggest that the stress fibers may function as contractile structures and contract in a manner analogous to muscle sarcomeres (i.e., via a sliding-filament mechanism). However, in order to observe the dynamic process of cell movement at the molecular level, techniques other than microscopic inspection of fixed cells must be utilized.

A novel approach to the problem of studying a dynamic intracellular process has recently been described in which fluorescently labeled actin was microinjected into the cytoplasm of the protozoans *Physarum polycephalum* and *Chaos carolinensis* (11). In this manner the distribution of fluorescent actin in the living organisms was obtained. Because of the relatively large size and thickness of these protozoans, though, it is difficult to examine the cells with fluorescence microscopy to determine the location of the fluorescent proteins injected or the location of the endogenous proteins by immunofluorescence. Mammalian cells such as fibroblasts are of the size that makes them especially suitable for fluorescence microscopy, but at the same time are of a sufficiently small size that microinjection of them is not as easily done as it is with the larger protozoans. However, with the development of the elegant techniques for the microinjection of mammalian cells by Graessmann and Graessmann (12), the problems of injecting small cells have been solved. In the present paper I describe the incorporation of fluorescently labeled α-actinin, a protein component of the Z-lines of muscle (13), into the functional pools of α-actinin in living fibroblasts after microinjection of the fluorescently labeled protein. Analysis of the injected cells by fluorescence microscopy shows the incorporation of the fluorescent protein into periodicities along the stress fibers as well as into the foci of the polygonal microfilament networks, types of structures that by immunofluorescent methods have been shown to contain α-actinin (7, 9, 14, 15).

MATERIALS AND METHODS

Cell Culture. Gerbil fibroma cells (CCL 146) originally obtained from the American Type Culture Collection were generously provided by W. E. Gordon III and S. H. Blose of this laboratory. The cells were grown in Dulbecco's modification of Eagle's medium containing 9% fetal calf serum, penicillin at 100 units/ml, and streptomycin at 100 μg/ml. For microinjection, cells were trypsinized (0.05% trypsin in 0.5 mM EDTA/phosphate-buffered saline) and reseeded onto glass coverslips (22 mm^2).

Purification of α-Actinin and Actin. α-Actinin was purified to homogeneity from chicken gizzard by a rapid procedure (unpublished results). Briefly, gizzard was homogenized and extracted for 30 min. The resultant extract was fractionated by Mg^{2+}-induced and $(NH_4)_2SO_4$-induced precipitations. α-Actinin was obtained by column chromatography on DEAE-cellulose and Sepharose 6B-CL resins. Actin was purified from acetone powders of rabbit skeletal muscle also by a rapid procedure (unpublished results). Basically, this procedure combines the most efficient steps from the procedures developed for actin purification by Seraydarian *et al.* (16) and Spudich and Watt (17) and is outlined below. Actin was extracted from the acetone powder as G-actin with dilute ATP/ascorbate buffer (rapid depolymerization) (16), treated with 3.3 M KCl (16), cycled one time from F-actin through G-actin to F-actin with the use of dilute ATP/ascorbate and 100 mM KCl, and finally treated, as F-actin, with 0.6 M KCl (17). This procedure takes only ≈30 hr and gives rise to homogeneous actin in high yields.

Fluorescent Labeling of α-Actinin. A solution of α-actinin (3 mg/ml) in 50 mM NaH_2PO_4 adjusted to pH 9 with $NaHCO_3$ was mixed with tetramethylrhodamine isothiocyanate (TMRITC) (Cappel Laboratories, Cochranville, PA) (15 μg of

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Abbreviation: TMRITC, tetramethylrhodamine isothiocyanate.

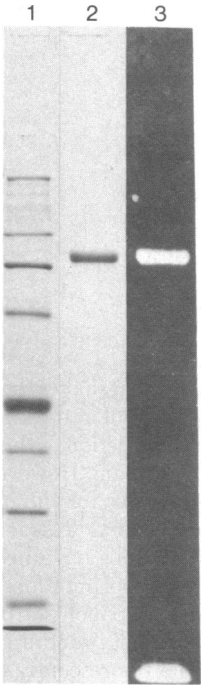

FIG. 1. TMRITC-labeled α-actinin. Approximately 15 μg of fluorescently labeled α-actinin was subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (lanes 2 and 3) as described by Blattler *et al.* (20) except that the buffer system of Laemmli (21) was used. Lane 2 shows the Coomassie brilliant blue staining pattern and lane 3 shows the fluorescence pattern (before Coomassie brilliant blue staining) obtained by ultraviolet light illumination of the gel. Molecular mass standards (in daltons) are shown in lane 1 and are (from top to bottom) myosin (200,000), β-galactosidase (130,000), phosphorylase b (94,000), bovine serum albumin (68,000), ovalbumin (45,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (21,000), and lysozyme (14,300).

dye per mg of protein) and kept at 0°C for 1 hr and at 4°C for 4 hr (18). The conjugated protein was dialyzed exhaustively against 20 mM Tris·acetate, pH 7.5/20 mM NaCl/15 mM 2-mercaptoethanol to remove excess reagent.

Microinjection. Fluorescently labeled molecules were injected into cells with the aid of a glass capillary needle drawn out to a tip of 0.5–1.0 μm in diameter (12) by a puller provided by A. Graessmann. Capillaries were treated with ethanol (100%) but not with HF or dimethylchlorosilane. A Leitz micromanipulator equipped with a vacuum and pressure device and an inverted phase-contrast microscope were employed for micromanipulation of the capillary and visualization of the microinjection process.

Indirect Immunofluorescence. Antibodies against bovine cardiac muscle α-actinin were a gift of K. Burridge of this laboratory and have been shown to be monospecific when tested against total protein from the cell line used in this study (K. Burridge, personal communication as shown by the technique of indirect immunoautoradiography (19). Cells were fixed with 3% formaldehyde in phosphate-buffered saline, made permeable with Triton X-100, and stained with rabbit anti-α-actinin antibody and subsequently with rhodamine-labeled goat anti-rabbit IgG as described by Lazarides (6).

Optical Methods. Cells were observed with a Zeiss Photomicroscope III equipped with a ×63 oil immersion lens on an epifluorescence nose piece. TMRITC was analyzed with the Zeiss narrow band pass interference filter G546 and the barrier filter LP590. Micrographs of cells were taken with either Ektachrome-400 or Tri-X film.

RESULTS

Tetramethylrhodamine Isothiocyanate-Labeled α-Actinin. Fluorescently labeled α-actinin migrated as a single band as shown by polyacrylamide slab gel electrophoresis in the presence of sodium dodecyl sulfate when stained with Coomassie brilliant blue (Fig. 1). Visualization of the slab gel by fluorescence methods (before Coomassie brilliant blue staining) showed a band coincident with the Coomassie brilliant blue staining band and a diffuse band running ahead of the dye front, which was most likely due to the presence of TMRITC

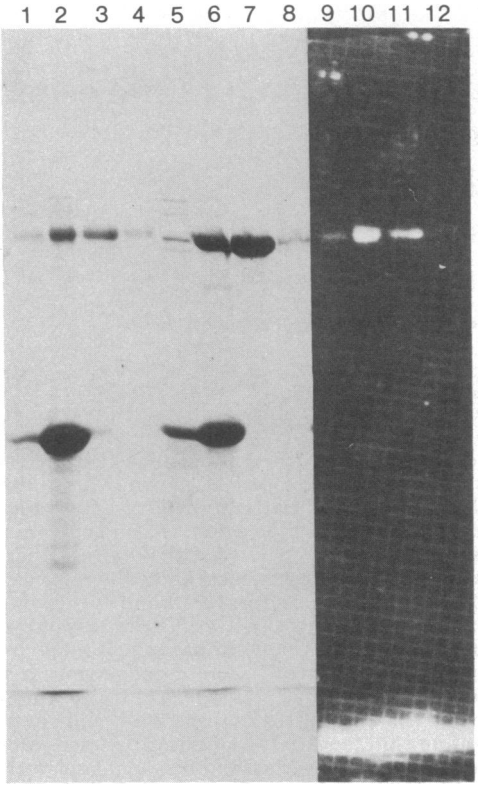

FIG. 2. Binding of TMRITC-label α-actinin to F-actin. Approximately 30 μg of TMRITC-α-actinin or 50 μg of control α-actinin was mixed with or without 75 μg of F-actin in the presence of 100 mM KCl for 1 hr at 4°C. The solutions were then centrifuged at 78,000 × *g* for 2 hr at 4°C. Aliquots of the supernatant fractions and the pellets representing equal volumes of each were subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Both Coomassie brilliant blue staining (lanes 1–8) and fluorescence (lanes 9–12) methods were used to visualize the proteins. The supernatants and pellets derived from the following solutions are shown: TMRITC-α-actinin with F-actin, lanes 1 and 2, respectively, and lanes 9 and 10, respectively; TMRITC-α-actinin without F-actin, lanes 3 and 4, respectively, and lanes 11 and 12, respectively; control α-actinin with F-actin, lanes 5 and 6, respectively; and control α-actinin without F-actin, lanes 7 and 8, respectively.

tightly but not covalently bound to the protein (i.e., nondialyzable). Experiments to determine the ability of the chemically modified α-actinin to bind to F-actin, a property of α-actinin, in comparison to control α-actinin were also carried out by determining whether or or not α-actinin would cosediment with F-actin under conditions in which α-actinin alone would not sediment (Fig. 2). Cosedimentation with F-actin reflected the binding of α-actinin to F-actin. Fluorescently labeled α-actinin possessed the property of actin-binding as shown in Fig. 2. The specificity of the interaction of TMRITC-α-actinin and F-actin was analyzed by performing the above studies in the presence of tropomyosin, a protein that inhibits the binding of α-actinin to F-actin at 37°C (22). The addition of tropomyosin decreased to a similar extent the amount of control and fluorescently labeled α-actinin that bound to F-actin (not shown). Thus, by these two criteria, fluorescently labeled α-actinin was functional *in vitro*.

Microinjection of Fibroblasts with TMRITC-α-Actinin. Shortly after microinjection of TMRITC-α-actinin into living fibroblasts, diffuse fluorescence was observed throughout the cytoplasm, but only faint fluorescence was observed apparently associated with the stress fibers (not shown). By 4 hr after injection, though, discrete patterns of fluorescence could be seen

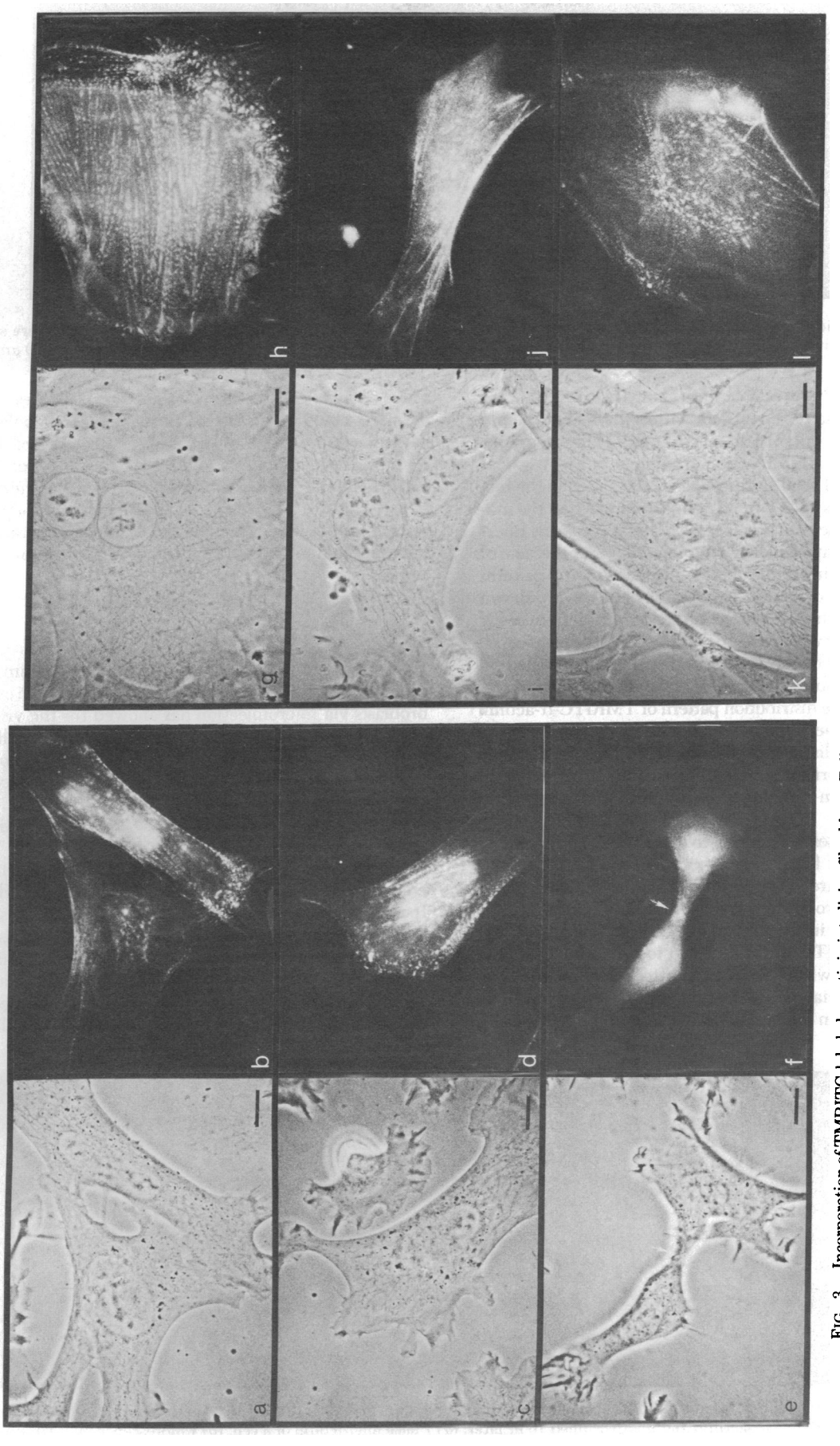

FIG. 3. Incorporation of TMRITC-labeled α-actinin into living fibroblasts. Fully spread gerbil fibroma cells were microinjected with TMRITC-α-actinin and photographed 4 hr later (*a–f*) or 16 hr later (*g–l*). Phase micrographs of the cells are shown in *a*, *c*, *e*, *g*, *i*, and *k*, and fluorescence micrographs of the same cells are shown in *b*, *d*, *f*, *h*, *j*, and *l*.

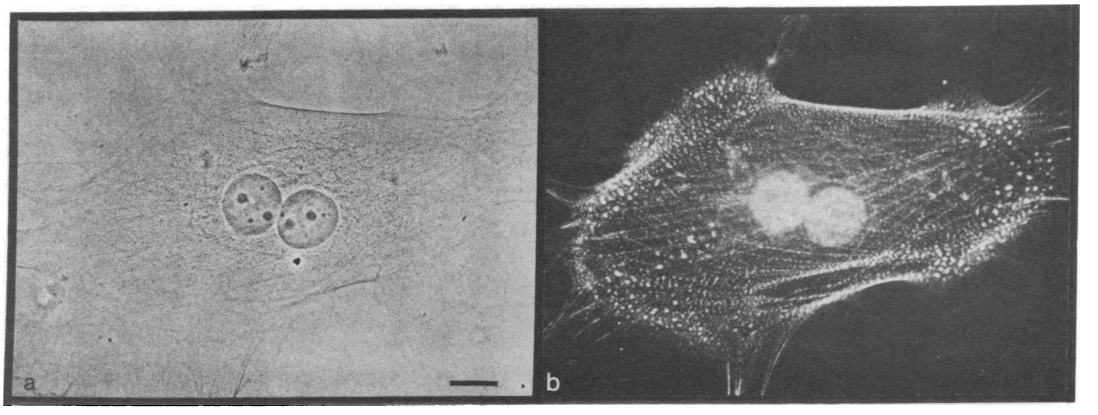

FIG. 4. Localization of α-actinin within fibroblasts by indirect immunofluorescence. Fully spread gerbil fibroma cells were stained for α-actinin by indirect immunofluorescence. (*a*) Phase micrograph of a cell. (*b*) Fluorescence micrograph of the same cell. Bar, 10 μm.

as a periodic distribution of α-actinin along the stress fibers as well as a distribution of the protein within the foci of the polygonal networks of microfilaments (Fig. 3 *a–f*). Micrographs of cells taken 16 hr after injection are shown in Fig. 3 *g–l*. It appears that most of the fluorescence was localized in the foci of the cells photographed 4 hr after injection whereas the majority of the fluorescence was localized along the stress fibers of the cells photographed 16 hr after injection. For the sake of comparison, the indirect immunofluorescence staining patterns for the endogenous α-actinin in the same cell type are shown in Fig. 4, which demonstrates the presence of α-actinin along the stress fibers and within the foci of the polygonal networks. Note the similarity of the α-actinin distribution revealed by indirect immunofluorescence. Also, as shown in Fig. 3*f*, it was possible to obtain the distribution pattern of TMRITC-α-actinin in mitotic cells. The diffuse distribution of α-actinin in the cytoplasm and the discrete localization of it within the cleavage furrow (Fig. 3*f*, arrow) are similar to the distributions of α-actinin found in mitotic cells by immunofluorescence methods (23).

Control experiments designed to determine the specificity of incorporation of fluorescence into the cells were also performed. In this regard, TMRITC-α-actinin preheated to 100°C for 2 min was microinjected into fibroblasts. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate showed that TMRITC was still bound to α-actinin after heat treatment (not shown). Within 16 hr after injection, the fluorescence was associated with organelles that appeared to be lysozomes located in the perinuclear region of the cell (Fig. 5). These data indicate that the incorporation of fluorescence along the stress fibers and in the foci of the polygonal microfilament networks depends upon the "native" structure of TMRITC-α-actinin and provides a control for the presence of noncovalently bound TMRITC in the TMRITC-α-actinin preparation. When free TMRITC, at approximately the same fluorescence intensity as the TMRITC-α-actinin preparation, was microinjected into cells, cell death occurred within 4–6 hr (not shown).

DISCUSSION

The introduction of fluorescently labeled α-actinin, a protein found in the contractile apparatus of muscle, into cultured fibroblasts via microinjection has allowed for the visualization of the protein in living cells. Because the localization of TMRITC-α-actinin in the living cells was similar to the localization of the protein by indirect immunofluorescence methods (Figs. 3 and 4), it is likely that the injected TMRITC-α-actinin was incorporated into the functional pools of α-actinin in the cells. Thus, within 4 hr after microinjection of TMRITC-α-actinin into the fibroblasts, fluorescent periodicities apparently along the stress fibers were visible, as well as fluorescent foci in the microfilament polygonal networks. These findings demonstrate a technique that allows for the direct visualization of the dynamics of proteins within living mammalian cells which complements techniques that require fixation of cells such as immunofluorescence and electron microscopy. That the procedures described herein involved mammalian cells is

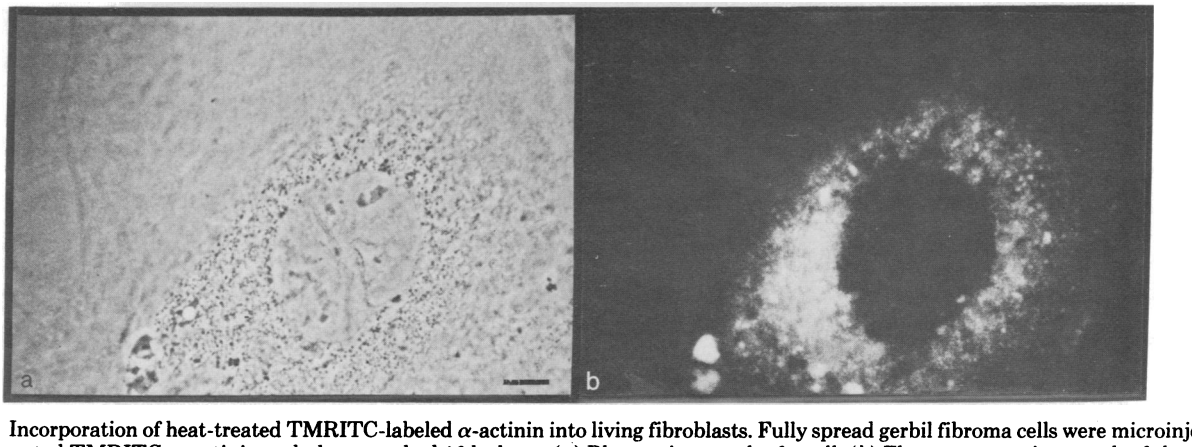

FIG. 5. Incorporation of heat-treated TMRITC-labeled α-actinin into living fibroblasts. Fully spread gerbil fibroma cells were microinjected with heat-treated TMRITC-α-actinin and photographed 16 hr later. (*a*) Phase micrograph of a cell. (*b*) Fluorescence micrograph of the same cell. Bar, 10 μm.

especially important because these cells are much more amenable to fluorescence microscopy than the protozoans *Physarum polycephalum* and *Chaos carolinensis* that were used by Taylor and Wang (11) for microinjection of fluorescent actin; therefore, studies of the cytoskeleton of mammalian cells may provide much more information regarding the molecular dynamics of cell motility.

In view of the ideas that stress fibers may function as contractile elements in nonmuscle cells (6, 10, 14) and that dense bodies, thought to be sites of α-actinin localization (7, 9, 24), may serve as the equivalent of the Z-lines found in striated muscle (7, 9, 25), it is interesting that α-actinin from smooth muscle was apparently incorporated into the functional pools of α-actinin within the fibroblasts (Fig. 3). These data provide evidence for the existence of some functional homology between α-actinins found in muscle (chicken gizzard) and nonmuscle (gerbil fibroma) cell types. Detailed studies of the movement of the TMRITC-α-actinin within living cells, necessary to determine whether or not the putative "sarcomeres" in nonmuscle cells contract, are currently in progress. Polygonal networks of microfilaments are structures found in some cultured cell lines and have been suggested to be precursors of stress fibers (14, 15) based upon their kinetics of appearance–disappearance during respreading and their molecular composition. The determination of the time-course of incorporation of fluorescently labeled components into both the polygonal networks and the stress fibers of respreading cells should provide a unique way of testing this hypothesis.

When heat-treated TMRITC-α-actinin was injected into the cells, the fluorescence became localized into structures that appeared to be lysozomes (Fig. 5), organelles involved in catabolism (26), indicating that the cells were able to discriminate between "native" (Fig. 3) and denatured (Fig. 5) α-actinin. These data provide not only an example of a control for the specificity of the labeling of structures with TMRITC-α-actinin but also for what appears to be autophagic segregation of cellular constituents, a process in which intracellular components are taken up by lysozomes (27). These results are similar to those obtained by Stacey and Allfrey in which several cytoplasmic proteins labeled with fluorescent probes became incorporated into autophagic vacuoles after microinjection (27).

Whether or not the approach taken in this paper will be generally useful for studies of other molecules of the contractile apparatus in cultured mammalian cells is not yet known, but it is clear that the technique requires (*i*) the availability of purified preparations of molecules, (*ii*) the retention of the functional activity of the molecule after fluorescent labeling, (*iii*) the discrete localization of the molecule within the cell at some time during the cell cycle, and (*iv*) a finite turnover of the molecule within the discrete localization areas. Preliminary attempts to study other proteins (i.e., actin and filamin) in living fibroblasts by these procedures have not as yet met with success; however, considering the numerous ways in which systems can be manipulated such that the above requirements are met, it seems likely that the technique should prove valuable in answering questions concerning the dynamics of many types of molecules and structures within living cells.

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