Mobility of Filamentous Actin in Living Cytoplasm

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Abstract. Filamentous actin in living cultured cells was labeled by microinjecting trace amounts of rhodamine-phalloidin (rh-pha) as a specific, high-affinity probe. The microinjection caused no detectable effect on cell morphology or cell division. The distribution of rh-pha–labeled filaments was then examined in dividing cells using image-intensified fluorescence microscopy, and the exchangeability of labeled filaments along stress fibers was studied during interphase using fluorescence recovery after photobleaching. rh-pha showed a rapid concentration at the contractile ring during cell division. In addition, recovery of fluorescence after photobleaching occurred along stress fibers with a halftime as short as 8 min. These observations suggest that at least some actin filaments undergo continuous movement and reorganization in living cells. This dynamic process may play an important role in various cellular functions.

EUKARYOTIC cytoplasm contains a dense network of filamentous structures (see, e.g., Gershon et al., 1985), which can potentially affect a wide spectrum of cellular properties including the mechanical characteristics of the cytoplasm, the movement of organelles, the assembly of macromolecular structures, and the rate of many biochemical reactions. Recent evidence indicates that this network, as well as a number of discrete structures formed by similar components, may be able to reorganize actively in living cells. For example, stress fibers, large bundles of actin filaments in cultured interphase cells, have been observed to undergo assembly, disassembly, merging, and splitting (Wang, 1984). In addition, actin subunits associated with the leading edge and with stress fibers appear capable of exchanging continuously with those in the ground cytoplasm (Kreis et al., 1982; Wang, 1985; Amato and Taylor, 1986).

However, not entirely clear is whether all the reorganizations of actin-containing structures involve de novo assembly of filaments, or whether intact actin filaments can directly move and reorganize. On the one hand, since most actin filaments in the network are probably longer than the diameter of the cytoplasmic pore (Jacobson and Wojcieszyn, 1984; Gershon et al., 1985; Luby-Phelps et al., 1986; Janmey et al., 1986), their thermal motion should be highly restricted. On the other hand, it is quite possible to move even long actin filaments in the cytoplasm through interactions with myosin and possibly other molecules.

To study the dynamic behavior of actin filaments, it is important not only to examine living cells but also to use probes that reveal specifically filamentous actin, since the cytoplasm also contains a high concentration of unpolymerized actin molecules (Bray and Thomas, 1976). In this study, I have microinjected a very small amount of rhodamine–phalloidin (rh-pha)[1] (Wieland, 1977; Faulstich et al., 1983) into living cells to label actin filaments, without inducing detectable changes in cellular morphology or behavior (Wehland et al., 1980; Wehland and Weber, 1981; Hamaguchi and Mabuchi, 1982). The probe binds filamentous actin with a high specificity and affinity, but has little affinity for monomeric actin molecules (Wieland, 1977; Faulstich et al., 1983). After microinjection, the distribution of fluorescence was recorded during cell division to determine whether rh-pha–labeled actin filaments can incorporate into the contractile ring. In addition, fluorescence recovery after photobleaching was used to determine the exchangeability of labeled actin filaments along stress fibers. The results suggest that at least some actin filaments in living cells can undergo dynamic movement and reorganization.

Materials and Methods

Cell Culture

Swiss 3T3 cells were obtained from American Type Culture Collection (Rockville, MD), and maintained in DME (KC Biological Inc., Lenexa, KS) with 10% calf serum (Colorado Serum Co., Denver, CO), 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were replated on injection/observation chambers (Wang, 1984) 12 h before experiments in F12K medium (KC Biological) with 10% FCS (KC Biological). PtK-1 cells were kindly supplied by Dr. J. R. McIntosh (University of Colorado, Boulder) and cultured in F12K medium with 10% FCS. They were replated on injection/observation chambers 48 h before experiments.

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1. *Abbreviations used in this paper:* fl-pha, fluorescein–phalloidin; rh-pha, rhodamine–phalloidin.

Microinjection and Processing of Cells

rh-pha was purchased from Molecular Probes Inc. (Eugene, OR) as a 3.3 µM solution in methanol. 3 ml of such solution was dried under N_2 and redissolved in 20 µl DMSO (Sigma Chemical Co., St. Louis, MO). The solution was then diluted with 150 µl of 2 mM Pipes (Research Organics, Cleveland, OH), pH 6.95, and clarified at 25,000 rpm for 30 min in a type 42.2 Ti rotor (Beckman Instruments, Inc., Palo Alto, CA). The final solution contained ~58 µM rh-pha and 12% DMSO. Microinjection of this solution was performed as described previously (Wang, 1984). The volume injected in all experiments was estimated to be <5% cell volume. Therefore, the final concentration of rh-pha in the cell was <3 µM.

After injection of rh-pha, 3T3 cells were incubated in a CO_2 incubator for 2–3 h before viewing. No intranuclear actin-containing aggregates, typical of cells incubated with DMSO (Fukui, 1978), were detected with injected rh-pha or after fixation and staining with fluorescein–phalloidin (fl-pha), presumably due to the low final concentration of DMSO and the ability of DMSO to diffuse rapidly out of the injected cell. For studies of cell division, PtK-1 cells with condensed chromosomes were identified and injected before the breakdown of nuclear envelope. Cells were then maintained on the microscope stage and the progress of mitosis monitored intermittently. The temperature of the microscope stage was maintained at 32°C for all experiments.

Complexes of rh-pha and actin filaments were prepared by polymerizing actin (Spudich and Watt, 1971) with 2 mM $MgCl_2$ and 60 mM KCl in the presence of rh-pha. The final solution had an actin concentration of 4 mg/ml and an actin–rh-pha molar ratio of 13.

Permeabilized cells for photobleaching were prepared in two different ways. First, coverslips were fixed with 3.7% formaldehyde, 0.05% Triton X-100 in PBS for 2 min, followed by further fixation for 10 min without Triton. The coverslips were then incubated with 0.5 ml of 0.1 µM rh-pha in PBS for 30–50 min before photobleaching. In a second approach, coverslips were treated directly with a mixture of 0.05% Triton X-100 and 1 µM rh-pha in PBS for 30–50 min.

The competition of rh-pha by unlabeled phalloidin was performed by treating rh-pha–injected cells for 1–2 min with a buffer containing 60 mM Pipes, 25 mM Hepes, 10 mM EGTA, 2 mM $MgCl_2$, pH 6.9 (Schliwa and van Blerkom, 1981), and supplemented with 0.15% Triton X-100. The coverslip was rinsed with the same buffer without Triton X-100 and then treated with 10 µM phalloidin dissolved in the same buffer (Boehringer Mannheim Biochemicals, Indianapolis, IN).

Fixation and staining of cells with fl-pha was performed as described previously (McKenna et al., 1985).

Fluorescence Microscopy and Photobleaching

Fluorescence microscopy was performed as described previously (McKenna et al., 1985; Meigs and Wang, 1986), using either a 100× or a 25× Neofluar objective. The 100 W quartz-halogen lamp for fluorescence excitation was operated at 5–6 V.

Photobleaching was performed as described by McKenna et al. (1985), using a 30-ms pulse at a power of 75 mW. The beam had a diameter of 3–4 µm. Acquisition of images and analysis of recovery halftimes were also performed as described by McKenna et al. (1985). Data on rapid recovery kinetics (Fig. 6) were collected by repeatedly averaging eight video frames, integrating intensities within the bleached area, and subtracting out dark currents in the same area. The action of shutters, the acquisition of images, and the integration of intensities were coordinated by the image processing computer.

Results

Microinjection of rh-pha and its Distribution during Cell Division

Small volumes of 58 µM rh-pha were microinjected into living 3T3 cells to label specifically filamentous actin. The cells, under both phase and fluorescence optics, assumed a normal morphology within 3 h after microinjection. Typically, injected interphase cells showed a well-defined array of stress fibers but no aggregates (Fig. 1), which were observed previously in cells injected with larger amounts of phalloidin (Wehland et al., 1977). After fixation and staining with fl-pha, injected and uninjected cells showed similar distributions and intensities of fl-pha fluorescence (Fig. 1), indicating that the normal distribution of actin filaments was not affected by the microinjection and that the binding sites for phalloidin were not saturated by the injected rh-pha. Furthermore, the cells showed no detectable decrease in rh-pha fluorescence after fixation and extraction, suggesting that the majority of rh-pha molecules were tightly bound. An interesting observation was the lack of binding of microinjected rh-pha in lamellipodia, but heavy staining in this region with fl-pha after fixation (Fig. 1, arrows). Similar observations were reported previously by Wehland and Weber (1981).

Some microinjected 3T3 cells were observed to enter mitosis. These cells had a rounded shape and contained no detectable stress fiber, typical of normal, unperturbed cells during division (not shown). The process of cell division was followed in greater detail with PtK-1 cells injected with rh-pha before the breakdown of the nuclear envelope. Before the initiation of cytokinesis, rh-pha showed an essentially uniform distribution in the cytoplasm (Fig. 2 a). However, during telophase, it became concentrated in the equatorial plane in as short as 5 min, coincident with the initiation of cytokinesis (Fig. 2 b). The intensity of the contractile ring reached a maximum at mid-cytokinesis, with nearly 30% of rh-pha lo-
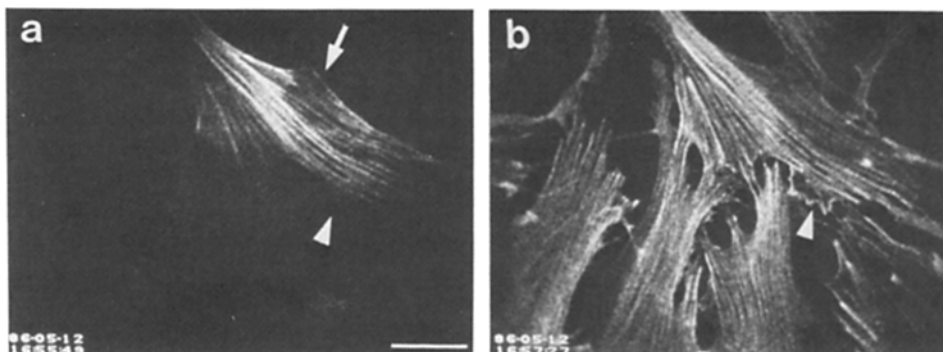

Figure 1. fl-pha staining of rh-pha-injected cells and uninjected cells. A number of 3T3 cells on a culture dish were first injected with rh-pha. After incubation, the dish was fixed, extracted, and stained with fl-pha. Observations were performed with filter sets selective for rhodamine (a) or fluorescein (b). rh-pha remained bound to structures in the injected cell after extraction (a, arrow). The pattern and intensity of fl-pha staining was similar for injected and uninjected cells (b). Note the heavy staining of lamellipodia with fl-pha but not with rh-pha (arrowheads). Bar, 20 µm.
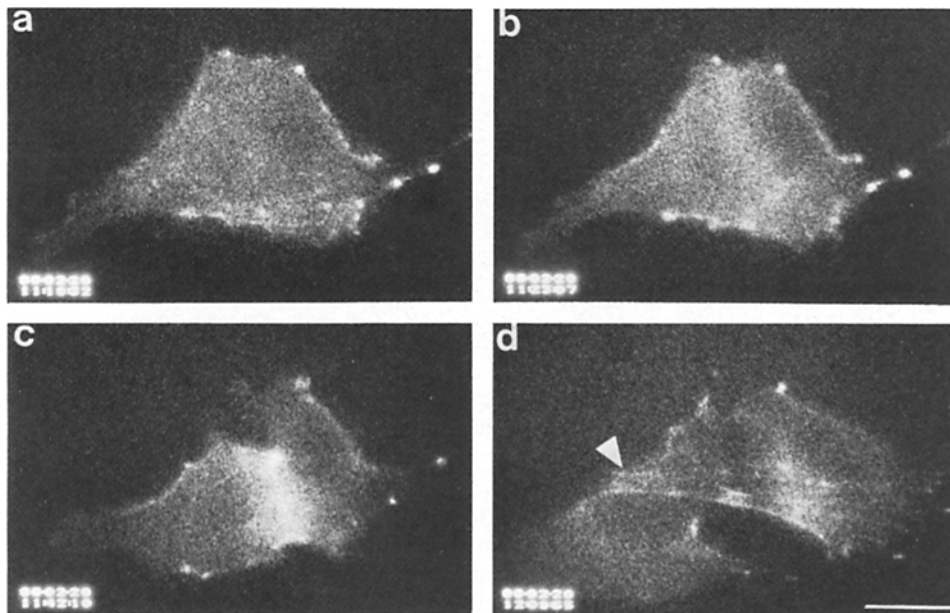
Figure 2. Time-lapse sequence of a rh-pha injected PtK1 cell during late mitosis. Images of the cell were shown 5 min before cytokinesis (*a*), immediately after the initiation of cytokinesis (*b*), 20 min after the initiation of cytokinesis (*c*), and after the completion of cell division (*d*). Thin stress fibers can be discerned in the last panel (*arrowhead*). Bar, 20 μm.

cated near the equatorial plane as estimated by integrating fluorescence intensities, and then decreased gradually. Near the end of cytokinesis, fluorescent stress fibers reappeared in the cytoplasm (Fig. 2 *d*). The time course of cell division for injected cells was similar to that for neighboring uninjected cells. These results suggest that rh-pha–labeled actin filaments can participate in the formation of transient actin-containing structures, such as the contractile ring, with no detectable disruptive effect.

Photobleaching Recovery of Microinjected rh-pha

2–3 h after microinjection of rh-pha into 3T3 cells, a laser beam 3–4 μm in diameter was used to photobleach fluorescence of stress fibers and the surrounding cytoplasm. As shown in Figs. 3 and 4, some recovery of fluorescence along stress fibers was detectable as early as 3 min after bleaching. The recovery was complete within 30 min (Fig. 3), with an estimated halftime of 500 ± 65 s. The recovery appeared to occur with no apparent decrease in the length of the bleached segment (Fig. 4), suggesting that it is caused more likely by the exchange of filaments in the stress fibers with those in the ground cytoplasm, than by filaments sliding along the length. The integrity of bleached stress fibers, as revealed by fl-pha staining, was unaffected by the laser beam (Fig. 5).

To determine whether the recovery of fluorescence was caused by a continuous association and dissociation of small, diffusible actin oligomers, the early phase of recovery in the ground cytoplasm was recorded by repeatedly integrating the
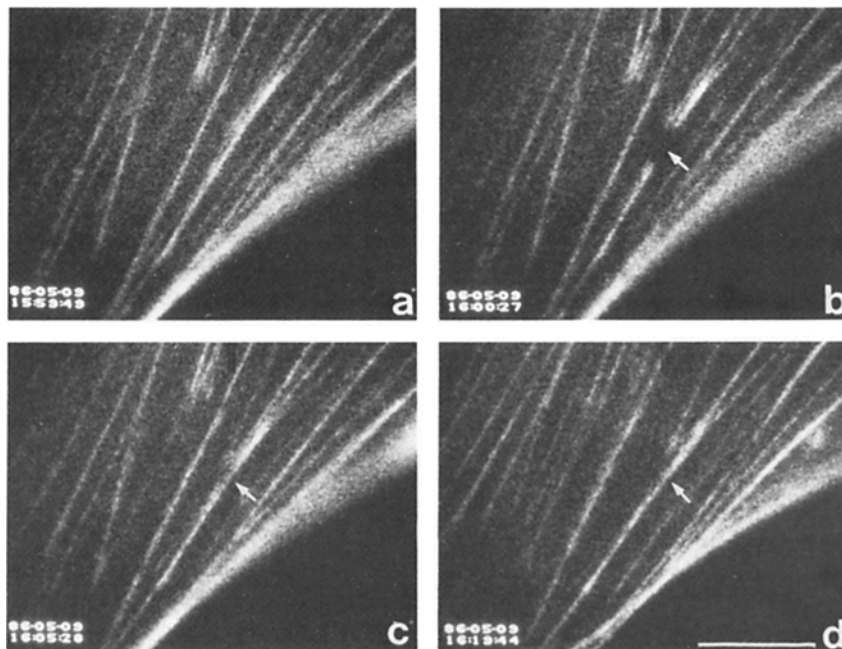

Figure 3. Recovery after photobleaching of rh-pha associated with stress fibers. A 3T3 cell was microinjected with rh-pha and fluorescence images were recorded immediately before (*a*), immediately after (*b*), 5 min after (*c*), and 20 min after (*d*) photobleaching a spot on a stress fiber (*arrows*). Extensive recovery was detected within the period of recording. Bar, 10 μm.
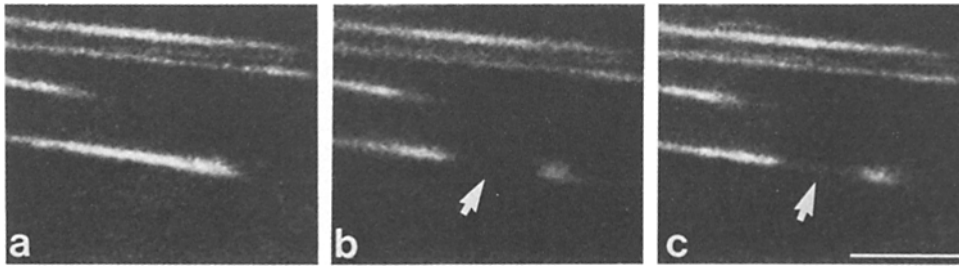
Figure 4. High magnification view of a bleached spot on a rh-pha–labeled stress fiber during early stages of photobleaching recovery. Fluorescence images were shown immediately before (*a*), immediately after (*b*), and 3 min (*c*) after photobleaching. A small extent of recovery was already detectable in *c*. The bleached segment (*arrows*) recovered without an apparent change in length. Bar, 5 μm.

fluorescence intensity in the bleached area. As shown in Fig. 6, no rapidly recovering component was detectable, indicating that the exchange involves only slowly mobile structures. The recovery in Fig. 6 reached a level of ∼16% over 100 s, consistent with a halftime of ∼400 s. However, the low level and the small change of signal, and the possible movement of bleached spot relative to the measuring spot, made it difficult to obtain accurate rates of recovery using this approach.

Since rh-pha binds actin filaments through noncovalent associations, several experiments were performed to rule out the possibility that the dynamic behavior of rh-pha reflects simply its direct exchange among actin filaments. In the first experiment, photobleaching was performed with permeabilized cells, with or without fixation, in the presence of excess rh-pha. Only very limited (20–30%) recovery was detected over a 30-min period after photobleaching (Fig. 7), indicating a relatively low rate of exchange of rh-pha. Similar results were obtained when cells microinjected with rh-pha were permeabilized and incubated with excess (10 μM) unlabeled phalloidin. The fluorescence again decreased by 20–30% over 30 min, consistent with a dissociation rate constant of ∼2 × 10⁻⁴ s⁻¹. Finally, actin filaments were prelabeled with rh-pha in vitro and microinjected into living cells. The filaments remained as amorphous or bundle-like aggregates for at least 3 h, possibly because of the great length and the entanglement of the filaments assembled in vitro. The fluorescence intensity of the aggregates decreased only slightly (<10%) over 30 min. Although the binding of rh-pha to exogenous actin may be different from its binding

to endogenous actin filaments, the result indicates that at least the dissociation of rh-pha from the injected complex is slow. Taken together, these observations suggest that the reorganization detected in living cells is unlikely to be caused by a slow, direct exchange of rh-pha molecules, and more likely reflects actual movement and reorganization of labeled actin filaments.

Discussion

Before attempting any interpretation of the present results, it is important to analyze the binding state of rh-pha after microinjection into living cells. First, injected rh-pha is nonextractable, indicating that most rh-pha molecules are tightly bound to actin filaments. Second, the spontaneous exchange of rh-pha among actin filaments is slow. Based on the results of permeabilized cells, it has a halftime close to 60 min, too slow to explain the dynamic behavior observed in living cells. rh-pha also dissociates very slowly from prelabeled filaments after microinjection into living cells. Finally, despite the intense labeling of stress fibers by injected rh-pha, lamellipodia are essentially unlabeled (Fig. 1), suggesting that rh-pha molecules undergo little exchange among available binding sites in living cells. Taken together, although it is difficult to rule out entirely the existence of unknown factors that may facilitate the exchange of rh-pha in living cells, these results support a relatively complete and stable association between rh-pha and actin filaments.
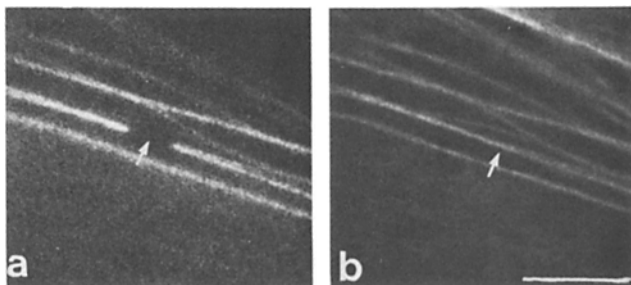

Figure 5. Integrity of stress fibers after photobleaching. An rh-pha–labeled stress fiber was photobleached and the dish was immediately fixed and stained with fl-pha. Images were then recorded using filter sets selective for rhodamine (*a*), or fluorescein (*b*). No discontinuity in fl-pha staining was detected along the bleached stress fiber. Arrows indicate the bleached region. Bar, 10 μm.
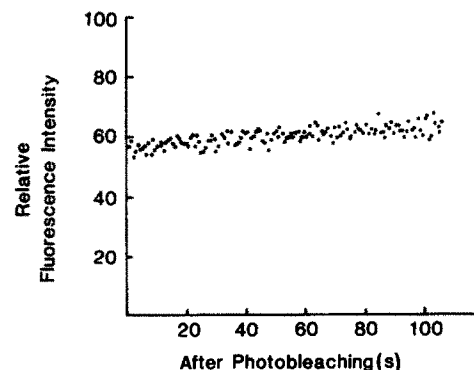

Figure 6. Recovery of fluorescence after photobleaching the ground cytoplasm between stress fibers. The fluorescence intensity before bleaching was arbitrarily designated 100. The time of photobleaching was designated 0. No rapidly recovering component was detected during the first 100 s after photobleaching. The recovery proceeds slowly and reaches 16% at the end of the observation period.
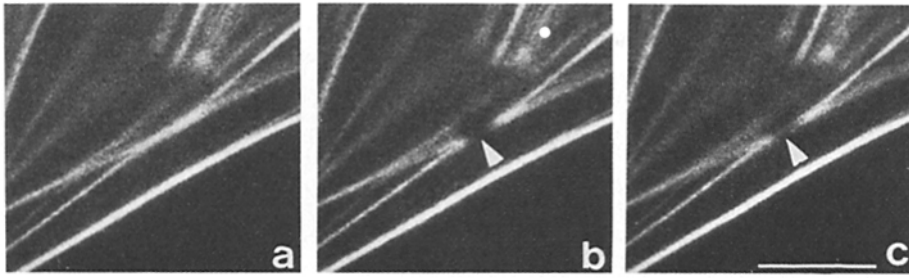
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Figure 7. Very slow recovery of rh-pha fluorescence in permeabilized cells. A dish containing $\sim 10^4$ 3T3 cells was fixed, permeabilized, and incubated with 0.5 ml of 0.1 μM rh-pha in PBS for 50 min. Images show a cell before photobleaching (*a*), immediately after photobleaching (*b*), and 20 min after photobleaching (*c*). Only very limited recovery was detected at the bleached spot (*arrowheads*). Bar, 10 μm.

Despite the tight binding of rh-pha, no more than 3% of actin subunits on the filaments should be labeled (assuming that the cytoplasm contains 200 μM actin subunits, of which 50% are in the filamentous form, and that rh-pha has a concentration of 3 μM). According to available biochemical data, this level of binding would stabilize the association of a small percentage (<9%) of actin subunits against disruptive treatments (Dancker et al., 1975). The filaments remain otherwise unaffected as regards interactions with tropomyosin, myosin, filamin, and alpha-actinin (Dancker et al., 1975; Wehland et al., 1980). The behavior of injected cells is in general consistent with these observations. Specifically, the morphology and the movement of the cell, the response of the cell to cytochalasins, and cell division are all unaffected by the microinjection (Figs. 1 and 2; Wehland and Weber, 1980).

If rh-pha molecules are indeed associated tightly with stabilized domains of actin filaments, then the results of photobleaching recovery along stress fibers can be explained most easily by an exchange of filamentous actin in the ground cytoplasm with those in the stress fiber. However, since only slowly mobile structures are detected in the ground cytoplasm (Fig. 6), it is unlikely that such exchange is mediated by small, diffusible oligomers. Instead, the results appear more compatible with a continuous, slow movement of actin filaments in the ground cytoplasm, coupled to a continuous association–dissociation of filaments with stress fibers.

The dynamics of actin along stress fibers has also been examined previously by photobleaching fluorescent actin analogue microinjected into living cells (Kreis et al., 1982; Amato and Taylor, 1986). These experiments revealed a rapidly mobile species and a slowly recovering species. The former shows a recovery ~ 100 times faster than that of rh-pha and probably represents actin monomers in the surrounding cytoplasm. However, both the pattern of recovery and the recovery halftime for the slow species are similar to those for rh-pha observed here. Thus although the slow recovery may reflect in part exchange of unpolymerized actin subunits with those in the filaments, as suggested by Kreis et al. (1982) and Amato and Taylor (1986), association–dissociation of actin filaments is also likely to be involved in this process.

Since actin filaments are probably the primary component of the cytoplasmic network, their movement implies that the entire network may be a dynamic structure undergoing constant local reorganization, rather than a stable "cytoskeleton." Such a dynamic framework can have a far-reaching effect on cellular functions. For example, it would allow the cell to maintain its unique viscoelastic properties, to change and maintain its shape, to reorganize microfilaments, microtu-

bules, and intermediate filaments independently, and to move even relatively large structures in the cytoplasm.

Dynamic reorganization would also allow cells to assemble new structures directly from existing actin filaments. One possible example is the contractile ring in dividing cells. It is presently unclear whether the assembly of contractile ring involves de novo polymerization or sequestration of actin filaments. However, rh-pha clearly becomes concentrated into this structure (Fig. 2). Because of the tight binding of rh-pha to actin filaments, it is unlikely that enough rh-pha molecules can dissociate from existing filaments and redistribute to a set of newly assembled filaments. Therefore, the result is more compatible with the sequestration mechanism, although it is difficult to rule out the involvement of de novo polymerization. This conclusion is also consistent with the nearly constant level of actin polymerization throughout the cell cycle (Heacock and Bamburg, 1983), and with a theoretical analysis using computer simulation (White and Borisy, 1983).

The incorporation of rh-pha into the contractile ring is in marked contrast to the lack of rh-pha binding in the lamellipodia (Fig. 1; Wehland and Weber, 1981), where actin is known to undergo a rapid turnover (Wang, 1985). If this turnover process involves predominantly actin monomers, which do not bind rh-pha, then few rh-pha molecules would become associated with the filaments. Thus, there may be two classes of actin-containing structures in the cell: one, such as the lamellipodia, assembled and/or maintained predominantly through polymerization of actin monomers; the other, such as the contractile ring, formed predominantly through reorganization of existing filaments.

The present study provides evidence that actin filaments may be able to move and reorganize in living cells. It will be worthwhile to perform direct observations by, for example, following rh-pha-labeled actin filament fragments microinjected into living cells. In addition, it will be important to identify the mechanism involved in the movement. Thermally driven diffusion, which should be hampered severely by the relatively small size of the pore of the cytoplasmic matrix and by the interactions of actin with accessory proteins, seems hardly adequate to account for the process. More likely, active force-generating mechanisms, such as actin–myosin interactions, may play an important role.

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