# Exchange of Actin Subunits at the Leading Edge of Living Fibroblasts: Possible Role of Treadmilling

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ABSTRACT Previous observations indicated that the lamellipodium ("leading edge") of fibroblasts contains a dense meshwork, as well as numerous bundles (microspikes) of actin filaments. Most, if not all, of the filaments have a uniform polarity, with the "barbed" end associated with the membrane. I investigated whether and how actin subunits exchange in this region by microinjecting living gerbil fibroma cells (IMR-33) with actin that had been labeled with iodoacetamidotetramethylrhodamine. After incorporation of the labeled actin into the lamellipodium, I used a laser microbeam to photobleach a 3–4-$\mu$m region at and surrounding a microspike, without disrupting the integrity of the structure. I then recorded the pattern of fluorescence recovery and analyzed it using a combination of TV image intensification and digital image processing techniques. Fluorescence recovery was first detected near the edge of the cell and then moved toward the cell's center at a constant rate of 0.79 ± 0.31 $\mu$m/min. When only part of the lamellipodium near the edge of the cell was photobleached, the bleached spot also moved toward the cell's center and through an area unbleached by the laser beam. These results indicated that steady state incorporation of actin subunits occurred predominantly at the membrane-associated end of actin filaments, and that actin subunits in the lamellipodium underwent a constant movement toward the center of the cell. I suggest that treadmilling, possibly in combination with other molecular interactions, may provide an effective mechanism for the movement of actin subunits and the protrusion of cytoplasm in the lamellipodium of fibroblasts.

The organization of actin filaments in many types of nonmuscle cells has been investigated extensively during the past years. Through a combination of light- and electron-microscopic observations, it is clear that actin filaments are capable of rapid rearrangement in nonmuscle cells (see, for example, reference 26). However, at the molecular level, little is known about how this dynamic reorganization is achieved.

In vitro, actin is capable of a wide spectrum of activities, including polymerization, gelation, bundling, fragmentation, and translocation, either by itself or in combination with other proteins (for reviews see references 5, 15, and 21). Of particular significance is the distinct polarity of the filaments, which not only causes myosin heads to interact with the filaments at a preferred angle but also accounts for the different rates of assembly at the two ends. The fast, or "barbed," end has a higher rate of assembly as compared with the slow, or "pointed," end (11, 31). In 1976, Wegner raised the possibility that the two ends could have different critical concentrations of polymerization, thus causing the subunits to "treadmill" continuously through the filament at steady state (29). Subsequent measurements of kinetic constants at the two ends indicated that treadmilling may indeed occur in vitro (4, 16). In addition, Kirschner pointed out that treadmilling may play various important roles, ranging from transport of organelles to regulation of filament stability, in living cells (10). However, without direct correlation with observations in vivo, it is not even certain whether this phenomenon actually occurs in living cells.

A particularly interesting area in which to study the dynamic behavior of actin in vivo is the lamellipodium ("leading edge") of fibroblasts. Previous electron microscopic observations demonstrated the presence of a meshwork of apparently continuous actin filaments, as well as numerous actin filament bundles (microspikes), extending from the edge of the cell

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toward the center (18–20). In addition, most, if not all, actin filaments in this region have a uniform polarity, with the fast, or "barbed" ends associated with the membrane (19). Myosin, on the other hand, is either absent or present in only a very small amount (7). The dynamic aspects of actin filaments in this region, however, remain largely unknown. Kreis et al. have studied the mobility of actin in embryonic chick fibroblasts (12). They measured the percentage and the apparent diffusion coefficient of the "mobile" fraction, which presumably corresponds to actin monomers or oligomers, in the lamellipodium. However, since the exchange of subunits would probably fall under the "immobile" (apparent diffusion coefficient $<3 \times 10^{-12}$ cm$^2$/s) category when their approach is used, few important questions about subunit exchange were answered. For example, does subunit exchange occur in this region? If so, does it occur along the length of the filament or only at the ends? Is the fast end, which appears to be blocked by the attachment to the membrane, active in subunit exchange?

I have been trying to answer these questions through the use of fluorescent analogue cytochemistry (22, 27), in conjunction with the technique of fluorescence recovery after photobleaching. In brief, my approach involves the microinjection of fluorescently labeled actin into living gerbil fibroma cells (IMR-33). After the incorporation of the fluorescent actin analogue into cellular structures, fluorescence in a small area of the lamellipodium is bleached with a laser microbeam without damaging the structure. The recovery of fluorescence is then measured. Since I am interested in not only the rate, but also the pattern, of recovery, I use a TV image intensifier to record the image (17), and analyze the data with digital image processing techniques. The results not only indicate a clear polarity in steady state subunit incorporation, favoring the membrane-associated end, but also reveal a constant flux of actin subunits toward the center of the cell. I suggest that treadmilling, possibly in combination with other molecular interactions, may play an important role in this region of the cell.

## MATERIALS AND METHODS

*Protein Preparation and Cell Culture:* Actin was prepared from rabbit skeletal muscle and labeled with iodoacetamidotetramethylrhodamine (Research Organics, Inc., Cleveland, OH) as described previously (26). The gerbil fibroma cell line IMR-33 was obtained from American Type Culture Collection (Rockville, MD), and maintained in F12K medium (KC Biological Inc., Lenexa, KS) containing 10% fetal calf serum (KC Biological Inc.), 50 U/ml penicillin, and 50 μg/ml streptomycin. The cells were replated 16–20 h before an experiment in Dulbecco's modified Eagle's medium (Gibco Laboratories, Grand Island, NY) containing 2% fetal calf serum and antibiotics as described above. This condition facilitates the formation of lamellipodium. The culture dish, formed by attaching a coverslip to the bottom of a Plexiglas plate, was described previously (26).

*Microinjection and Photobleaching:* Cells were maintained on the microscope stage under a Plexiglas incubator (26). Microinjection was performed as described by Graessman et al. (6). The intracellular concentration of labeled actin was estimated to be 5–10% that of endogenous actin. The culture dish was returned to the regular $CO_2$ incubator for 1 h after microinjection to allow complete dispersion and incorporation of injected molecules.

Photobleaching was performed with a laser microbeam at a wavelength of 515 nm, generated from a 2-W argon ion laser (model 94; Lexel Corp., Palo Alto, CA). The optical components for the formation of microbeams were essentially as described by Jacobson et al. (8). The duration of the laser pulse was controlled by an electronic shutter and a shutter timing control unit (Vincent Associates, Rochester, NY). Cells were bleached with a power of 50–75 mW for 20–50 ms. The bleached area had a diameter of 3–4 μm. Similar results were obtained from cells bleached to various extents.

*Image Recording and Analyses:* Fluorescence images were observed with a Zeiss IM35 microscope equipped with an epi-illuminator and either a 100X/numerical aperture 1.30 Neofluar or a 63X/numerical aperture 1.25 Neofluar objective (Carl Zeiss Inc., Thornwood, NY). A 100-W quartz–halogen lamp, operated at an input of 6–7 V, was used as the light source. No cell damage was detectable during image recording. Images were recorded using an intensified silicon intensified target video camera (model ISIT-66; Dage-MTI Inc., Michigan City, IN) coupled to an image processing computer (G. W. Hannaway & Associates, Boulder, CO). The signal-to-noise ratio of the image was improved dramatically by averaging 128 frames. Averaged images were then stored on a hard disk (Fujitsu America Inc., Santa Clara, CA). Each image was stored in 512 pixel × 480 pixel × 8 bit. With a 100X objective, each pixel corresponded to an area of 0.1 × 0.08 μm.

Distance measurements were performed by specifying points on the monitor using a graphics tablet (GTCO Corp., Rockville, MD) and calculating the distance based on the number of pixels between the points, taking into account the rectangular shape of the pixel. To obtain the intensity profile along the spike, background intensity immediately outside the edge of the cell was first averaged and subtracted from the entire image. The cursor, controlled using the graphics tablet, was then moved along the image of the spike on the monitor, and intensities in a 5 × 5 pixel area surrounding various points were averaged. Phase-contrast images were recorded with a vidicon camera (model TC2521; RCA Electro-Optics & Devices, Lancaster, PA) then contrast enhanced using a shadow-casting convolution algorithm.

*Phalloidin Staining:* Cells were injected and photobleached as described above. Immediately after photobleaching, an averaged image was stored, the exact position of the cell was marked, and the coverslip was removed for fixation. The interval between photobleaching and fixation was ~1–2 min. Fixation was performed as described by Small et al. (20), using a mixture of 0.5% glutaraldehyde and 0.3% Triton X-100 during the first step and 1% glutaraldehyde during the second step. The coverslip was rinsed after the second fixation, treated with sodium borohydride, and then stained with fluorescein-phalloidin (Molecular Probes, Junction City, OR) without further extraction in Triton. Fluorescence of fluorescein-phalloidin was observed with a Zeiss 48 77 16 filter set and an extra barrier filter (cut-off wavelength 550 nm). No crossover of rhodamine fluorescence was detectable. Fluorescence of tetramethylrhodamine-labeled actin was observed using a Zeiss 48 77 15 filter set, which excludes any fluorescein fluorescence to a level beyond detection.

## RESULTS

When cultured under appropriate conditions, >80% of the IMR-33 cells showed prominent leading lamellipodium. Most cells, however, exhibited little net translocation during the period of observation (<10 min). A pair of photographs of a living cell microinjected with tetramethylrhodamine-actin, taken with phase optics and fluorescence optics, respectively, are shown in Fig. 1. The lamellipodium, 2–6 μm wide, was discerned under phase optics as a slightly phase-dense band. In addition, microspikes, either embedded within the lamellipodium or projecting out of the cell, were readily detected. The stress fibers were located in the more central region of the cell. Under fluorescence optics, the lamellipodium showed an increased concentration in microinjected actin compared to the adjacent cytoplasm, as well as distinctly fluorescent microspikes. The general morphology of the lamellipodium was similar to that described by Abercrombie et al. (2) and Small et al. in chick embryonic fibroblasts (18, 20).

Because of the discrete morphology of the microspikes, which facilitates the analysis and interpretation of data, photobleaching was performed predominantly at microspikes and adjacent areas. However, similar results were also obtained in areas of diffuse fluorescence through the lamellipodium. The microspikes were highly dynamic structures, with a lifetime usually shorter than 10 min. Besides assembly and disassembly, reorientation, merging, and splitting of microspikes were frequently observed. The rapid change in microspikes has limited the period of time for the observation of fluorescence recovery after photobleaching. However, as described below, since the recovery process was fast, I was able to follow

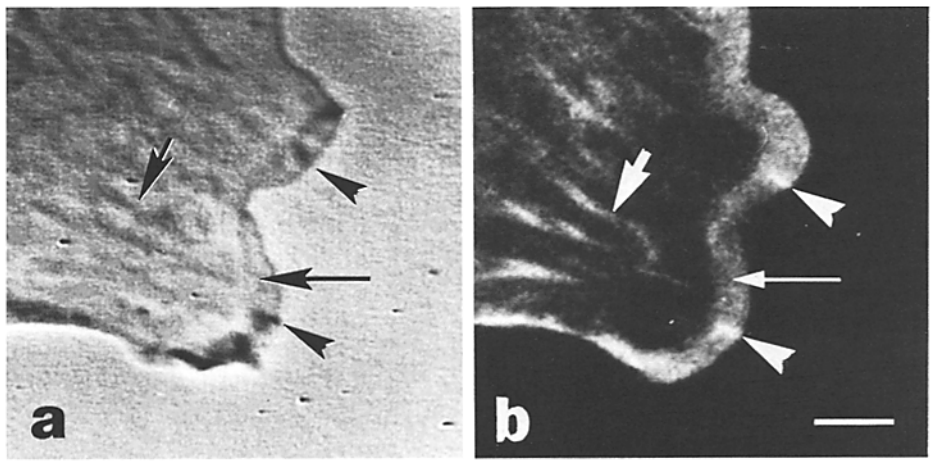


FIGURE 1 A living IMR-33 cell cultured under the specified condition and microinjected with iodoacetamidotetramethylrhodamine-labeled actin. The lamellipodium (long arrow) and microspikes (arrowheads) are evident in *a*, the computer-enhanced phase-contrast image, and in *b*. Stress fibers are located in the more posterior region of the cell (short arrow). Fluorescence image of injected actin is shown in *b*. Some changes at the lamellipodium occurred during the time required to change from the vidicon camera to the intensified silicon intensified target image intensifier. Bar, 5 μm. × 1,800.

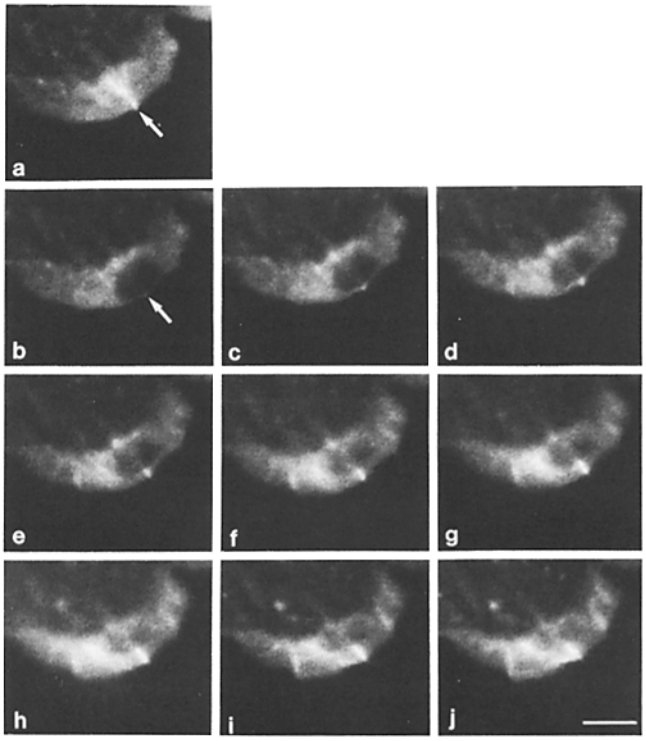


FIGURE 2 Fluorescence recovery of an almost completely bleached microspike. The cell before photobleaching is shown in *a*. A microspike (arrow) and its surrounding area are photobleached at $t = 0$. Images are then recorded at $t = 35$ s (*b*), 63 s (*c*), 95 s (*d*), 123 s (*e*), 160 s (*f*), 188 s (*g*), 217 s (*h*), 245 s (*i*), and 275 s (*j*). The recovery begins at the membrane and moves toward the center of the cell. Bar, 5 μm. × 1,570.

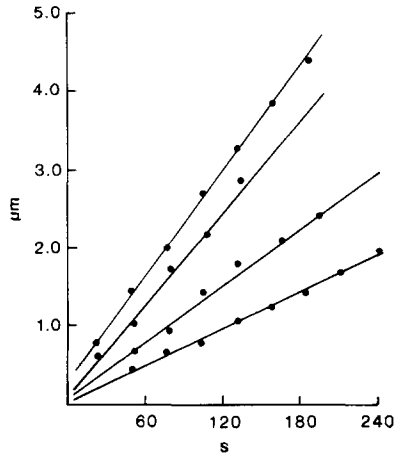


FIGURE 3 Time course of the fluorescence recovery. Lengths of the microspikes during recovery are measured and plotted as a function of time. The lines are obtained by least-square fitting. Data from four different cells are shown here.

microspikes through most of the recovery processes.

Through the use of a laser microbeam, an area 3–4 μm in diameter was bleached at and surrounding a microspike. The recovery of fluorescence after photobleaching followed a highly polar pattern. It was first detected near the edge of the cell and then moved toward the cell's center (Fig. 2). Both the microspikes and the surrounding diffuse area have similar kinetics, forming a uniform boundary during recovery. When the length of the recovered segment of the microspike was plotted against time, a straight line was obtained. From nine such plots, I obtained a rate of movement of $0.79 \pm 0.31$ μm/min (Fig. 3).

If the laser beam was aligned so that only the distal portion of the microspike was photobleached, the subsequent recovery appeared to involve a translocation of the bleached spot toward the center of the cell and through an area unbleached by the laser beam (Fig. 4). This was clearly demonstrated with the intensity profiles of the microspike during various stages of recovery (Fig. 5).

The pattern and kinetics of recovery were specific for actin. When tetramethylrhodamine-labeled ovalbumin was microinjected into the cell, it entered the lamellipodium with a slightly higher intensity than it entered the adjacent cytoplasm. This was probably due to a larger accessible volume in the lamellipodium (27). However, when the fluorescence was photobleached, the recovery was so fast that no bleached spot could be detected even in the first averaged image immediately after photobleaching (not shown).

To ensure that the recovery of fluorescence reflected normal exchange of actin subunits rather than repair of structures damaged by the laser beam, a microspike was partially bleached at the membrane-associated end. Immediately after the recording of the bleached image, the cell was extracted and fixed using a protocol that preserves the lamellipodium (20). Actin in the remaining cytoskeleton was stained with fluorescein-phalloidin. As shown in the image of injected actin (Fig. 6 *c*), some recovery occurred near the edge of the cell before fixation. However, the bleached spot was still clearly

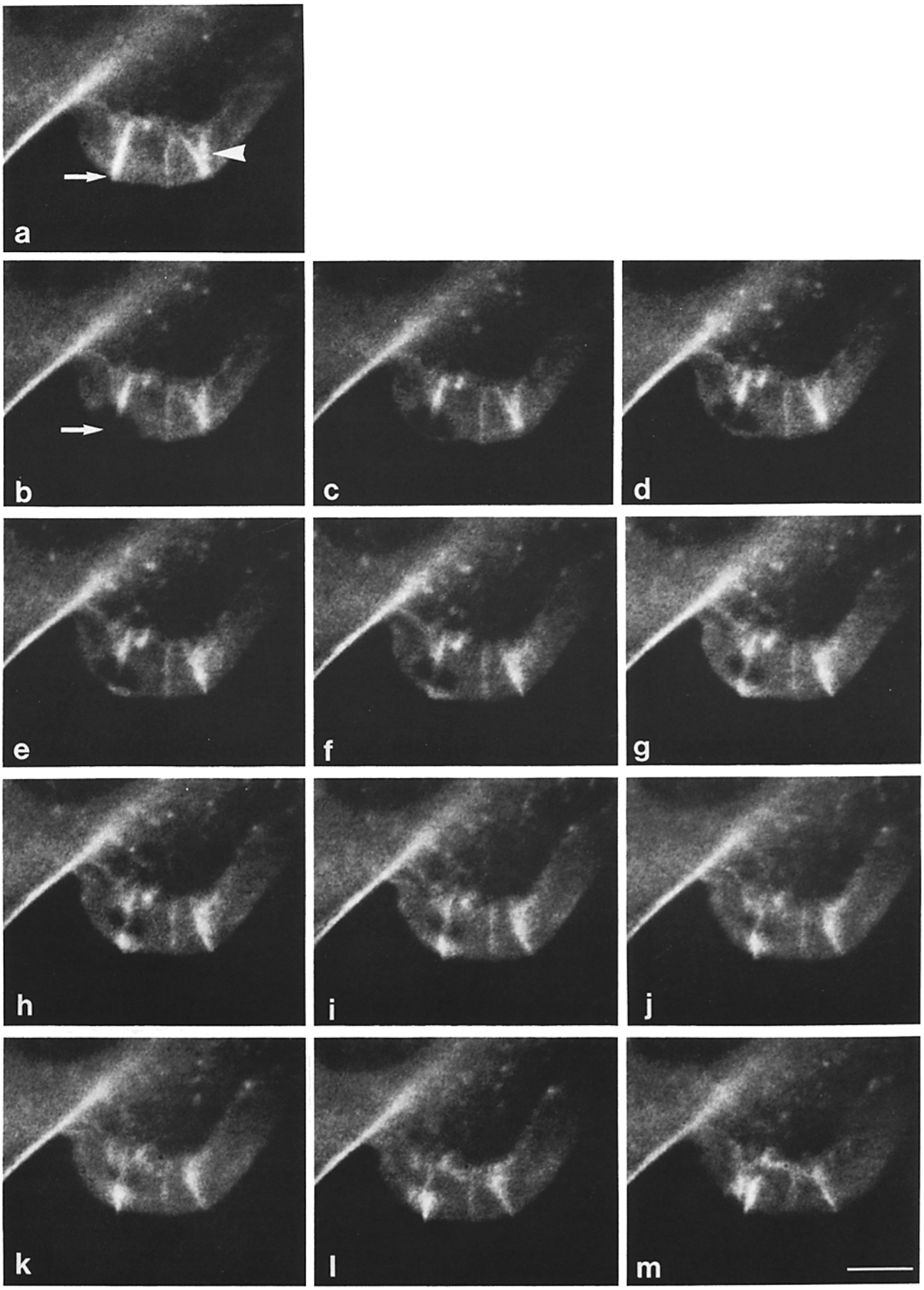


FIGURE 4 Fluorescence recovery of a microspike partially bleached near the edge of the cell. The cell before bleaching is shown in *a*. A small portion of a microspike (arrow) near the edge of the cell is bleached at $t = 0$. Images are then recorded at $t = 24$ s (*b*), 50 s (*c*), 77 s (*d*), 105 s (*e*), 133 s (*f*), 159 s (*g*), 185 s (*h*), 212 s (*i*), 241 s (*j*), 274 s (*k*), 307 s (*l*), and 438 s (*m*). The microspike later splits into multiple small microspikes (*m*). Two neighboring unbleached microspikes merge and subsequently undergo partial disassembly during the period of observation (arrowhead). Bar, 5 μm. × 3,700.

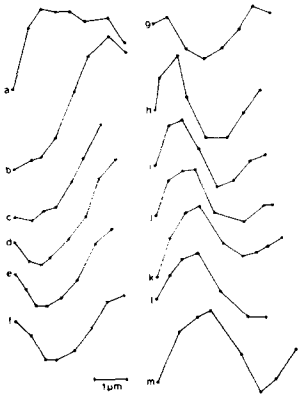

FIGURE 5 Intensity profiles of the microspike shown in Fig. 4. Intensities in a 5 × 5 pixel area surrounding various points along the microspike are averaged and plotted as a function of the distance from the membrane-associated end. Each line represents the profile of the microspike in the corresponding micrograph shown in Fig. 4.

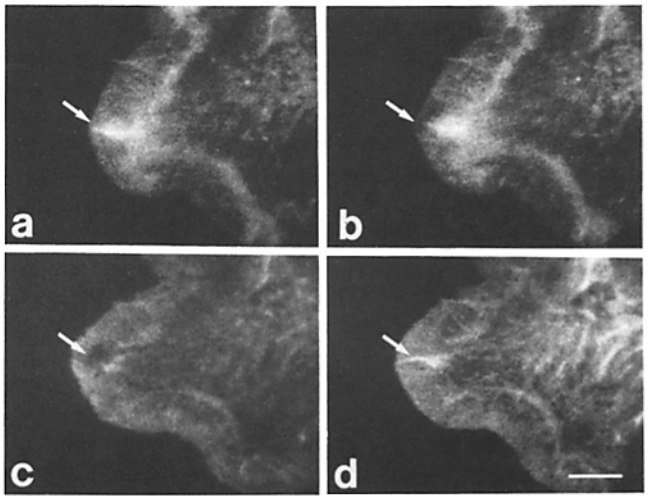


FIGURE 6 Phalloidin staining of a photobleached microspike. The living cell and the microspike (arrow) before bleaching are shown in *a*. The microspike was partially bleached near the edge of the cell (*b*). The cell was then fixed, extracted, and stained with fluorescein-phalloidin. The bleached spot is still readily detectable in the image of injected actin after staining, although it had moved toward the center of the cell before the cell was fixed (*c*). The image of fluorescein-phalloidin indicates that the integrity of the microspike is maintained during recovery (*d*). Bar, 5 μm. × 1,180.

detectable. The image of fluorescein-phalloidin, on the other hand, showed an intact microspike similar to that before photobleaching (Fig. 6 *d*).

## DISCUSSION

One important concern for experiments that involve photobleaching recovery is the possibility of structural damage induced by the intense laser beam. Although it is difficult to rule out entirely the possibility of a laser-induced artifact, several arguments indicate that it is unlikely. First, the results with phalloidin-stained Triton cytoskeletons indicate that the microspike maintains its structural integrity, its resistance to Triton extraction, and its phalloidin binding sites after a partial photobleaching at the membrane-associated end. If recovery involved a repair of the damaged part of the spike rather than an exchange of subunits, then during recovery of partially bleached spikes one would expect to detect a discontinuity along the length of the spike with phalloidin staining. This is not the case here. In addition, if the physical continuity of the spike were disrupted at a level beyond the resolution of fluorescence microscopy, it would be difficult to explain the transport of the bleached spot along the microspike toward the center of the cell, as shown in Figs. 4 and 5. Finally, the pattern and the rate of recovery are not affected by the extent of photobleaching. If photobleaching damaged the actin in a way that affects its dynamic behavior, one would expect to detect a change in recovery dynamics and/or pattern as a function of the extent of photobleaching.

An explanation of the observed pattern of fluorescence recovery should consider several possible mechanisms. First, actin filaments in the lamellipodium may continuously detach from the edge of the cell and move toward the center. In addition, assembly of new filaments may occur constantly near the edge. This mechanism is improbable, since, under the electron microscope, most filaments in this region appear to associate with the edge of the cell and extend all the way across the lamellipodium (18, 20). Alternatively, if filaments are indeed continuous and maintain a constant length during recovery, the highly biased pattern of recovery can only be generated by a highly biased incorporation of subunits into the membrane-associated end, even though this end appears to be blocked by the membrane association. In addition, since both the microspikes and the surrounding area recover at a similar rate, forming a uniform boundary, it is likely that the entire lamellipodium is covered with filaments of similar kinetic properties.

At least in three specialized systems, similar accessibility of actin filaments at the fast, membrane-associated end has been suggested. First, Mooseker et al. observed that when isolated intestinal brush border was incubated with excess actin monomers, the core microfilaments, which have a uniform polarity with the fast ends associated with the membrane, can incorporate additional actin subunits at the membrane-associated end (14). Second, Tilney and Inoué analyzed the extension of the acrosomal process of the *Thyone* sperm (25). The elongation of actin filaments slowed with time, consistent with a requirement for the subunits to diffuse to the distal, fast end. Finally, Tilney et al. examined the pattern of the elongation of actin bundles in differentiating *Limulus* spermatids (24). Since the number of filaments of the bundle during early stages of differentiation was similar to that at the slow end in the mature sperm, they suggested that it was probably the fast, membrane-associated end that elongated during differentiation. The present experiments indicate that accessibility and exchangeability of subunits may be a general property of the fast, membrane-associated end of actin filaments, and may be involved in functions more general than those in microvilli or sperm.

The recovery at the fast end of the filament could be mediated through two possible mechanisms: same-end association-dissociation and opposite-end association-dissociation (treadmilling). As Zeeberg et al. (32) and Salmon et al. (17) pointed out, the two mechanisms have very different kinetics. If the recovery occurs predominantly through same-end association-dissociation, the movement of the boundary of recovery should slow rapidly as it reaches into the interior of the filaments. On the other hand, if treadmilling is the predominant mechanism, then a constant movement should be detected. Our data are clearly more consistent with the second mechanism. However, they do not exclude the possibility that same-end association-dissociation also occurs to some extent.

Another way to distinguish the two mechanisms is to photobleach only the distal portion of the spikes. If recovery occurs through association-dissociation at the membrane-

associated end, the bleached subunits should not move appreciably during the recovery. On the other hand, if treadmilling occurs, then the flux generated should carry the bleached subunits toward the center of the cell into unbleached areas, and eventually result in the dissociation of those subunits at the end away from the membrane. Our results, again, are consistent with the mechanism of treadmilling.

According to published kinetic constants (4, 16), one can calculate that treadmilling of pure actin filaments in vitro has a flux rate of 0.06 to 0.11 $\mu$m/min, which is much slower than the movement of the bleached spot (0.79 ± 0.31 $\mu$m/min). Therefore, if treadmilling does represent the mechanism of the movement of actin subunits in the lamellipodium, its rate is probably enhanced significantly by the interactions with other proteins and/or by the local ionic environment. For example, the rate of flux can be increased in the presence of profilin, which preferentially inhibits the addition of subunits to the pointed end (23). The proteins that mediate the association of the filament with the membrane may further promote the addition, or inhibit the dissociation, of subunits at the barbed end. Finally, since the exchange of subunits in vitro is highly dependent on ionic conditions (4, 16, 28, 30), the environment in the cytoplasm may further facilitate the treadmilling process.

The centripetal flux and possible treadmilling of actin subunits may play an important role in cell motility. First, based on simple microscopic observations, Abercrombie pointed out many years ago the possibility of a "steady retreat" of actin in the leading lamella (1), which is consistent with the present observation. Second, it has been observed that on the surface of lamellipodium there is a continuous centripetal flow of receptors and associated particles (3, 9). If the membrane components are directly or indirectly connected to the underlying actin filaments, a flux of actin subunits would cause a similar flux of membrane components and associated particles. Third, if treadmilling does occur in the lamellipodium, it may provide an effective mechanism for the protrusion of the cytoplasm in this region. During the insertion of subunits to the membrane-associated end of the filament, either the entire filament must move toward the center of the cell, or the membrane must yield and protrude to offer the extra space required. The force involved may be generated as a result of treadmilling alone or in combination with other (e.g., actin-myosin) molecular interactions. The resulting cell movement may then be regulated by the rigidity of the membrane relative to the submembranous cortex. Finally, as Kirschner (13) and Mooseker et al. (19) pointed out, treadmilling would allow fine regulation of the stability of actin filaments, through the binding of capping proteins to the fast end. When this end is capped, the filament will continue to disassemble at the opposite end until all the subunits are removed and reassembled onto other uncapped filaments. This would allow the cell to use efficiently its energetic and mechanical resources, and assemble and disassemble its motile machinery in response to specific signals.

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