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Disruption of microfilament organization in living nonmuscle cells by microinjection of plasma vitamin D-binding protein or DNase I

(actin-binding proteins/stress fibers/cell shape/cytoskeleton)

JEAN M. SANGER*, G. DABIRI†, B. MITTAL*‡, M. A. KOWALSKI†, J. G. HADDAD†, AND J. W. SANGER*§

Departments of *Anatomy and †Medicine, University of Pennsylvania, School of Medicine, Philadelphia, PA 19104-6058

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ABSTRACT **Plasma vitamin D-binding protein (DBP), which binds to monomeric actin, causes the breakdown of stress fibers when it is microinjected into nonmuscle cells. Disruption of the stress fiber network is also accompanied by shape changes in the cell that resemble those seen after cytochalasin treatment. When DBP was coinjected with fluorescently labeled α-actinin, no fluorescent stress fibers or attachment plaques were visible 30 min after injection. Twelve hours later the cells regained their flattened shape and their stress fibers. Fluorescently labeled DBP causes the same reversible changes in cell shape as the unlabeled protein. Upon injection, the labeled DBP diffuses throughout the cytoplasm, becoming localized by 12 hr in a punctate pattern, presumably due to lysozomal sequestration. Similar injections of DBP into skeletal myotubes and cardiac myocytes did not lead to shape changes or breakdown of nascent and/or fully formed myofibrils, even though DBP has a 2-fold higher binding affinity for muscle actin over that of the nonmuscle isoactins. Similar differential effects in nonmuscle cells were also observed after the microinjection of DNase I, another protein capable of binding monomer actin. The effects of these microinjected monomer actin-binding proteins imply that an accessible pool of monomer actin is needed to maintain stress fiber integrity in nonmuscle cells but not the integrity of the nascent or fully formed myofibrils in muscle cells.**

The major actin–myosin cytoskeletal elements in tissue culture cells as well as in some somatic cells *in situ* are stress fibers (1), and in skeletal and cardiac muscle cells, myofibrils are the major cytoskeletal elements. Microinjection of trace amounts of fluorescently labeled actins (2, 3) and myosins (4, 5) into living nonmuscle and muscle cells have indicated how rapidly these labeled molecules are incorporated into stress fibers and myofibrils. The cycling of cells through the cell cycle also has revealed that the cells can disassemble and reassemble their stress fibers or myofibrils (6, 7). Microinjection of proteins that bind to (or cap) the barbed ends of actin filaments led to the reversible disassembly of stress fibers in nonmuscle cells (8). These observations suggested that the ability of actin filaments to incorporate monomer at their barbed ends is required for the stability of stress fibers (8).

Plasma vitamin D-binding protein (DBP; 58,000 daltons), also called Gc-globulin, is found in the blood serum at a level of about 300 mg/liter (≈6 μM) where it binds to vitamin D and its metabolites (9). It also binds to monomer actin and can cause the disassembly of fibrous actin *in vitro* by binding the monomers, which are in equilibrium with F-actin (10). Infusions of G-actin into the circulation system of a rat at concentrations that saturated the plasma actin-binding capacity of DBP led to the formation of intravascular actin filament formation, microthrombi, and endothelial injury (11). In this paper we report that DBP, when microinjected into living nonmuscle cells above a certain concentration, induces the complete disassembly of stress fibers and dramatic shape changes, both of which are reversible with time. Injections of DBP into skeletal myotubes and cardiac myocyte did not lead to shape changes or breakdown of nascent and/or fully formed myofibrils. Similar differential effects in nonmuscle versus muscle cells were also observed after the microinjection of DNase I (31,000 daltons), another protein capable of binding monomer actin. Our experiments indicate that an accessible pool of monomer actin of a certain critical size is necessary for the stability of the actin–myosin cytoskeleton and cell shape in nonmuscle cells but not in muscle cells. The lack of an effect of these monomer-binding proteins in muscle cells supports the view that nascent sarcomeres are precursors of adult sarcomeres and are not stress fibers.

MATERIALS AND METHODS

Cells. Nonmuscle cell lines (PtK2 and gerbil fibroma cells) were obtained from the American Type Culture Collection and grown in tissue culture as described (4). Fibroblasts as well as skeletal and cardiac muscle cells were obtained from chicken and quail embryos and grown as described (4). Some cells were permeabilized by using cold acetone or detergents (12).

Proteins. α-Actinin was purified from frozen and fresh chicken gizzards and labeled with fluorescent dyes as reported (12). DBP was isolated from the blood of human volunteers (13). Some of this protein was coupled to lissamine–rhodamine–sulfonyl chloride. The labeled protein was dialyzed and then passed through a Sephadex G-25 column to remove unbound dye. The labeled DBP was then passed through an actin-linked column (13). The labeled DBP was subsequently released from the bound actin by passage of 3 M guanidinium hydrochloride. The labeled DBP was then dialyzed to remove any traces of the guanidinium hydrochloride. The DBP was placed in a vacuum concentrator to attain a final concentration of 250 μM. This stock solution was diluted to various concentrations with a low-salt buffer. Purified DNase I was obtained from Worthington and further purified by passage through a hydroxyapatite column (14). The fractions containing the DNase I were collected and concentrated to 150 μM against a solution containing 5 mM Hepes (pH 7.0).

Microinjection. The labeled and unlabeled proteins were microinjected into living cells with a pressure injection system previously described (15). Images were recorded by the use of video cameras (Nuvicon and SIT cameras, Dage-MTI, Michigan City, IN) coupled to an image-processing unit

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Abbreviation: DBP, vitamin D-binding protein.

‡Present address: Sanjay Gandhi Post-Graduate Institute of Medical Sciences, Lucknow, India.
§To whom reprint requests should be addressed.

(Image I-IVS, Concord, MA) and an optical memory disc recorder (Panasonic, Secaucus, NJ). Images were photographed with 35-mm film (100 ASA) off a high-resolution monitor (15).

RESULTS

When DBP or DNase I was microinjected into nonmuscle cells of the PtK2 or gerbil fibroma lines in amounts estimated to be 5–10% of a cell's volume, no detectable effects on cell structure were observed with stock concentrations of the proteins below 125 μM. At or above that concentration, marked changes in cell shape occurred (Figs. 1–3). Within the first minutes after injection, vacuoles appeared in the cytoplasm (Fig. 1 *a* and *b*), followed by a gradual loss of normal cell contour that culminated after 30 min in extensive retractions of the cytoplasm (Fig. 1 *c* and *d*). Coinjection of fluorescently labeled α-actinin and unlabeled DNase I (Fig. 3) also showed a complete loss of cytoplasmic stress fibers. The drastic changes in morphology and cytoskeletal organization induced by DBP were fully reversible 10–12 hr after injection (Fig. 2*c*). The respread cells had the normal distribution of α-actinin in stress fibers and attachment plaques and were able to divide and reform stress fibers after cytokinesis. Although some DNase I-injected cells recovered their stress fibers and normal cell shape within 10–12 hr, nuclear abnormalities were often detected in the PtK2 cells at this time, presumably because of the long-term nuclear effects of DNase I.

When a fluorescent label was coupled to DBP or DNase I and the labeled protein was microinjected into cells, we were able to follow the DBP (or DNase I) in the living cells (Fig. 2 *d–f*). Initially the DBP diffused throughout the cytoplasm and was excluded from the nucleus. DNase I (31,000 daltons), in contrast to DBP (58,000 daltons), was able to diffuse into the nucleus. Twelve hours later there was a decreased amount of diffuse, labeled DBP in the cells, part of it diffusely distributed and part sequestered in small round particles that were presumably lysosomes (Fig. 2*f*). When these cells were fixed and stained with fluorescein-labeled phalloidin, stress fibers could be seen throughout the cytoplasm.

Labeled and unlabeled DBP or DNase I were also injected into skeletal and cardiac muscle cells as well as into neighboring fibroblasts in these cultures. No effects were observed on the muscle cells, whereas the injected fibroblasts underwent dramatic shape changes and loss of stress fibers (Fig. 4). Unlabeled DBP or DNase I (needle stock concentrations up to 250 μM) plus labeled α-actinin (needle stock concentrations of 0.5–1 mg/ml) had no effect on the sarcomeric structures in the muscle cells. Both the fully formed sarcomeres and the shorter nascent sarcomeres in both skeletal and cardiac muscle cells were unaffected by the presence of the DBP or DNase I (Fig. 4). Myotubes were given multiple injections along their lengths to inject a comparable volume in relation to their greater size. Normal contractions occurred in the skeletal and cardiac muscles in the myofibrils containing fully formed sarcomeres. Injections of the same concentrations of the DBP (and α-actinins) into neighboring fibroblasts induced their rounding-up and gross change of shape (Fig. 4*d*). Nevertheless, these fibroblasts recovered their flattened shapes and stress fibers after 10–12 hr. Long-term observations of the adjacent muscle cells revealed no disruption of the myofibrils, either nascent or fully formed. Permeabilized muscle and nonmuscle cells that were exposed to various concentrations of the labeled and unlabeled monomer actin-binding proteins showed no loss of stress fibers, attachment plaques, or nascent or fully formed sarcomeres.

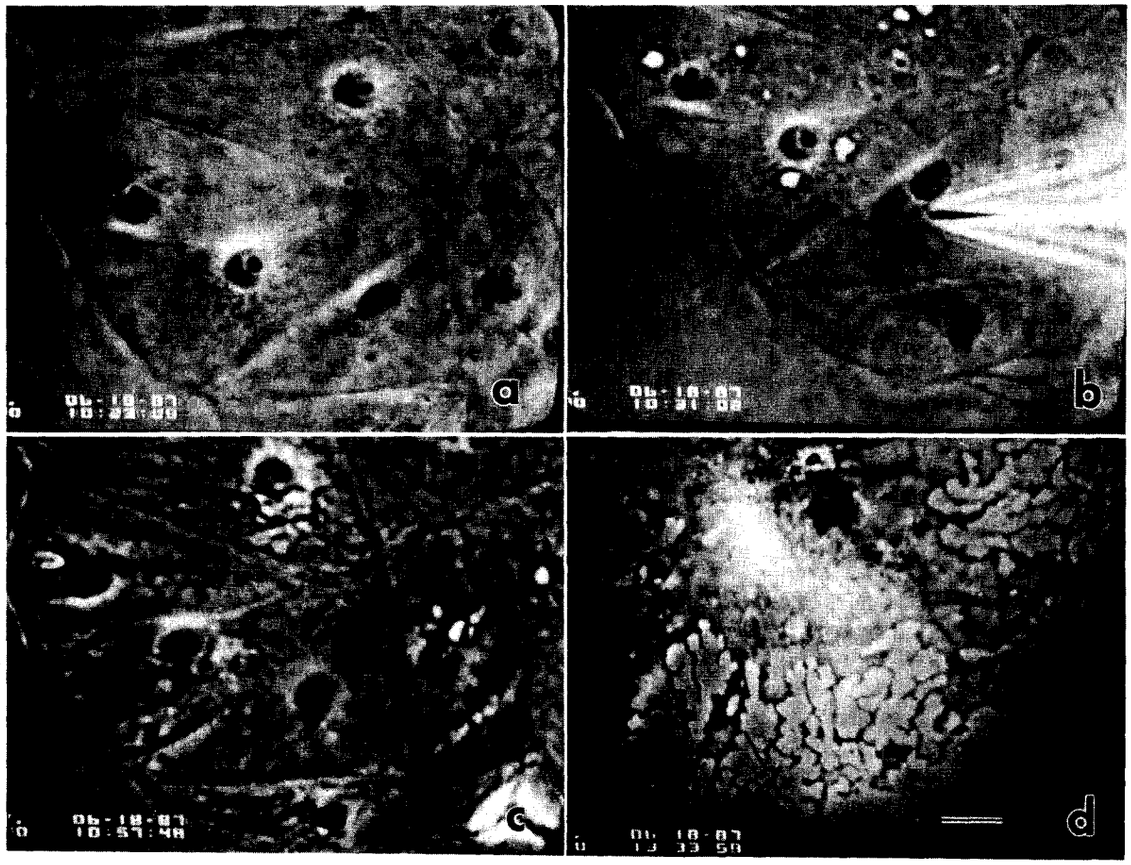

FIG. 1. PtK2 cells before (*a*) and 2–3 min after (*b*) an injection of DBP. Needle concentration was 200 μM. Vacuoles appear within 30 sec of injection. (*c*) Thirty minutes after injection, the cells are losing their smooth shape. (*d*) Typical appearance of an injected cell 3 hr later. (Bar = 10 μM.)

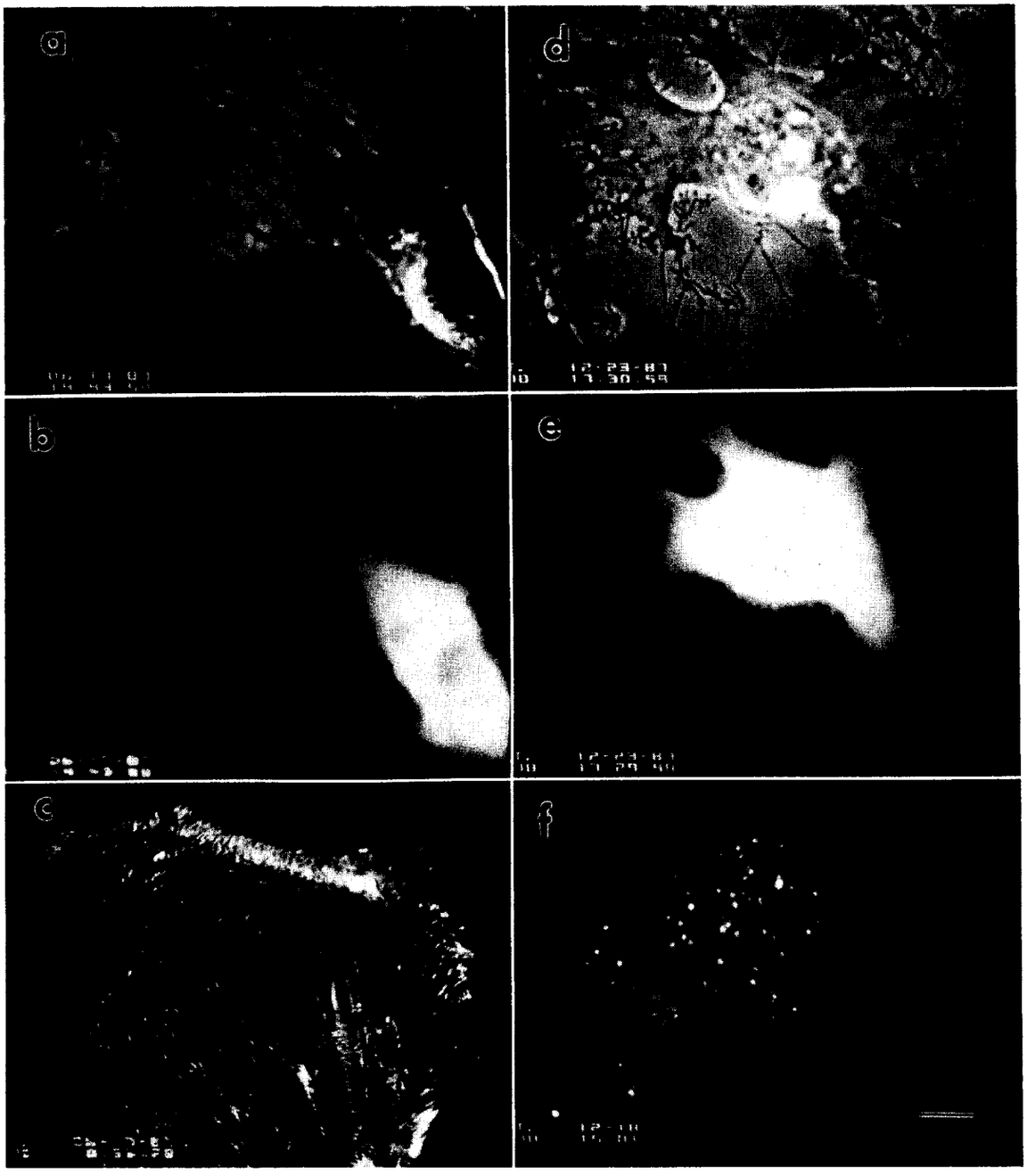

FIG. 2. Phase-contrast (*a*) and fluorescent (*b*) micrographs of a PtK2 cell that had been injected with labeled α-actinin and unlabeled DBP (needle concentration, 125 μM) 3 hr earlier. The cell has lost its normal shape and stress fibers. (*c*) A sister cell 15 hr after the coinjection has now recovered its shape and its stress fibers. (*d* and *e*) Phase contrast (*d*) and fluorescent (*e*) micrographs of a PtK2 cell that had been injected 3 hr previously with labeled DBP (needle concentration, 125 μM), showing its loss of shape. (*f*) A sister cell 15 hr after the injection has regained its normal shape, and some labeled DBP is concentrated in vacuoles. (Bar = 10 μm.)

DISCUSSION

The injection of DBP or DNase I has a profound effect on the cell shape and the actin–myosin cytoskeleton in nonmuscle cells but no observable effects on muscle cells. The injection of DBP, a monomer actin-binding protein, has no long-term effects on the cell and therefore is a good probe to test its effect on the cytoskeleton. The effects of these injected monomer-binding proteins are concentration dependent to the extent that stock needle concentrations <125 μM had no effect in nonmuscle cells. Nonmuscle cells are thought to have actin concentrations ≤ 200 μM, with possibly half of this actin in a monomer form (i.e., a nonfilamentous state) (16). It had been thought that there was enough of the monomer-binding protein, profilin, to bind all of the cell's nonfilamentous actin (17); however, there may not be as much profilin as originally thought, and additional monomer actin-binding molecules (e.g., gelsolin) may also bind some of the nonfilamentous actin (18). In our injections into nonmuscle cells, we estimate that the volume microinjected is 5–10% of the cell's volume, resulting, therefore, in the introduction of DBP or DNase I at about 6–12 μM. This is of considerable interest because this concentration is similar to the concentration of DBP in plasma (i.e., 6–8 μM) (9, 11). If half of the nonmuscle cell's actin were in a monomer form (i.e., 100 μM nonfilamentous actin), one DBP or DNase I molecule per 8–17 nonfilamentous actin molecules would be sufficient to cause the whole system of stress fibers and attachment plaques, and, presumably, much of the cortical actin system to collapse. We are clearly not injecting enough DBP or DNase I to convert or depolymerize all of the filamentous actin to a nonfilamentous state.

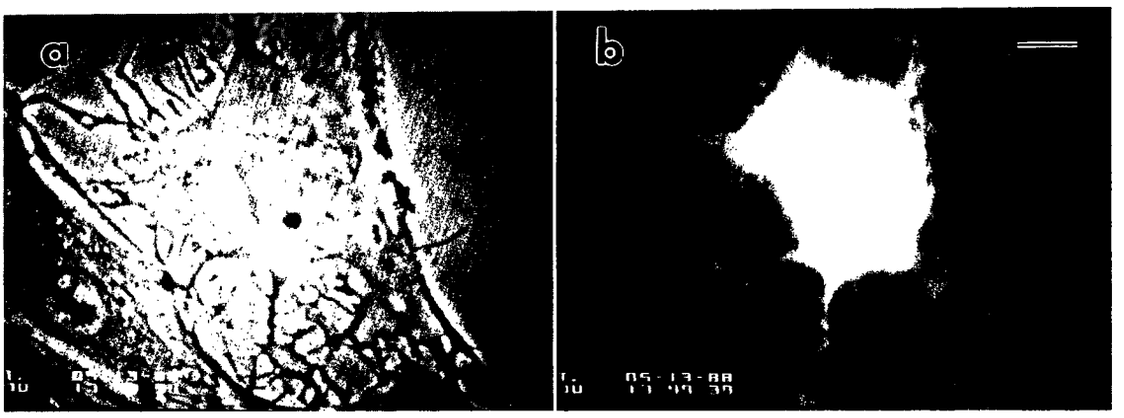

FIG. 3. Phase contrast (*a*) and fluorescent (*b*) micrographs of a PtK2 cell injected several hours earlier with labeled α-actinin and unlabeled DNase I (needle concentration, 125 μM). Note the loss of cell shape and stress fibers. (Bar = 10 μm.)

These gross cytoskeletal effects are similar to those of cytochalasins (19) and to those produced by the microinjection of actin-capping proteins (8), both of which are effective at micromolar levels. Brain capping proteins attach to the barbed ends of the actin filaments (8), which in stress fibers are embedded in the α-actinin-containing dense bodies (20). Fuechtbauer *et al.* (8) reported that injection of brain capping protein at a needle concentration of only 0.2 mg/ml (or 3 μM) induced the reversible disassembly of the radial stress fibers in PtK2 cells. If we assume the average length of actin filaments in PtK2 cells to be 0.5 μm (21) (i.e., 185 monomers per 0.5 μm of polymer) and the amount of filamentous actin to be 100 μM, then the concentration of actin filaments would be about 0.54 μM. If one assumes a 1:10 dilution in the cells of the injected stock (0.3 μM), then one would need only one molecule of brain capping protein per two actin filaments to induce stress fiber disassembly. A similar calculation for the monomer actin-binding proteins used in this study indicates one needs 10–20 molecules per actin filament to get disassembly. These calculations indicate that brain capping protein is much more effective at inducing the disassembly of stress fibers than DBP or DNase I.

There are at least two ways in which these monomer-binding proteins may induce the disassembly of the stress fibers and other components of the actin cytoskeleton. First, the introduction of these proteins may cause the shortening

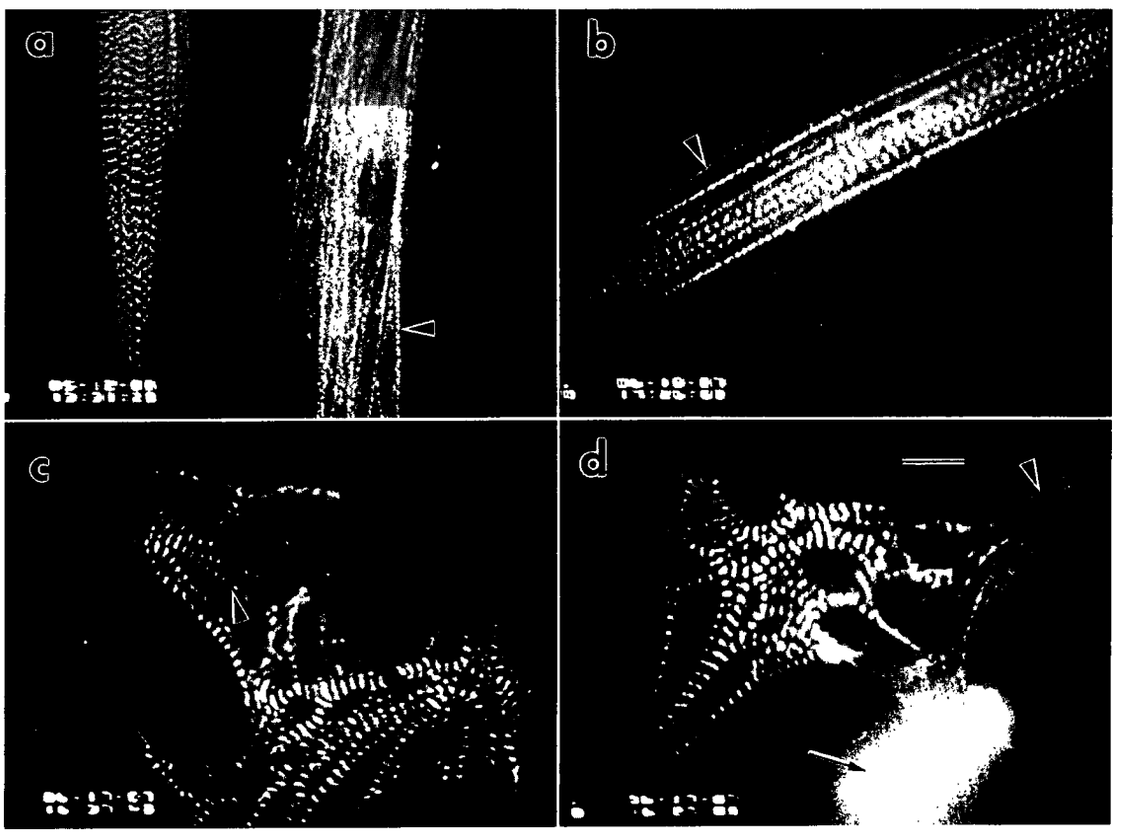

FIG. 4. Coinjections of labeled α-actinin and unlabeled monomer actin-binding proteins (needle concentrations, 250 μM) produce no effects on nascent sarcomeres (arrowheads) or fully formed sarcomeres in these fluorescent micrographs. (*a*) Distribution of α-actinin and DNase I in a quail myotube. (*b*) α-Actinin and DBP in a chicken embryo myotube. (*c*) α-Actinin and DBP in a chicken embryo cardiomyocyte. (*d*) α-Actinin and DBP in a chicken embryo cardiomyocyte and in an adjacent fibroblast (arrow). Note the rounding of the fibroblast due to the DBP injection. (Bar = 10 μm.)

of the individual actin filaments (containing 185 monomers per 0.5-μm length) to slightly shorter filament lengths (185 minus 11–23 subunits that now bind the DBP or DNase I), which are unstable. This seems unlikely. The second possibility is that the monomer-binding protein or the complex formed by DBP or DNase I to monomer actin prevents the addition of monomer actin to the barbed ends of the actin filaments embedded in α-actinin. There is no evidence that monomer actin bound to DBP or DNase I binds to the barbed end of the actin filaments. While there is some evidence that DNase I can bind to the barbed ends of the actin filaments (14), there is no such evidence for DBP. However, there is evidence that α-actinin (found in the dense bodies in which the barbed actin ends are embedded) can induce the removal of profilin from profilactin and allow monomer actin to polymerize (22). It has been suggested that profilin might be removed from monomer actin in the region where α-actinin is concentrated so that the newly released monomer actin could then be added to the barbed end of the actin filament (22). Future work will have to determine if monomer actin bound to DBP or DNase I competitively inhibits this process.

There are currently two theories of how myofibrils form and grow (23). In one (24), myofibrils are proposed to form alongside a stress fiber template, where the muscle thin and thick filaments assemble into sarcomeres of adult length ($\approx$2.3 μm). The stress fibers then disassemble, leaving the myofibril behind. In a second model of myofibrillogenesis, sarcomeres are proposed to form by the assembly of contractile proteins into nascent sarcomeric units (α-actinin spacings of 0.4–1.0 μm), which grow in length into fully formed sarcomeres (α-actinin spacings of $\approx$2.3 μm) via further incorporation of proteins (25, 26). Such sarcomeric growth has been seen in invertebrate cross-striated muscles (27, 28). In those sarcomeres, the myosin filaments also grew in length during myofibrillogenesis. In avian muscles, however, there is no evidence that myosin filament length increases. Myosin antibody staining shows a continuous pattern along fibers that have closely spaced bands of α-actinin, indicating that the thick filaments overlap one another in the nascent myofibril (33). If the fibers with the smaller periodicites of α-actinin in the developing skeletal and cardiac muscles were stress fibers, they should have been sensitive to DBP and DNase I as were fibroblasts in the same cultures. Even though DBP has a 2-fold higher binding affinity for muscle actin (α-actin) than for the nonmuscle isoactins (β, γ) (29), needle concentrations of DBP up to 250 μM and injection volumes of up to 25% cell volume had no effect on the fibers with short spacings of α-actinin in the muscle cells (Fig. 4). This indicated to us that these fibers are nascent sarcomeres as opposed to stress fibers. These stable nascent sarcomeres (and fully formed sarcomeres, too) also behave differently from the labile stress fibers in cells exposed to dimethyl sulfoxide, cytochalasins, and depleted ATP levels—all of which have no effect on the nascent or fully formed myofibrils but induce a reversible disassembly of the stress fibers (23). Thus, the nascent sarcomeres do not behave like stress fibers under a variety of conditions. These experiments support the view that the closely spaced bands of α-actinin represent nascent sarcomeres in embryonic muscle and are not parts of stress fibers (23, 25, 26).

The lack of effect of DBP or DNase I on nascent and fully formed sarcomeres suggests, furthermore, that an accessible or particular size pool of monomer actin is not necessary for the stability of these structures. These nascent and fully formed sarcomeres are not static structures, as microinjected labeled actin and tropomyosin are readily incorporated into these structures (3, 30). Nevertheless, the reaction of nonmuscle versus muscle cells to the injection of monomer-binding proteins suggests that the nonmuscle actin filaments may be more dynamic than actin filaments in muscle cells. This could be achieved by differences in the capping proteins of the barbed and pointed ends of the muscle actin filaments. Although a number of barbed-end capping proteins are known in both muscle and nonmuscle cells (31), it is unclear if pointed-end capping proteins exist in either muscle or nonmuscle cells (32).

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