Distribution of Fluorescently Labeled α-Actinin in Living and Fixed Fibroblasts

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ABSTRACT The distribution of fluorescently labeled α-actinin after microinjection into fibroblasts has been determined in both living and fixed cells. We have found that the distribution of the injected tetramethylrhodamine isothiocyanate-labeled protein (TMRITC-α-actinin) in living cells, which is in ruffling membranes, actin microfilament bundles, and polygonal microfilament networks (Feramisco, 1979, *Proc. Natl. Acad. Sci. U. S. A.* 76:3967–3971), was virtually unaffected by the fixation (3.5% formaldehyde) and extraction (absolute acetone) used for the preparation of the cells for immunofluorescence. Also, these patterns were found to coincide with the α-actinin revealed by immunofluorescence. These findings offer, for the first time, evidence indicating the validity of the immunofluorescence technique in the localization of α-actinin in cultured cells. With the combination of the injection procedure and the immunofluorescence localization of endogenous structural proteins, it was determined that nearly all of the actin stress fibers were decorated in a periodic manner with the injected α-actinin. Endogenous tropomyosin in the injected cells was found to be distributed with a periodic pattern along the stress fibers that was antiperiodic to the pattern observed for the microinjected α-actinin. The tropomyosin antibody stained the polygonal microfilament networks and was excluded from the foci, whereas the microinjected α-actinin was incorporated into the foci of the networks. Thus, the microinjected fluorescent derivative of α-actinin appears to be incorporated into the functional pools of α-actinin within the living cell and to be utilized by the cell with fidelity.

To study the dynamics of structural proteins within living cells, techniques amenable to studies of living cells must supplant those of immunofluorescence or electron microscopy, which require fixation of the cells. An obvious approach for overcoming the problem of fixation is the microinjection into living cells of "native" structural proteins that have been fluorescently labeled. The distribution of fluorescently labeled actin in living protozoans has been observed by this technique (24, 25). The large size and thickness of these cells, though, makes it difficult to visualize the injected fluorescent proteins or the distribution of the endogenous proteins by immunofluorescence with fluorescence microscopy. Recently, this type of approach has been applied to living fibroblasts (i.e., cells that are ideally suited for fluorescence microscopy) into which fluorescently labeled α-actinin (8), a 130,000-dalton protein from smooth muscle (3), or actin (16) was microinjected. Microinjection of mammalian cells (5, 7, 15), combined with the use of fluorescently labeled proteins, promises to have many useful applications for the study of the dynamic behavior of structural proteins in living cells.

In the present study we have addressed several questions concerning this approach: (*a*) What is the relative distribution of microinjected fluorescent α-actinin in relation to other structural proteins in the cell? (*b*) Does microinjected fluorescent α-actinin fail to incorporate into any particular area(s) in the cell that contains endogenous α-actinin? (*c*) Is the distribution of injected protein within the living cell significantly altered by the treatments normally used for the preparation of cells for immunofluorescence (i.e., fixation and extraction)? With the microinjection technique combined with immunofluorescence and double-label fluorescence microscopy we have found that microinjected fluorescent α-actinin coincides with the α-actinin revealed by immunofluorescence, is distributed in a periodic pattern along the actin stress fibers which is antiperiodic to tropomyosin, and is localized in the foci of the actin polygonal microfilament networks that are exclusive of tropomyosin. Furthermore, we have determined that fixation and extraction have little effect on the distribution of the injected protein. These findings suggest that the microinjected, fluorescently labeled α-actinin faithfully reflects the distribution of α-actinin

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in the living cells and offer, for the first time, some evidence indicating the validity of the immunofluorescence technique in the localization of α-actinin in cultured cells.

MATERIALS AND METHODS

Cell Culture

Gerbil fibroma cells (CCL 146) were cultured in Dulbecco's modification of Eagle's medium containing 9% fetal calf serum as previously described (8). For microinjection the cells were treated with trypsin (0.05% trypsin in 0.5 mM EDTA-phosphate-buffered saline) and reseeded onto glass coverslips.

Tetramethylrhodamine Isothiocyanate-labeled α-Actinin

Homogeneous α-actinin purified from chicken gizzard (9) was chemically modified with the fluorescent reagent tetramethylrhodamine isothiocyanate (TMRITC) (8), except that the unreacted reagent was removed by gel filtration (Sephadex G-50) in 20 mM Tris-acetate (pH 7.5), 20 mM NaCl, 15 mM 2-mercaptoethanol, and 0.1 mM EDTA. These procedures give rise to fluorescent α-actinin that retains the ability to bind to actin filaments (8). The stoichiometry of labeling was estimated to be 2-4 mol dye/mol native α-actinin, based upon the A_{554} (21) and the protein concentration of the conjugate.

Microinjection

TMRITC-α-actinin was injected into cells with a glass capillary drawn out to a tip of 0.5-1.0 μm using the technique of Graessmann and Graessmann (15). The capillaries were treated with ethanol (100%) and connected to a Leitz micromanipulator (E. Leitz, Inc., Rockleigh, N. J.) equipped with a vacuum and pressure device (8, 15). With this method ~90% of the cells that were injected were alive and had incorporated the fluorescently labeled protein into endogenous cytoskeletal structures.

Indirect Immunofluorescence

Antibodies against α-actinin (4) and actin (2) were used as previously characterized. Antibodies were prepared against chicken gizzard tropomyosin. Tropomyosin was purified from ethanol-ether powders of chicken gizzard by isoelectric precipitation and ammonium sulfate fractionation (6) as modified by Fine et al. (10). Antibodies to tropomyosin were elicited in guinea pigs and shown to be specific by the formation of a single precipitin band in immunodiffusion plates (not shown), the staining of myofibril I segments (Fig. 1), and the periodic staining of microfilament bundles (Fig. 4).

Microinjected cells grown on coverslips were washed with phosphate-buffered saline, pH 7.4, at 20°C and fixed with 3.5% formalin in phosphate-buffered saline for 30 min at 20°C. Coverslips were then washed by immersing them 10 times in buffered saline and once in deionized water; then they were extracted with acetone at −20°C for 10 min and rinsed in buffered saline. The actin antibody was used at a dilution of 1:100 and the tropomyosin antibody was diluted 1:20. For the staining with antibody against α-actinin, the antibody was affinity purified and used as described previously (4). The coverslips were incubated in a humidified atmosphere at 37°C for 30 min. After being washed in an excess of buffered saline the coverslips were stained for 30 min with fluorescein-labeled goat anti-rabbit or goat anti-guinea pig IgG diluted 1:20 or 1:60 for the coverslips stained first with actin antibody. After being washed thoroughly in buffered saline, the coverslips were mounted on a glass slide in 16% (wt/vol) Gelvatol 20-30 (polyvinyl alcohol, Monsanto Polymers & Petrochemicals Co., St. Louis, Mo.) and 33% (vol/vol) glycerol in 0.14 M NaCl, 0.01 M KH_2PO_4–$Na_2HPO_4 \cdot 12H_2O$, and 0.1% sodium azide, final pH 7.2.

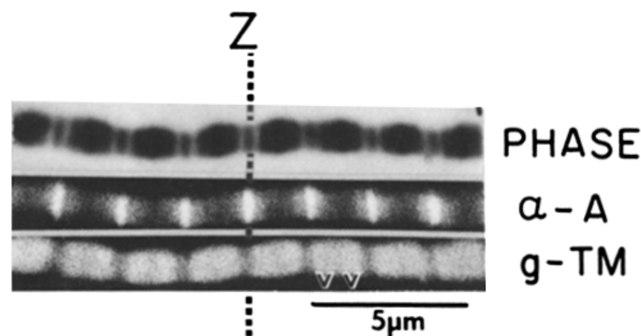


FIGURE 1 Double-label immunofluorescence localization(of α-actinin and tropomyosin in a skeletal muscle myofibril. A glycerinated myofibril prepared from chicken thigh muscle was incubated with rabbit anti-α-actinin and guinea pig antitropomyosin and subsequently with FITC-goat anti-rabbit IgG and FITC-goat anti-guinea pig IgG. The anti-α-actinin (*α-A*) stained the Z line (*Z*, dotted line) and the antitropomyosin (*g-TM*) stained the I segments between the Z lines (chevrons). The micrograph was made with a Zeiss Neofluar ×100 oil phase 3 lens (NA 1.3), giving an image magnification of ×420 at the film plane.

Microscopy

Cells were photographed on a Zeiss epifluorescence photomicroscope III with a Zeiss 63 × oil phase 3 lens (NA 1.4). Rhodamine (microinjected labeled α-actinin) was analyzed with a Zeiss G546 (narrow band pass interference filter, 546 ± 2 nm) excitation filter and LP590 barrier filter, and fluorescein (labeled antibodies) was analyzed with a Zeiss dichroic excitation filter BP485/20 and barrier filter LP520. Phase micrographs were recorded on Kodak High Contrast Copy Film (5069) and fluorescence micrographs were recorded on Kodak Tri-X Film (5063) as previously described (1, 8). All of the light was diverted to the film plane, giving exposure times of 5-10 s for immunofluorescence and 5-20 s for microinjected protein and image magnification of × 270 with a resolution of ~0.2 μm (0.19 μm for fluorescein isothiocyanate [FITC] and 0.21 μm for TMRITC).

RESULTS

Effect of Fixation and Permeabilization on the Distribution of TMRITC-α-Actinin

As was previously demonstrated, within 2-4 h after injection of TMRITC-α-actinin into the cytoplasm of cultured fibroblasts, the fluorescence localizes within the ruffled membrane region of the cell's leading edge, in the polygonal microfilament networks, and as periodicities along what appears to be microfilament bundles (8). This gave us the unique opportunity to observe the effects of treatments normally used in the preparation of cells for immunofluorescence on the intracellular distribution of the injected, fluorescently labeled α-actinin, for which cells were injected with TMRITC-α-actinin and photographed as living cells 4 h after injection. The same cells were immediately fixed and permeabilized as described in Materials and Methods. After being mounted in Gelvatol, the cells were rephotographed. An example of a cell treated in this manner is given in Fig. 2, with the live cell shown in panels *A* and *B* and the fixed and permeabilized cell shown in panels *C* and *D*. Very little difference was found in the fluorescence patterns of the living cell (Fig. 2 *B*) and the treated cell (Fig. 2 *D*), including the patterns of the ruffled membrane regions and the microfilament bundles. The amount of diffuse fluorescence in the perinuclear region (Fig. 2, *bottom*) of the living cell (Fig. 2 *B*) was greater than that found in the treated cell (Fig. 2 *D*). It is interesting to note that the phase micrograph of the living cell (Fig. 2 *A*) shows virtually no stress fibers, whereas the treated cell (Fig. 2 *C*) shows prominent phase-dense structures corresponding to the microfilament bundles.

The Relationship of Injected TMRITC-α-Actinin to Endogenous Structural Proteins

Knowing that the treatments of formaldehyde fixation and acetone extraction have little effect on the distribution of fluorescently labeled α-actinin found in the living cell (Fig. 2), we carried out studies to compare the distribution found for the injected protein with that found for α-actinin by the immunofluorescence method. Whereas the former would show only the injected smooth muscle α-actinin, the latter would be

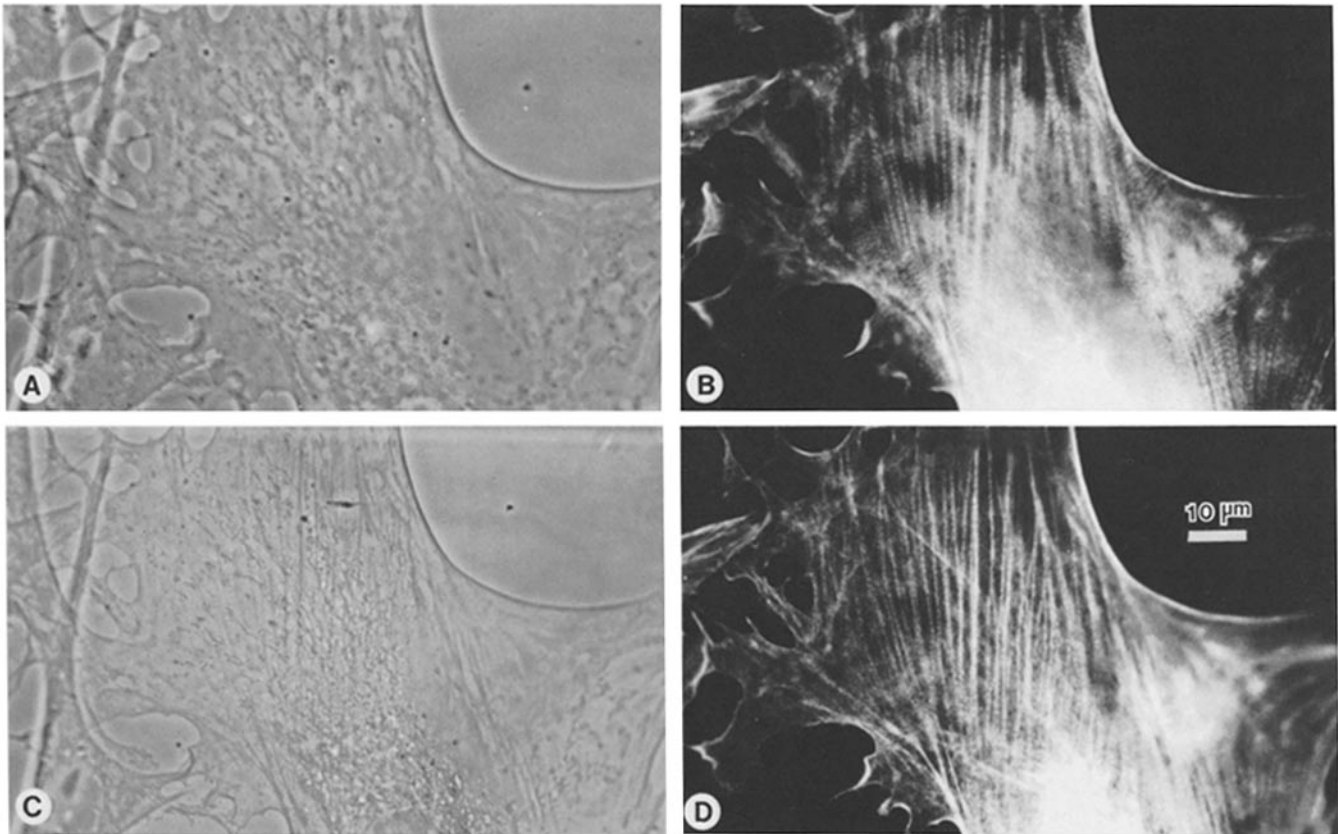


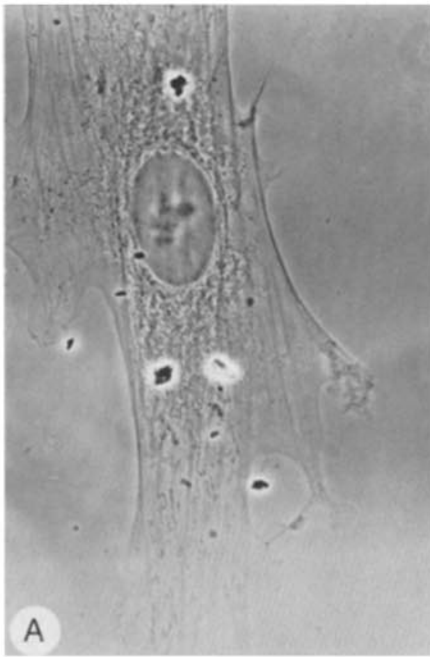
FIGURE 2 Phase and fluorescent micrographs of a cell microinjected with TMRITC-α-actinin before and after fixation-extraction. Gerbil fibroma cells were injected with TMRITC-α-actinin and photographed 4 h later, while still living, with phase (*A*) and fluorescence (*B*) optics. The cells were immediately submerged in 3.5% formalin for 30 min, washed extensively in phosphate-buffered saline, rinsed briefly in H_2O, and extraced in absolute iketone for 5 min(it −20°C. After rehydration in H_2O and phosphate-buffered saline the cells were mounted in Gelvatol. The same cell shown in *A* and *B* was then rephotographed with phase (*C*) and fluorescence (*D*) optics.

expected to reveal both the injected and the endogenous α-actinin, whereby, it could be determined whether the injected protein (purified from smooth muscle) was excluded from any areas of the cell that contained endogenous α-actinin.

When cells that had been injected with TMRITC-α-actinin were stained by indirect immunofluorescence for α-actinin using FITC-labeled antibodies, it was found that all areas (at least at the level of fluorescence microscopy) of the cells that contained α-actinin, as revealed by immunofluorescence, contained the injected α-actinin (Fig. 3). Thus, phase-dense stress fibers (Fig. 3 *A–F*), foci of polygonal microfilament networks (Fig. 3 *G–I*, single arrowheads), and ruffled membrane regions (Fig. 3 *G–I*, double arrowheads) showed coincident patterns for the immunofluorescence-localized α-actinin and the injected protein.

To determine the interrelationships between the microinjected α-actinin and the endogenous structural proteins tropomyosin or actin, cells microinjected with TMRITC-α-actinin were stained by indirect immunofluorescence for tropomyosin (Fig. 4) or for actin (Fig. 5) using FITC-labeled antibodies. An intimate relationship within supramolecular structures was found between the microinjected smooth muscle protein and the endogenous structural protein in both cases. In polygonal microfilament networks (14, 17), the injected α-actinin was

FIGURE 3 Phase and fluorescent micrographs of cells microinjected with TMRITC-α-actinin and indirectly stained for α-actinin with antibodies labeled with FITC. Gerbil fibroma cells were microinjected with TMRITC-α-actinin and 4 h after injection were fixed, permeabilized, and incubated, first with affinity purified rabbit anti-α-actinin and then with FITC-labeled goat anti-rabbit IgG. *A*, *D*, and *G* show phase-contrast micrographs of the cells. *B*, *E*, and *H* show the injected cells viewed selectively for rhodamine fluorescence to allow the microinjected α-actinin to be visualized. *C*, *F*, and *I* show the same fields seen in *B*, *E*, and *H*, respectively, except they are viewed selectively for fluorescein fluorescence to allow the distribution of α-actinin to be visualized by indirect immunofluorescence. Note the periodic distribution of fluorescence in *B* and *C* for both the microinjected α-actinin and the α-actinin revealed by immunofluorescence. In *D*, *E*, and *F* only one cell of the three cells in the field of view was injected. It should be noted that the fluorescence intensity of the three cells stained with anti-α-actinin is similar, suggesting that the injected cell did not contain an overwhelming excess of α-actinin. It should also be noted that in *E* the two cells that were not injected were essentially invisible when viewed with the rhodamine optics, indicating that there was little or no fluorescein fluorescence showing through in the rhodamine optic system. In *H* and *I* a ruffled membrane region is marked with a double arrowhead, and the foci of polygonal microfilament networks are marked with a single arrowhead. Both structures incorporated the microinjected α-actinin (*H*) and stained for α-actinin with immunofluorescence (*I*).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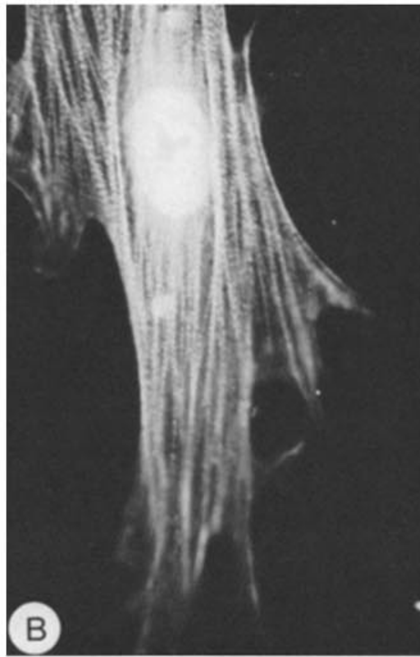
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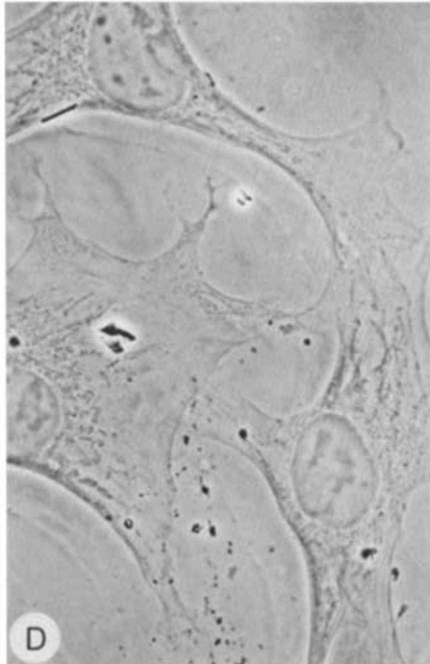
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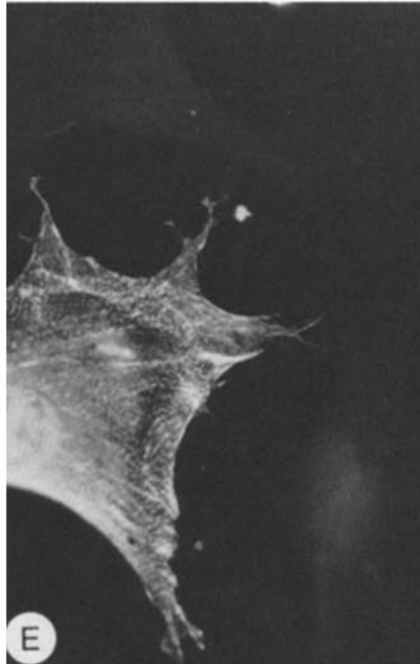
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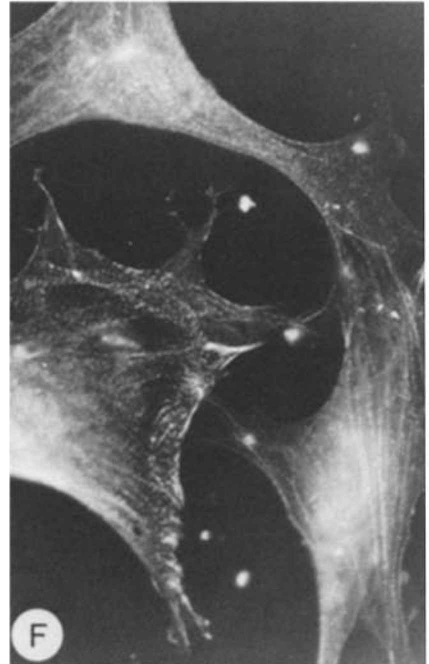
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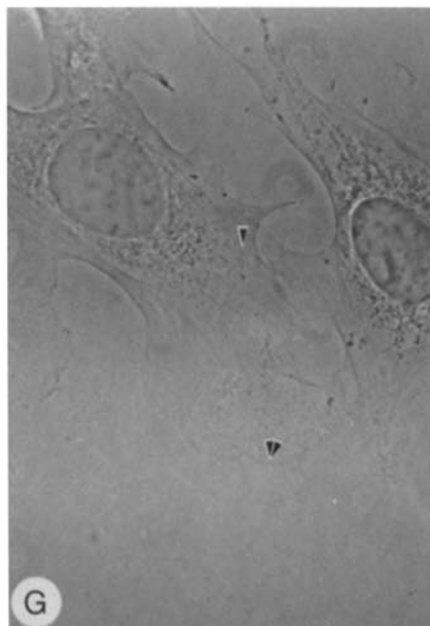
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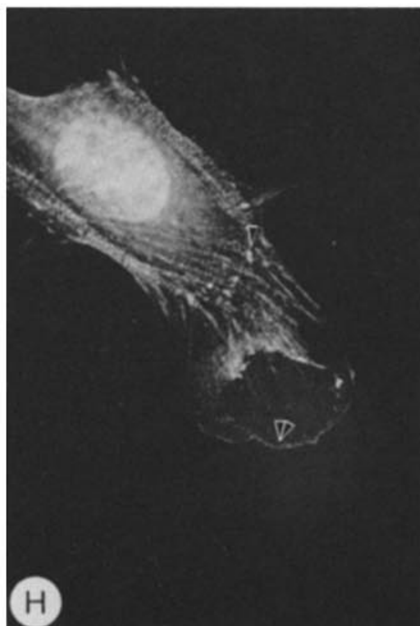
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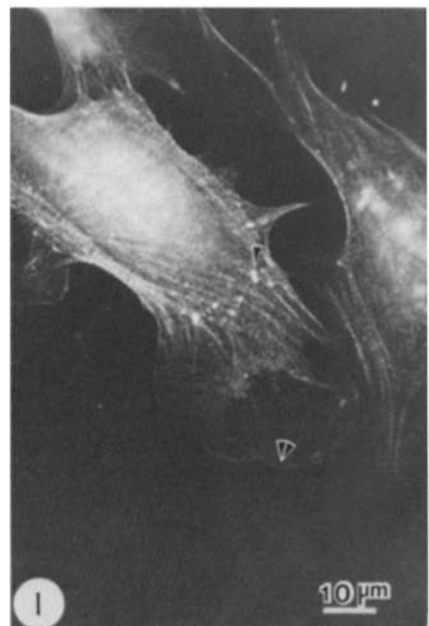
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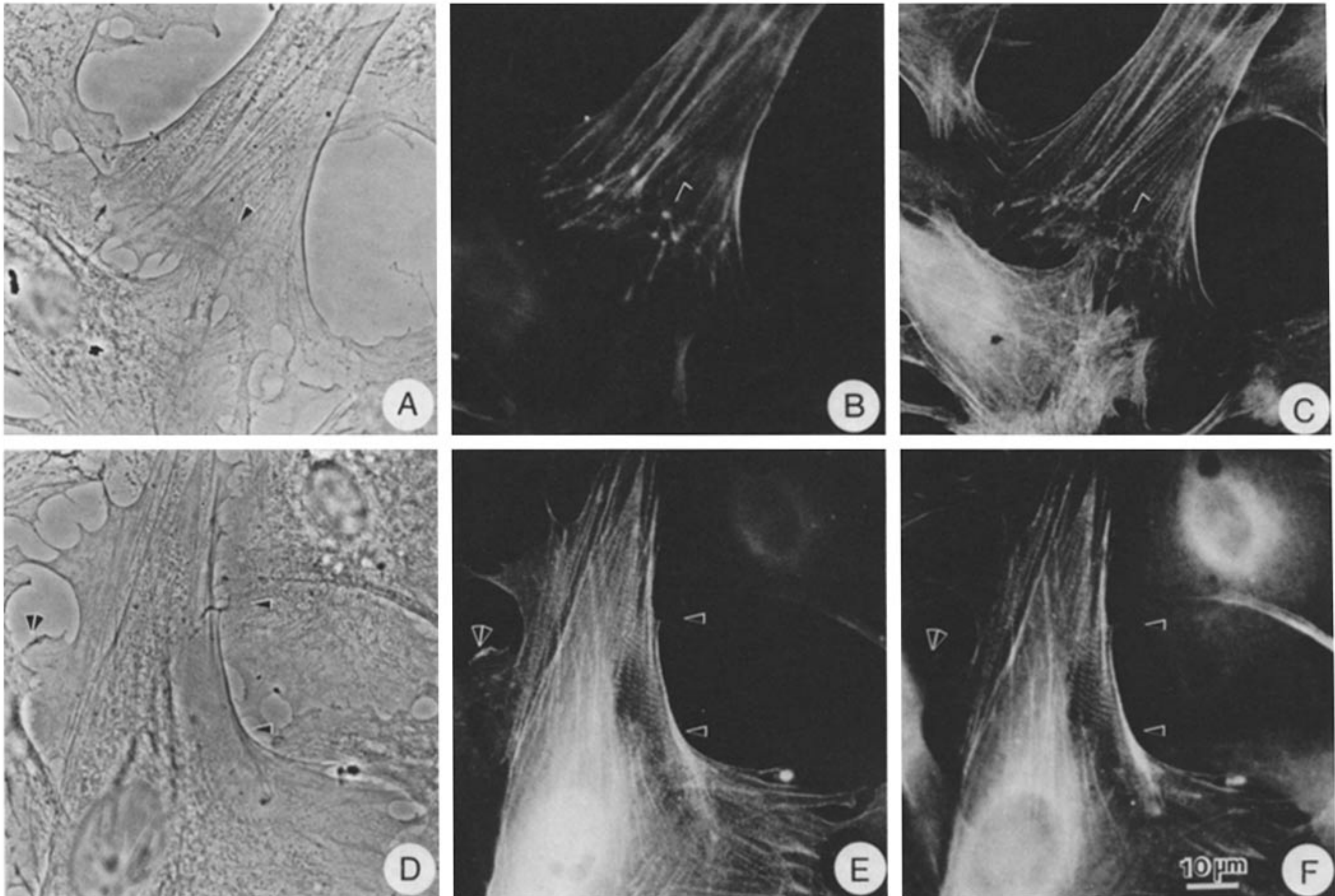
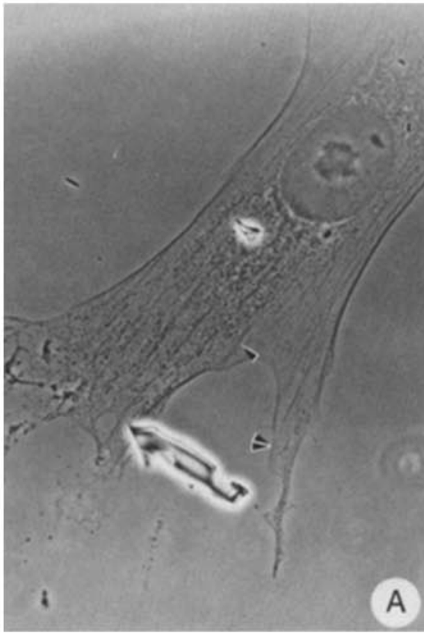


FIGURE 4 Phase and fluorescent micrographs of cells microinjected with TMRITC-α-actinin and indirectly stained for tropomyosin with antibodies labeled with FITC. Gerbil fibroma cells were microinjected with TMRITC-α-actinin and at 4 h after injection were fixed, permeabilized, and incubated, first with guinea pig antitropomyosin and then with FITC-labeled goat anti-guinea pig IgG. *A* and *D* show phase-contrast micrographs of the cells. *B* and *E* show the injected cells viewed with optics selective for rhodamine to visualize the injected TMRITC-α-actinin. *C* and *F* show the same fields seen in *B* and *E*, respectively, except they were viewed with optics selective for fluorescein to allow the distribution of tropomyosin to be visualized by immunofluorescence. In *A*, *B*, and *C* the center of a polygonal microfilament network is marked with a single arrowhead. This center incorporated the microinjected TMRITC-α-actinin (*B*) but is not labeled by the antitropomyosin antibody (*C*). In *D*, *E*, and *F* a ruffled membrane region is marked with a double arrowhead. Again, this structure incorporated the injected TMRITC-α-actinin but shows little or no staining with the antitropomyosin antibody (*E* and *F*, respectively). Higher magnifications of the polygonal microfilament networks in *B* and *C* are shown in Fig. 6. Higher magnifications of the region bounded by the single arrowheads in *E* and *F* are shown in Fig. 7. Note that the periodicities seen in *E* and *F* by fluorescence optics can also be resolved with phase-contrast optics in *D*.

incorporated into the foci of the vertices and gave a periodic pattern along the radial connecting fibers (Figs. 4 *B*, and 5 *E*, single arrowheads). The endogenous tropomyosin was found to be excluded from the foci of the networks and to be in an antiperiodic relationship to α-actinin along the connecting fibers (Fig. 4 *C*). These patterns can be seen more clearly in a higher magnification micrograph of the polygonal network (Fig. 6). Endogenous actin was localized along the connecting fibers of the polygonal networks (Fig. 5 *F*, single arrowheads). With respect to the stress fibers, the injected α-actinin was found to be localized along nearly all of the endogenous actin fibers (Fig. 5) and was found to be arranged in an antiperiodic

FIGURE 5 Phase and fluorescence micrographs of cells microinjected with TMRITC-α-actinin and indirectly stained for actin with antibodies labeled with FITC. Gerbil fibroma cells were microinjected with TMRITC-α-actinin and at 4 h after injection were fixed, permeabilized, and incubated, first with rabbit anti-actin and then with FITC-labeled goat anti-rabbit IgG. *A*, *D*, and *G* show phase-contrast micrographs of the cells. *B*, *E*, and *H* show the injected cells viewed with optics selective for rhodamine to allow the injected TMRITC-α-actinin to be visualized. *C*, *F*, and *I* show the same fields seen in *B*, *E*, and *H*, respectively, except they were viewed with optics selective for fluorescein to allow the distribution of actin to be visualized by immunofluorescence. Note that the actin stress fibers revealed by immunofluorescence (*C*, *F*, and *I*) incorporated the microinjected α-actinin periodically along their lengths (*B*, *E*, and *G*). A prominent phase-dense stress fiber is marked with a single arrowhead in *A*, *B*, and *C*. Ruffled membrane regions in *A*, *B*, and *C*, and in *G*, *H*, and *I* are marked with single arrowheads. These structures incorporated the injected TMRITC-α-actinin and are labeled with the anti-actin antibody. In *D*, *E*, and *F* a region of microfilament polygonal networks is marked with a single arrowhead. The foci of these networks incorporated the injected α-actinin (*E*), whereas the actin antibody labels both the foci and the interconnecting spokes of the networks (*F*).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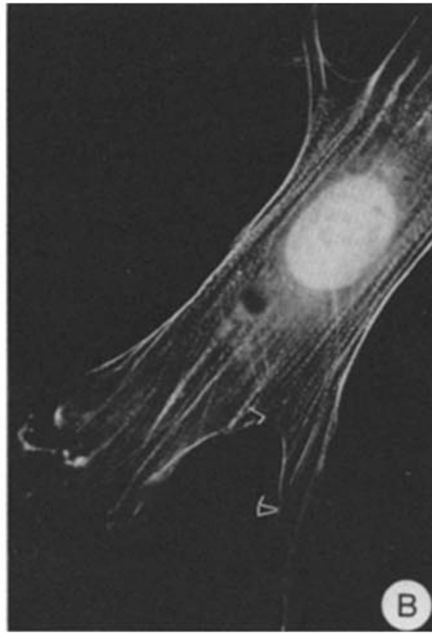
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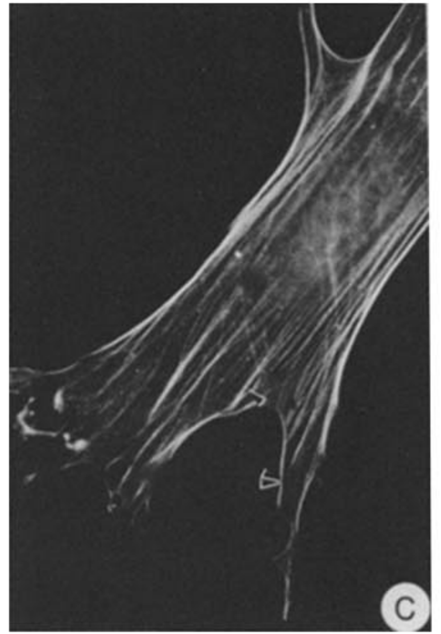
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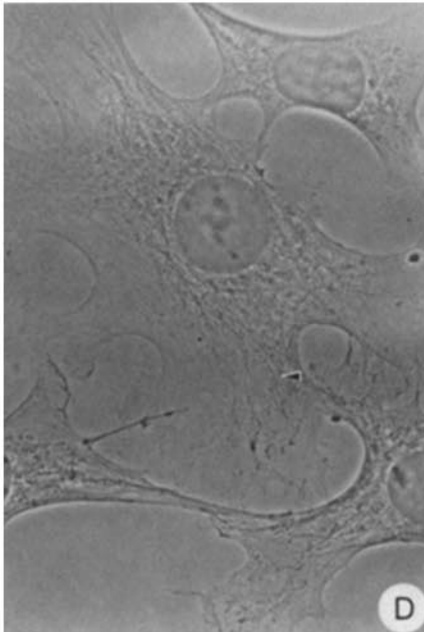
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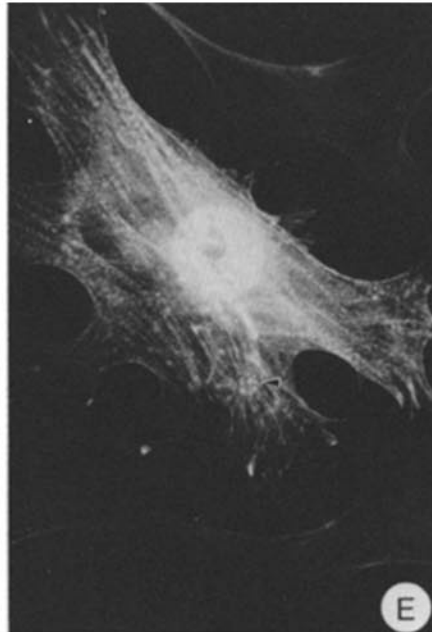
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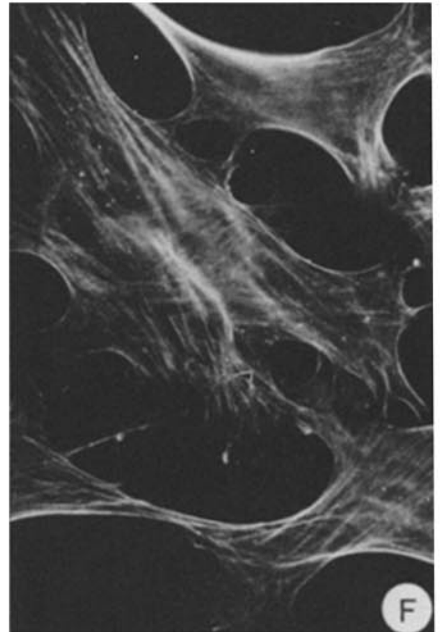
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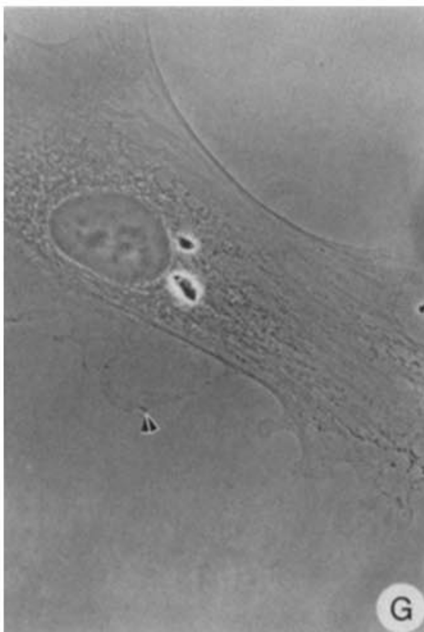
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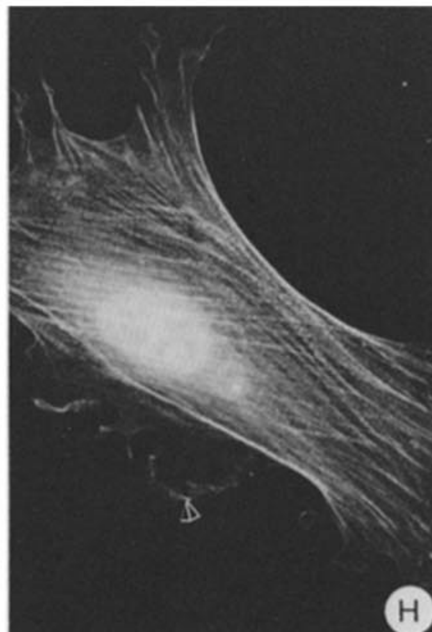
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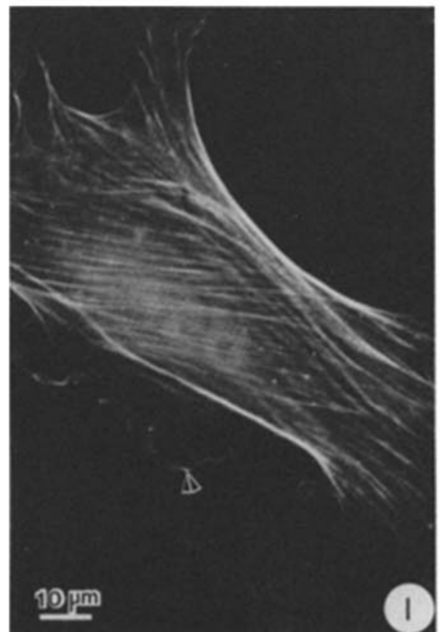
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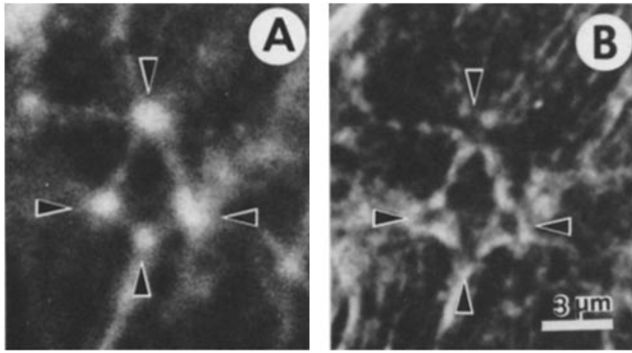

FIGURE 6 Higher magnification of the polygonal microfilament network indicated in Fig. 4 *B* and *C*. This cell was microinjected with TMRITC-α-actinin and stained for tropomyosin by indirect immunofluorescence with FITC-labeled antibodies as described in the legend to Fig. 4. *A* shows a region of the polygonal network viewed with optics selective for rhodamine to allow the injected α-actinin to be visualized. Note that the four foci of this network incorporated the injected α-actinin (*A*). This is in contrast to the distribution found for tropomyosin (viewed with optics selective for fluorescein in *B*), in which the foci of network are not stained, but the interconnecting spokes are stained with the antibody.

manner relative to the endogenous tropomyosin along the stress fibers (Fig. 4 *D–F*). It is interesting to note that these phase-dense periodicities can be seen in the phase micrograph (Fig. 4 *D*). The counter-periodicity of α-actinin and tropomyosin can be seen more clearly by the overlaying of pin-registered, black-and-white transparencies of the region in Fig. 4 *D–F* (bounded by the single arrowheads in Fig. 7). Also, as expected (19), ruffled membrane regions contained the injected α-actinin and endogenous actin (Fig. 5, double arrowheads) but not tropomyosin (Fig. 4, double arrowheads).

DISCUSSION

Fluorescently labeled α-actinin from chicken gizzard microinjected into fibroblasts is incorporated into ruffled membranes, foci of polygonal microfilament networks, and periodically along the actin stress fibers. We showed that fixation and permeabilization of the injected cells did not significantly alter the distribution of the fluorescently labeled protein seen in the living cells (Fig. 2). Moreover, immunofluorescence staining for α-actinin with FITC-labeled antibodies, in comparison to the pattern of TMRITC-α-actinin in the fixed, permeabilized cells, showed the two distributions to be identical at this level of resolution (~0.2 μm; Fig. 3). These results provide, for the first time, evidence indicating the validity of the immunofluorescence technique in the localization of at least the α-actinin integrated into supramolecular structures in cultured cells. It is now necessary to determine what effect fixation or permeabilization procedures other than those employed here (e.g., methanol [22] or glutaraldehyde [26] fixation, Triton extraction [4]) have on the distribution of α-actinin in the live cell, and more importantly, to determine the effect of fixation or permeabilization on other proteins that are routinely localized by immunofluorescence or immunoelectron microscopy.

From these studies, it appears that α-actinin from smooth muscle can be incorporated into nonmuscle cells into the diverse areas that are known to contain α-actinin. We could not determine, however, whether there were areas that contained microinjected α-actinin but not endogenous α-actinin because the immunofluorescence staining would be expected to show both the injected and the endogenous α-actinin. We inferred this from an estimation of the number of molecules of α-actinin injected into each cell in comparison to the number of intrinsic α-actinin molecules in the cell (i.e., 0.6×10^6 molecules injected vs. 6×10^6 endogenous molecules.[1] Also, as seen in Fig. 3, the relative intensity of fluorescence given by antibody staining for α-actinin in both injected cells and non-injected cells is similar, which may indicate that there is not much more α-actinin in the injected cells than in the control cells.

As revealed by immunofluorescence staining for actin and tropomyosin of the cells injected with fluorescent α-actinin, the injected α-actinin is localized along the actin bundles with a periodic distribution (Fig. 5), and that the periodicity of this distribution alternates with the distribution of tropomyosin (Fig. 7). The fact that the combined techniques of microinjection of fluorescent α-actinin and immunofluorescence staining of tropomyosin presented here indicate the complimentary periodic localization of these components of stress fibers (Fig. 7), found previously by either single-label or double-label immunofluorescence for these proteins, (11, 13, 20, 27) provides additional evidence that the living cell apparently faithfully uses the microinjected fluorescent α-actinin.

In this light, we have found that both the microinjected α-actinin and the antibody staining for α-actinin localize α-actinin in the nucleus or at the nuclear membrane. We have noted this apparent distribution of α-actinin by immunofluorescence previously but have usually dismissed it as an artifact. Nuclear staining by α-actinin antisera can also be seen in previously published immunofluorescent micrographs (4, 8, 20). That the microinjected α-actinin sometimes localizes at the nucleus or nuclear membrane (8) suggests that this could be a real location of this protein, a possibility which should be investigated further.

Finally, several investigators have raised a question (e.g., 11, 18) concerning the potential artifactual nature of the periodic distribution of α-actinin along the stress fibers, as seen by immunofluorescence methods (12, 20, 23, 27), which is due to either extraction or masking of the antigen. Inasmuch as we have observed similar periodic distribution patterns for TMRITC-α-actinin in living cells as well as in cells treated for indirect immunofluorescence (Figs. 2 and 3), it seems likely that the observed periodic distribution of α-actinin along the stress fibers is of physiological importance. The fact that we can now study the dynamics of α-actinin in living cells may elucidate the function(s) of this protein.

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[1] Assuming that fibroblasts have ~0.75 ng of total protein, which is composed of 0.25% (wt/wt) α-actinin, per cell, there would be ~6×10^6 molecules of intrinsic α-actinin per cell. The injection of ~5×10^{-14} liters (15) of a 5 mg/ml solution of α-actinin into each cell would introduce ~0.6×10^6 molecules of α-actinin per cell. Thus, the number of injected α-actinin molecules would be ~10% of the number of endogenous α-actinin molecules.

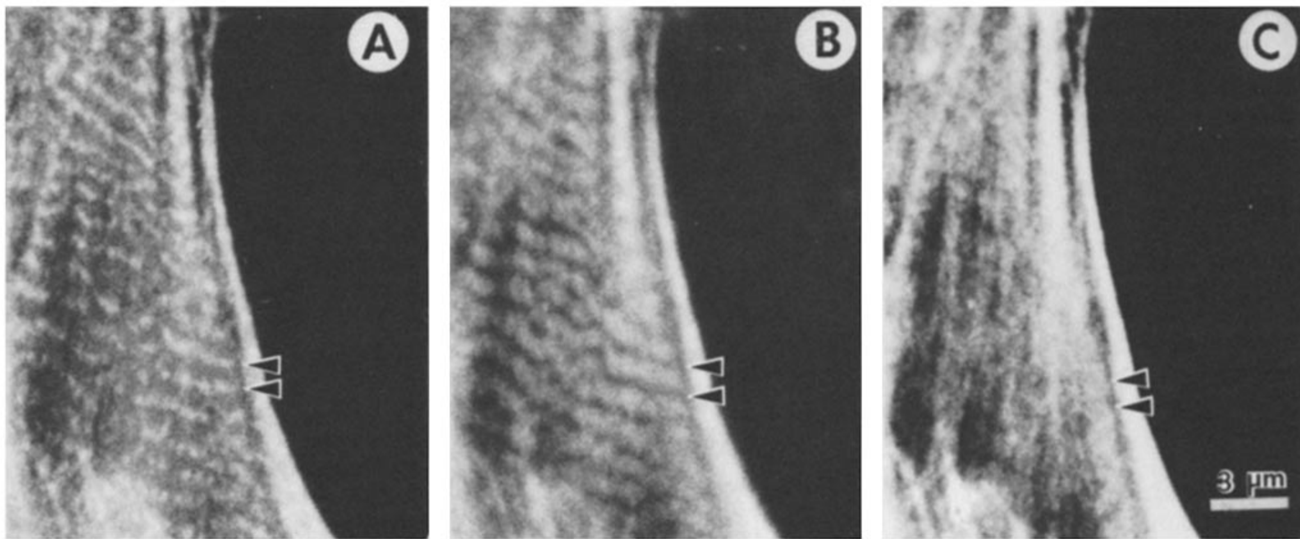

FIGURE 7 Higher magnification of the region of stress fibers indicated in Fig. 4 *E* and *F*. This cell was microinjected with TMRITC-α-actinin and stained for tropomyosin by indirect immunofluorescence with FITC-labeled antibodies as described in the legend to Fig. 4. *A* shows a region of the stress fibers viewed with optics selective for rhodamine to allow the injected α-actinin to be visualized. The same region is seen in *B*, except it is viewed with optics selective for fluorescein to allow the tropomyosin to be visualized by immunofluorescence. In *C*, pin-registered, black-and-white, 8 × 10-inch transparencies corresponding to Fig 4 *E* and *F* were photographed and the negative was printed at twice the exposure time. Note that the periodicities for both the α-actinin and the tropomyosin disappear in *C*, confirming that the periodicities in *A* and *B* were antiperiodic. Two arrowheads are used to mark successive lines of α-actinin in *A*, *B*, and *C*.

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