

Possible Translocation of Actin and Alpha-actinin along Stress Fibers

NANCY M. McKENNA¹ and YU-LI WANG^{2,*}

¹*Department of Molecular and Cellular Biology, National Jewish Center for Immunology and Respiratory Medicine, Denver, CO 80206* and ²*Department of Biochemistry, Biophysics and Genetics, University of Colorado Health Sciences Center, Denver, CO 80220, USA*

We have employed fluorescent analogue cytochemistry and fluorescence photobleaching to study the mobility of actin and α -actinin along stress fibers. Rhodamine-labeled actin or α -actinin microinjected into embryonic chick cardiac fibroblasts soon became incorporated into stress fibers. A pulse of a laser microbeam was used to photobleach small spots on the fluorescent stress fibers. Images of the bleached fiber were recorded with an intensified image processing system at 2–3-min intervals. The distance between the bleached spot and the terminus of the stress fiber, which remained stationary throughout the experiment, was then measured in the successive images. Movement of bleached spots was detected along stress fibers located in the apparently trailing processes of polygonal fibroblasts, and only occurred in one direction: away from the distal tip of the stress fiber. The rate of movement calculated for α -actinin-injected cells was $0.24 \pm 0.12 \mu\text{m}/\text{min}$, for actin-injected cells, $0.29 \pm 0.11 \mu\text{m}/\text{min}$. The rate did not seem to be affected by the location of the spot relative to the distal end of the stress fiber unless the spot was located within the most distal $5 \mu\text{m}$ of the stress fiber. Anti-myosin antibody staining indicated that stress fibers which demonstrated translocation were relatively depleted of myosin. The apparent translocation of proteins along stress fibers, possibly generated by stretching, may be related to the retraction of cell processes during locomotion. © 1986 Academic Press, Inc.

Stress fibers are bundles of actin filaments possibly involved in maintaining the spread morphology of many cultured cells. These structures are highly dynamic and are capable of contraction [14] and rapid reorganization. For example, changes in the number and orientation of stress fibers occur during wound healing *in vivo* [7, 28], and in response to stimuli such as wounds, electric fields, shear forces and replating *in vitro* [4, 9, 17, 23]. Time-lapse studies of fluorescently labeled stress fibers in living fibroblasts have further revealed that the reorganization may involve processes such as elongation, merging and fragmentation [26]. Consistent with these observations is the finding that at least some of the proteins which comprise the stress fiber, such as actin and α -actinin, have relatively high rates of exchange with their soluble intracellular pools [15, 19].

In this paper, we describe a novel aspect of stress fiber dynamics: the apparent directional translocation of structural components. We have used a laser micro-

* To whom offprint requests should be sent. Address: Department of Molecular and Cellular Biology, National Jewish Center for Immunology and Respiratory Medicine, 1400 Jackson st., Denver, CO 80206, USA.

beam to photobleach small spots on stress fibers in living cells which have incorporated microinjected fluorescently labeled actin or α -actinin [15, 19, 27]. Analysis of the subsequent locations of the bleached spots indicate that bleached spots can move along the stress fiber away from the distal tip, even though the stress fiber and its tip remain stationary. The simplest interpretation of these data is that actin and α -actinin translocate either actively or passively along these stress fibers toward the nucleus. Such translocation, possibly generated by stretching, may represent a prelude to the retraction of the trailing end of the cell during locomotion.

MATERIALS AND METHODS

Preparation of Fluorescent Analogues

Smooth muscle α -actinin was isolated and labeled with iodoacetamidotetramethylrhodamine (IATR, Molecular Probes, Eugene, Ore.) as described previously [19]. Skeletal muscle actin was purified according to Spudich & Watt [24] and was labeled with lissamine rhodamine B sulfonyl chloride (Molecular Probes) [18].

Cell Culture, Microinjection, and Fluorescence Microscopy

Monolayer cultures of cardiac fibroblasts were obtained by trypsinizing hearts of 7-day chick embryos [3]. Cells were plated on glass coverslips and maintained in F12K medium (KC Biological, Lenexa, Kans.) with 5% fetal bovine serum (KC Biological) and antibiotics. Some 3–7 days after plating, cells were microinjected as described previously [18–19]. After microinjection, the culture dish was incubated for 1–3 h before the initiation of observation to allow for incorporation of injected molecules. During observation, culture dishes were placed on the heated stage of a Zeiss IM-35 inverted microscope in a humidified atmosphere containing an appropriate level of CO₂. Fluorescence images were observed using a 100 \times Neofluar objective (N.A. 1.30) and epi-illumination. Immunofluorescence was performed according to Amato et al. [1]. Antibody against platelet myosin was a gift of Dr K. Fujiwara. Its preparation and characterization have been described [5]. Fluorescein-labeled secondary antibody was purchased from Miles Scientific (Naperville, Ill.).

The apparatus for laser photobleaching was similar to that described by Jacobson [11]. Cells were photobleached with the 515 nm line of an argon ion laser. The laser pulse, which had a power of 75 mW and a duration of 50 ms, did not disrupt the structural integrity of the stress fiber [19].

Image Processing

Fluorescence images were recorded with a Dage-MTI (Michigan City, Ind.) ISIT image intensifier coupled to a digital image processor [19, 26, 27]. The distance between two points in an image was measured using a graphics tablet (GTCO, Rockville, Md.). The contrast of the image was enhanced by image processing to facilitate the visualization of the bleached spot. The location and boundaries of bleached spots on stress fibers were usually easily discernible for 5–10 min in actin-injected cells and for up to 20 min in α -actinin-injected cells. Movement of bleached spots was detected by measuring the distance between the center of the bleached spot and the distal tip of the stress fiber in several (>3) successive images. Distance calculations were based on the number of pixels between points: each pixel is equivalent to an area 0.1 \times 0.08 μ m. Since the stage usually remained stationary throughout the experiment, any movement of the stress fiber tip was easily detected. Only cells in which the distal tip of the stress fiber could be reliably identified and in which no movement of the stress fiber as a whole was detected were analysed. Repeated measurements of a distance between the bleached spot and the stress fiber tip usually varied by less than 0.25 μ m. Cells which were processed for anti-myosin staining were fixed as soon as enough images had been collected to determine whether the bleached spot was moving.

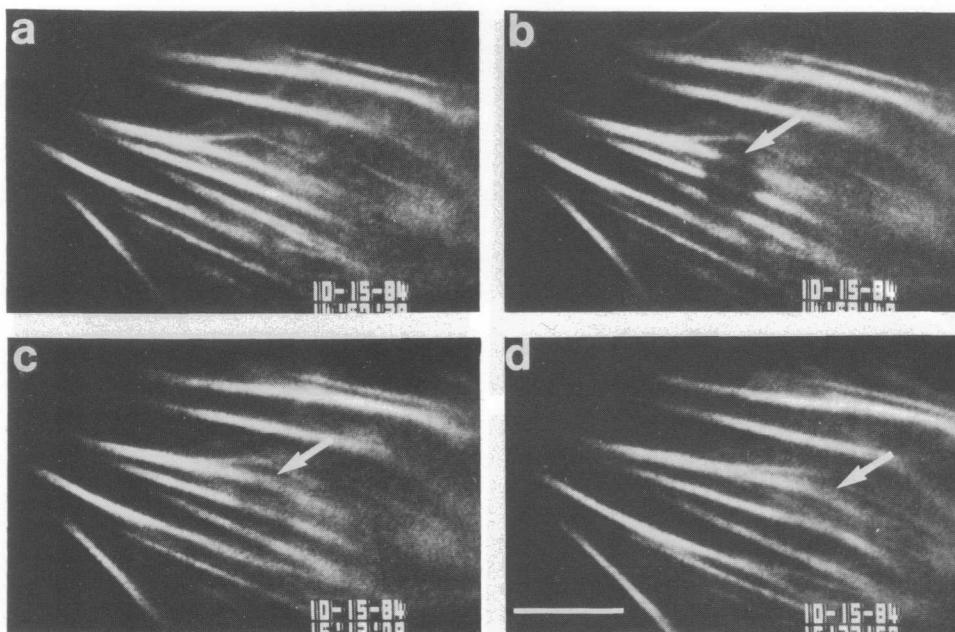


Fig. 1. (a) Fluorescent stress fibers in an IATR α -actinin-injected fibroblast. (b) A photobleached spot (arrow) is initially very well defined. (c) Thirteen min and (d) 34 min later, considerable recovery has occurred, but the location of the spot is still recognizable by the slightly less intense fluorescence in that area. Comparison of (b), (c) and (d) indicates that the spot has translocated toward the center of the cell (right) at a rate of approx. $0.18 \mu\text{m}/\text{min}$, while the distal end of the stress fiber does not move. Bar, $10 \mu\text{m}$.

RESULTS

Alpha-actinin

Injected α -actinin soon become localized in stress fibers [19]. When a spot on a stress fiber was photobleached, the bleached spot was easily discernible for about the first 10–20 min. Thus the distance between the center of the bleached spot and the distal end (the end closest to the edge of the cell) of a well defined stress fiber or the edge of the cell could be measured accurately. Data were collected only from stress fibers whose distal ends remained stationary throughout an experiment. The proximal ends (the ends closer to the nucleus) of many stress fibers were poorly defined and underwent rapid changes, and could not be used as reference points. Although spots bleached on stress fibers in most cells did not move relative to the distal end of the stress fiber, clear translocation away from the distal end of the stress fiber was detected along some stress fibers in the apparently trailing processes (processes which extend in a direction opposite or nearly opposite to that of the largest ruffle) of elongated, polygonal cells. Stress fibers located near the largest expanse of ruffle in such cells did not exhibit the phenomenon. Generally, 25–50% of the bleached stress fibers in the appropriate

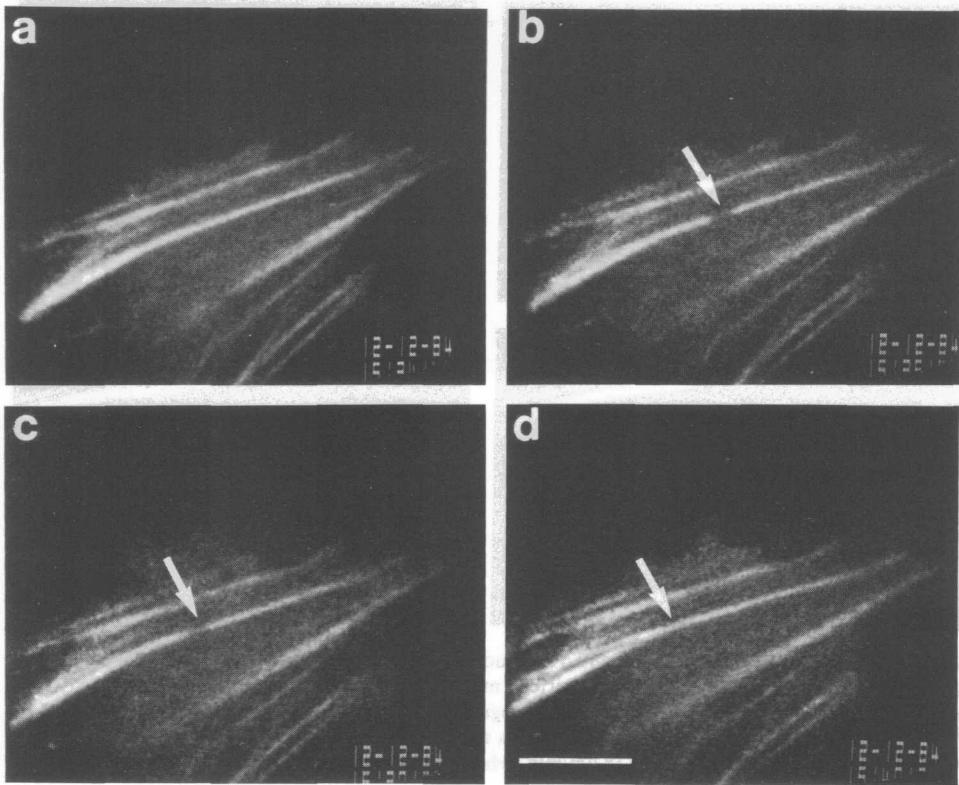


Fig. 2. (a) Fluorescent stress fibers in an LRB actin-injected cell before bleaching. (b) Immediately after bleaching, the bleached spot is well-defined (arrow). It is still recognizable, despite rapid recovery, 3 min (c) after bleaching. (d) Five min after bleaching, the bleached spot is almost invisible to the eye, although computer analysis indicates that fluorescence recovery is still incomplete. Comparison of (b), (c) and (d) indicates that the bleached spot has moved centripetally along the stress fiber at a rate of $0.37 \mu\text{m}/\text{min}$. Bar, $10 \mu\text{m}$.

location of the appropriate cell type exhibited measurable translocation of the bleached spot. We did not detect this phenomenon in very well spread fibroblasts or in wedge-shaped or half-moon-shaped cells. The latter generally have few or poorly defined stress fibers and are generally thought to be rapidly locomoting.

Fig. 1 shows an example of a bleached spot which moved centripetally along a stress fiber. The rate of translocation ranged from 0.10 to $0.47 \mu\text{m}/\text{min}$ ($0.24 \pm 0.12 \mu\text{m}/\text{min}$ from 25 stress fibers in 25 cells). Bleached spots on adjacent, parallel stress fibers usually translocated at similar rates (fig. 1). However, frequently, processes contained both stress fibers which demonstrated translocation of bleached spots and stress fibers which did not.

The rate of movement of a bleached spot was independent of its location along a large segment of the stress fiber. The mean rate of translocation for spots

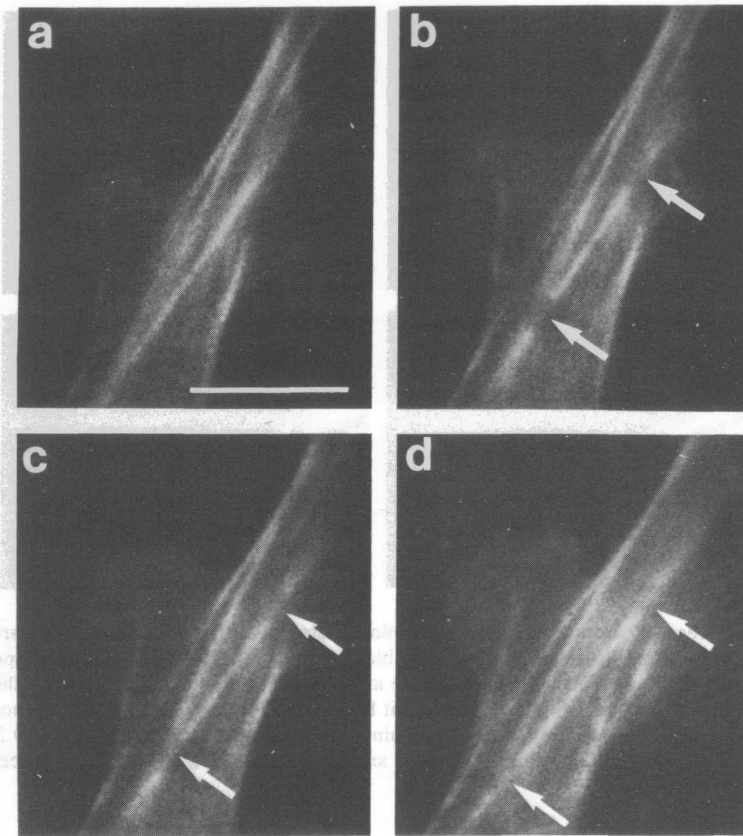


Fig. 3. (a) Stress fibers in an α -actinin-injected cell before two spots are bleached on a single stress fiber. Locations (arrows) of the bleached spots (b) immediately after second bleaching, (c) 3 min and (d) 10 min later. The distance between the bleached spots in (b), 11.3 μm , has increased to 12.3 μm in (c) and to 15 μm in (d). The rate of movement calculated for the proximal bleached spot, 0.47 $\mu\text{m}/\text{min}$, is greater than that calculated for the distal bleached spot, 0.10 $\mu\text{m}/\text{min}$. Bar, 10 μm .

bleached 5–10 μm from the distal end (0.22 ± 0.10 $\mu\text{m}/\text{min}$, $n=16$) was essentially identical with the rate for spots bleached more than 10 μm from the distal end (0.21 ± 0.10 $\mu\text{m}/\text{min}$, $n=16$). However, as will be discussed below, spots bleached very close to the distal end of the stress fiber often moved slowly.

Actin

Injected rhodamine-labeled actin also became localized along stress fibers rapidly [18, 26]. When a spot was bleached on an actin-labeled stress fiber, results similar to those for α -actinin were obtained (fig. 2). Translocating bleached spots moved centripetally at rates of 0.17–0.42 $\mu\text{m}/\text{min}$ (0.29 ± 0.11 $\mu\text{m}/\text{min}$ from six stress fibers in five cells), which were similar to the rates obtained with α -actinin.

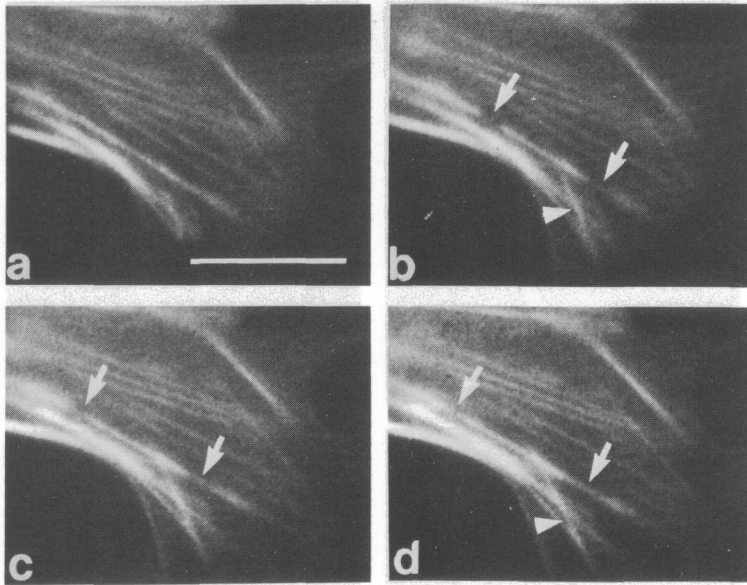


Fig. 4. (a) Fluorescent stress fibers in an α -actinin-injected fibroblast before two spots are bleached on a single stress fiber. (b) Immediately after bleaching, two well defined bleached spots can be observed (arrows) $8.2 \mu\text{m}$ apart. (c) One minute after bleaching, the distance between the bleached spots is $8.4 \mu\text{m}$, while 8 min (d) after bleaching, it has increased to $9.6 \mu\text{m}$. The rate of movement for the distal spot during this period was $0.11 \mu\text{m}/\text{min}$; the rate for the proximal spot was $0.29 \mu\text{m}/\text{min}$. One stress fiber (arrowhead) is elongating and its angle relative to the long axis of the process changes during this sequence. Bar, $10 \mu\text{m}$.

Two Bleached Spots on a Stress Fiber

One possible explanation for the centripetal translocation of bleached spots along a stress fiber is that the portion of the fiber between the bleached spot and the distal end is undergoing stretching, perhaps as a prelude to retraction. To test this hypothesis, two spots were bleached on the same fiber, and the distance between them was measured as a function of time. When both spots were located more than $5 \mu\text{m}$ from the distal end, no change in distance between them was detectable. This was consistent with the relatively constant rates of movement mentioned above. However, when one spot was located close to the distal end ($<5 \mu\text{m}$) and the other spot was several μm closer to the nucleus, clear increases in distance were detected in some stress fibers. Attempts to follow spots bleached at the very tip of stress fibers were unsuccessful, mainly because the rapid recovery of fluorescence in this region resulted in the loss of definition of the bleached spots [19].

An example of such an increase in distance between two bleached spots is shown in fig. 3. The rate of movement for the bleached spot closest to the distal end was $0.10 \mu\text{m}/\text{min}$, almost too low to be considered to be moving, whereas the rate for the more central spot was $0.47 \mu\text{m}/\text{min}$. Similarly, in a second example

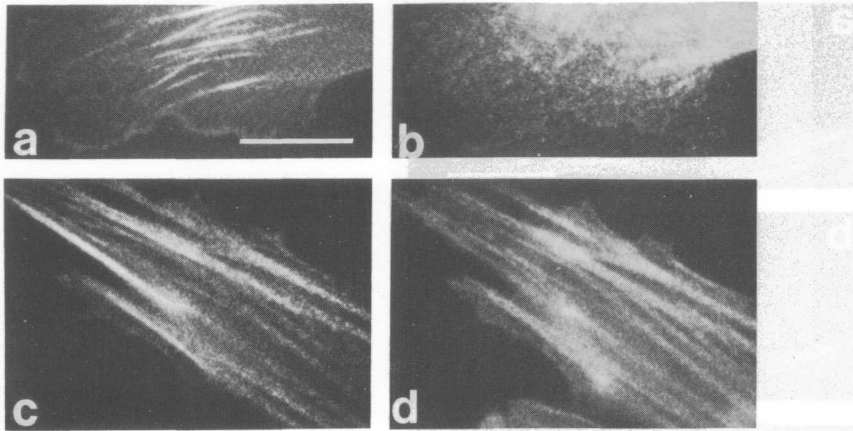


Fig. 5. Alpha-actinin-injected cells which were fixed in 3.76% formaldehyde, extracted in acetone, and stained with rabbit antimyosin antibody and fluorescein-labeled goat anti-rabbit antibody. Some stress fibers which have incorporated α -actinin (a), do not stain at all with anti-myosin antibody (b), whereas others which have incorporated α -actinin (c), stain heavily with anti-myosin antibody (d). Bar, 10 μ m.

(fig. 4), the rate for the more distal spot was 0.11 μ m/min, whereas the rate for the more central spot was 0.29 μ m/min. The diameter of the distal bleached spot also increases in size from 1.6 μ m in fig. 4b to 2.6 μ m in fig. 4d. In this same figure, changes in the position of a stress fiber which was apparently attached to the bleached fiber suggest that the fiber is being pulled into a position parallel to the long axis of the process.

Sequences such as those shown in figs 3 and 4 suggest that stretching may occur between the bleached spots along some stress fibers. However, in many double bleach experiments, the distance between the two spots did not change appreciably, and the rates of movement for both bleached spots were very similar. These negative results may be explained if stretching occurs only near the distal end of the stress fiber. However, other mechanisms cannot be ruled out (see Discussion).

Myosin Immunofluorescence

The translocation of bleached spots along stress fibers suggests that there might be a gradient of contractile force along some stress fibers. Therefore, we examined the localization of myosin, which is presumably responsible for the generation of force along stress fibers. Alpha-actinin-injected cultures were processed for myosin indirect immunofluorescence using fluorescein-labeled secondary antibodies. The images of rhodamine α -actinin were then compared with those of the fluorescein antibody. Although the great majority of α -actinin

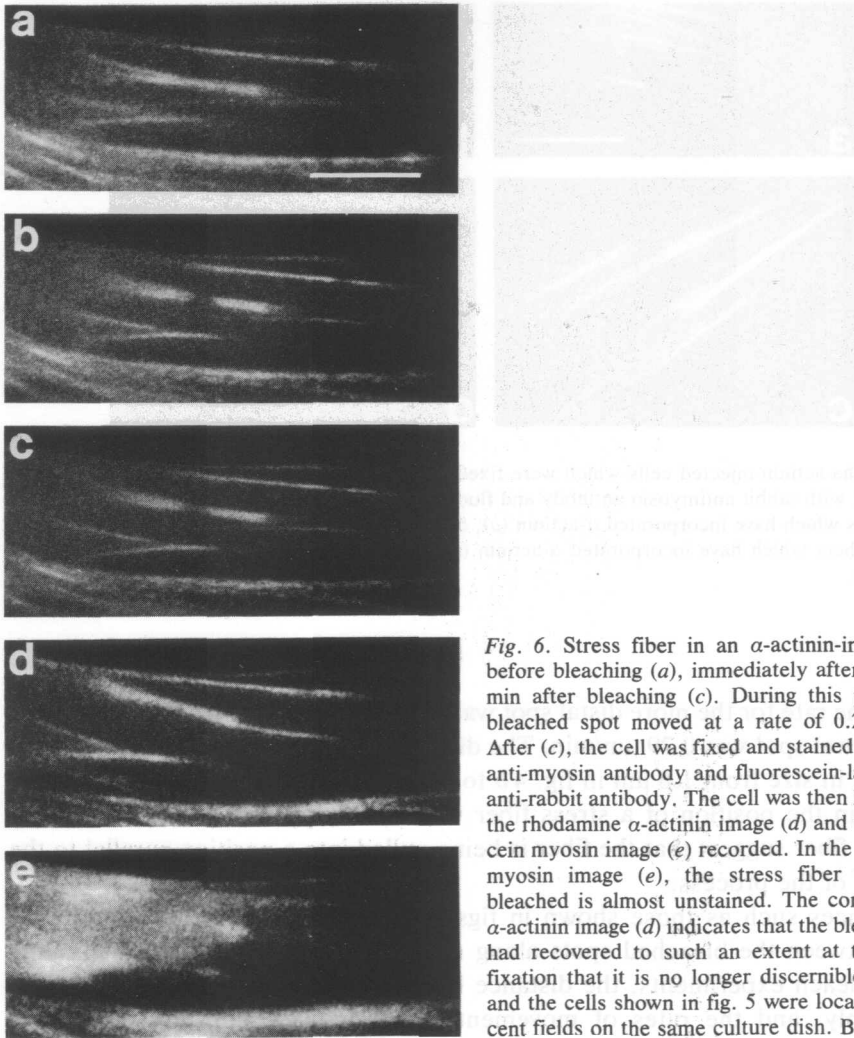


Fig. 6. Stress fiber in an α -actinin-injected cell before bleaching (a), immediately after (b), and 4 min after bleaching (c). During this period, the bleached spot moved at a rate of $0.25 \mu\text{m}/\text{min}$. After (c), the cell was fixed and stained with rabbit anti-myosin antibody and fluorescein-labeled goat anti-rabbit antibody. The cell was then located and the rhodamine α -actinin image (d) and the fluorescein myosin image (e) recorded. In the fluorescein myosin image (e), the stress fiber which was bleached is almost unstained. The corresponding α -actinin image (d) indicates that the bleached spot had recovered to such an extent at the time of fixation that it is no longer discernible. This cell and the cells shown in fig. 5 were located in adjacent fields on the same culture dish. Bar, $10 \mu\text{m}$.

containing stress fibers in our cultures were well-stained with anti-myosin antibody (fig. 5c, d), some were only lightly stained or were completely unstained (fig. 5a, b). A single cell might contain only well-stained fibers, only unstained fibers, or even both types of fiber. Completely unstained fibers were often observed in the vicinity of ruffles, a region in which we did not observe translocation. When we fixed and stained cells in which translocation had been detected, five of six stress fibers which exhibited translocation before fixation were lightly stained with anti-myosin antibody (fig. 6).

DISCUSSION

The simplest explanation for these results is that actin and α -actinin can move either actively or passively along some stress fibers toward the center of the cell. Several arguments support the idea that such translocation represents a normal physiological process rather than a laser-induced artifact. First, we have demonstrated previously that the integrity of stress fibers is not affected by photo-bleaching, as revealed by staining with fluorescent phalloidin [19]. Second, if the translocation were induced by the laser pulse, one would not expect it to be limited to a discrete population of stress fibers or for its rate to be unaffected by multiple bleaches on the same stress fiber. Nor would one expect it to be unidirectional, especially since the repair of stress fibers severed by intense laser irradiation is not directional [13].

Since the rate of movement of the bleached spot is similar in both actin- and α -actinin-injected cells, it is likely that this process involves the entire macromolecular assembly along the stress fiber. One mechanism which could cause proteins to apparently translocate along stress fibers is passive stretching. Stress fibers are similar to skeletal muscle fibers in their protein composition and their structural organization [8, 10, 21], characteristics which permit muscle fibers to be passively stretched. When a stress fiber is placed under tension, actin filaments may slide along each other as do filaments in muscle fibers. Stretching is consistent with the observed increase in distance between two spots bleached on the same stress fiber and the increase in diameter of some bleached spots. A stretching mechanism would also predict that the stress fiber would increase in length, but since the proximal ends of stress fibers are often poorly defined, data on the total length of these stress fibers are unreliable. That a stress fiber can actually be stretched *in situ* is, however, shown in fig. 4, where a short fiber changes its position and seems to become elongated as a result of association with a neighboring fiber which exhibits translocation of a bleached spot.

A second mechanism which could account for the directional movement of actin and α -actinin toward the nucleus is 'treadmilling'. Observations consistent with 'treadmilling' have been reported for actin filaments in the leading edge of gerbil fibroma cells [27]. Although actin filaments along the length of stress fibers are of mixed polarity [2, 20], and cannot support a directional translocation through treadmilling, the membrane-associated tips of stress fibers are comprised of actin filaments arranged in a specific polarity with the 'fast' ends of filaments associated with the membrane [20]. The preferential addition of new actin subunits at the membrane-associated end of the stress fiber could displace older subunits and their associated proteins away from the membrane. The mixed polarity of the more interior filaments, which may be achieved through continuous random exchange with actin outside stress fibers, would not interfere with this process, since only the short membrane-associated region would be responsible for the observed movement.

Differences in the staining of stress fibers with anti-myosin antibody have been described briefly by Fujiwara & Pollard [6]. Further characterization of the extent of variation in the myosin content of stress fibers and the significance of this variation should be very interesting. Clearly, stress fibers that exhibit centripetal translocation of bleached spots are relatively depleted in myosin. Thus, if stretching is responsible for the translocation of actin and α -actinin, it is probably achieved either passively through forces exerted by other structures or through the residual myosin molecules still associated with the fiber. In addition since myosin may act to stabilize actin-containing structures, its depletion may destabilize these stress fibers and allow stretching or treadmilling to occur. The recent report that microinjected fluorescently labeled calmodulin becomes associated with some stress fibers and not with others [16] also suggests that there may be different populations of stress fibers, which vary either in their content of certain proteins or in their abilities to incorporate proteins.

The physiological significance of the translocation of proteins along stress fibers is not clear. However, it is interesting to speculate why this process occurs predominantly at the apparently trailing end of the cell. It is possible that the processes of fibroblasts may create different amounts of tension during their extension or retraction, resulting in a sort of tug of war among the various processes. Stress fibers within the retracting end would more likely be stretched and pulled toward the nucleus. The importance of tension as an organizational force has been postulated for neurites [12] and for fusing myoblasts [22, 25]. Alternatively, centripetal translocation of proteins along stress fibers may be part of an active mechanism responsible for the retraction of the trailing end during cell movement.

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