

Localization and Dynamics of Nonfilamentous Actin in Cultured Cells

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Abstract. Although the distribution of filamentous actin is well characterized in many cell types, the distribution of nonfilamentous actin remains poorly understood. To determine the relative distribution of filamentous and nonfilamentous actin in cultured NRK cells, we have used a number of labeling agents that differ with respect to their specificities toward the filamentous or nonfilamentous form, including monoclonal and polyclonal anti-actin antibodies, vitamin D-binding protein (DBP), and fluorescent phalloidin. Numerous punctate structures were identified that bind poorly to phalloidin but stain positively with several anti-actin antibodies. These bead structures also stain with DBP, suggesting that they are enriched in nonfilamentous actin. Similar punctate structures were observed after the microinjection of fluorescently

labeled actin into living cells, allowing us to examine their dynamics in living cells. The actin-containing punctate structures were observed predominantly in the region behind lamellipodia, particularly in spreading cells induced by wounding confluent monolayers. Time-lapse recording of cells injected with fluorescent actin indicated that they form continuously near the leading edge and move centripetally toward the nucleus. Our results suggest that at least part of the unpolymerized actin molecules are localized at discrete sites, possibly as complexes with monomer sequestering proteins. These structures may represent transient storage sites of G-actin within the cell which can be transformed rapidly into actin filaments upon stimulation by specific signals.

MANY motile activities of nonmuscle cells, including cytokinesis, cell locomotion, and growth cone extension are associated with the reorganization of actin filaments and the formation of new actin-containing structures (Satterwhite and Pollard, 1992; Heath and Holifield, 1991; Mitchison and Kirschner, 1988). At least some of these processes, most notably cell locomotion, have been shown to involve the *de novo* polymerization of actin filaments (Wang, 1985; Theriot and Mitchison, 1991). Since it is known that cultured cells maintain a high concentration of unpolymerized actin (Bray and Thomas, 1976; Blikstad et al., 1978), through association with various monomer sequestering factors (Cooper, 1991; Hartwig and Kwiatkowski, 1991; Safer, 1992; Nachmias, 1993), the polymerization of new filaments presumably requires the release of sequestered actin subunits followed by the assembly of subunits onto nucleation sites or filament ends. Therefore, to understand the mechanism of the regulation of actin assembly, it is crucial to gain information concerning the distribution of nonfilamentous actin, the regulation of sequestration factors, and the activity of assembly sites.

Although the distribution of filamentous actin is well characterized in many types of cultured cells, little is known about the distribution of nonfilamentous actin. It is commonly assumed that these unpolymerized molecules are freely diffusible in the cytoplasm, and upon encountering proper signals and/or active assembly sites, become incorporated into filaments. However, equally attractive is the possibility that some sequestered actin molecules may be

concentrated near active sites of assembly or the site of the release of second messengers. Such concentration of unpolymerized actin may facilitate the regulation and increase the rate of assembly in response to localized signals. To date the concentration of nonfilamentous actin has only been reported in two highly differentiated and specialized systems. First, in *Thyone* sperm, unpolymerized actin is known to be concentrated at the "acrosomal cup", and undergoes rapid assembly into filaments during elongation of the acrosomal process following sperm activation (Tilney, 1976*a,b*, 1978). Second, in unfertilized sea urchin eggs, nonfilamentous actin is found to concentrate in the cortex and is thought to assemble rapidly into cortical filaments following fertilization (Spudich et al., 1988; Bonder et al., 1989). These observations raise the interesting possibility that other cell types may use a similar strategy of localizing a storage form of nonfilamentous actin in regions of high assembly activities.

To address the possible existence of discrete nonfilamentous actin structures in cultured cells, we have performed a systematic study on the distribution of filamentous and nonfilamentous actin with a number of probes. These include anti-actin antibodies that bind to G- and F-actin (Lin, 1981; Lessard, 1988), fluorescent phalloidin that binds specifically to F-actin (Wieland, 1977; Wulf et al., 1979) and vitamin D-binding protein (DBP)¹ that binds specifically to G-actin (Van Baelen et al., 1980; Goldschmidt-Clermont et

1. *Abbreviations used in this paper:* DBP, vitamin D-binding protein; NRK, normal rat kidney.

al., 1985; Lee and Galbraith, 1992). In addition, using multiple probes and ratio imaging, we have been able to map the relative distribution of nonfilamentous actin in motile cells. We show that at least part of the nonfilamentous actin molecules are present as discrete beadlike structures. The bead structures are prominent in locomoting cells, especially in those responding to monolayer wound, and are localized in the region behind the leading lamellipodia. These discrete structures of nonfilamentous actin may represent precursors for the cortical actin meshwork that undergo continuous turnover in locomoting cells.

Materials and Methods

Cell Culture and Microinjection

A subclone of normal rat kidney epithelial cells (NRK-52E; American Type Culture Collection, Rockville, MD) were cultured in F-12K medium (JRH Bioscience, Lenexa, KS) containing 10% FBS (JRH Bioscience, Lenexa, KS), 50 $\mu\text{g}/\text{ml}$ streptomycin, and 50 $\mu\text{g}/\text{ml}$ penicillin. Cells were plated onto special coverslip dishes for 36–48 h before experiments (McKenna and Wang, 1989). In some experiments, monolayers were wounded by gentle scratching with a Pasteur pipet.

Microinjection was performed as described by Wang (1992), with air pressure regulated by a custom-designed electronic control system. The solution was microinjected conservatively, and the volume delivered was estimated to be <5% of the cell volume. No cell damage was visible under phase optics. Cells were cultured on the microscope stage during microinjection and subsequent period of observation (McKenna and Wang, 1989).

Preparation of Fluorescently Labeled Vitamin D-binding Protein and Actin

DBP (Gc-Globulin; Calbiochem-Behring Corp., San Diego, CA) was dissolved in 2 mM Pipes (pH 7.0) at a concentration of 5 mg/ml and dialyzed against the same buffer overnight at 4°C. After centrifugation at 100,000 *g* for 30 min, the DBP solution was mixed with a solution of carboxyfluorescein succinimidyl ester (Molecular Probes, Eugene, OR) in 50 mM Pipes, pH 7.0, at a molar ratio of 1:30. After incubation at room temperature for 2.5 h, the reaction was stopped by adding DTT to 10 mM. The conjugate was purified from free dye molecules by passing through a Sephadex G-25 column and was concentrated with Centricon-30 (Amicon Corp., Danvers, MA).

G-actin was purified and fluorescently labeled with tetramethylrhodamine following a protocol modified from that described previously (Wang, 1984). Actin was reacted in the polymerized form with tetramethylrhodamine iodoacetamide for 2 h at room temperature. After dialysis overnight against a low salt buffer to depolymerize the filaments, labeled actin was purified by passing through a Sephadex G-25 column and one cycle of polymerization–depolymerization. The actin analog was microinjected at a concentration of 2–3 mg/ml. Similar results of microinjection were obtained with either non-column purified actin or with actin purified through a G-150 column before fluorescent labeling.

Staining of Cells with Fluorescent Antibody, Phalloidin, and DBP

Cells were rinsed twice with warm PBS and fixed with 4% formaldehyde (16% stock solution, EM grade; Electron Microscopy Sciences, Fort Washington, PA) in PBS for 10 min. After rinsing twice with PBS and once with H₂O, cells were extracted in acetone at –20°C for 5 min and blocked with PBS containing 1% BSA (Boehringer Mannheim Biochemicals, Indi-

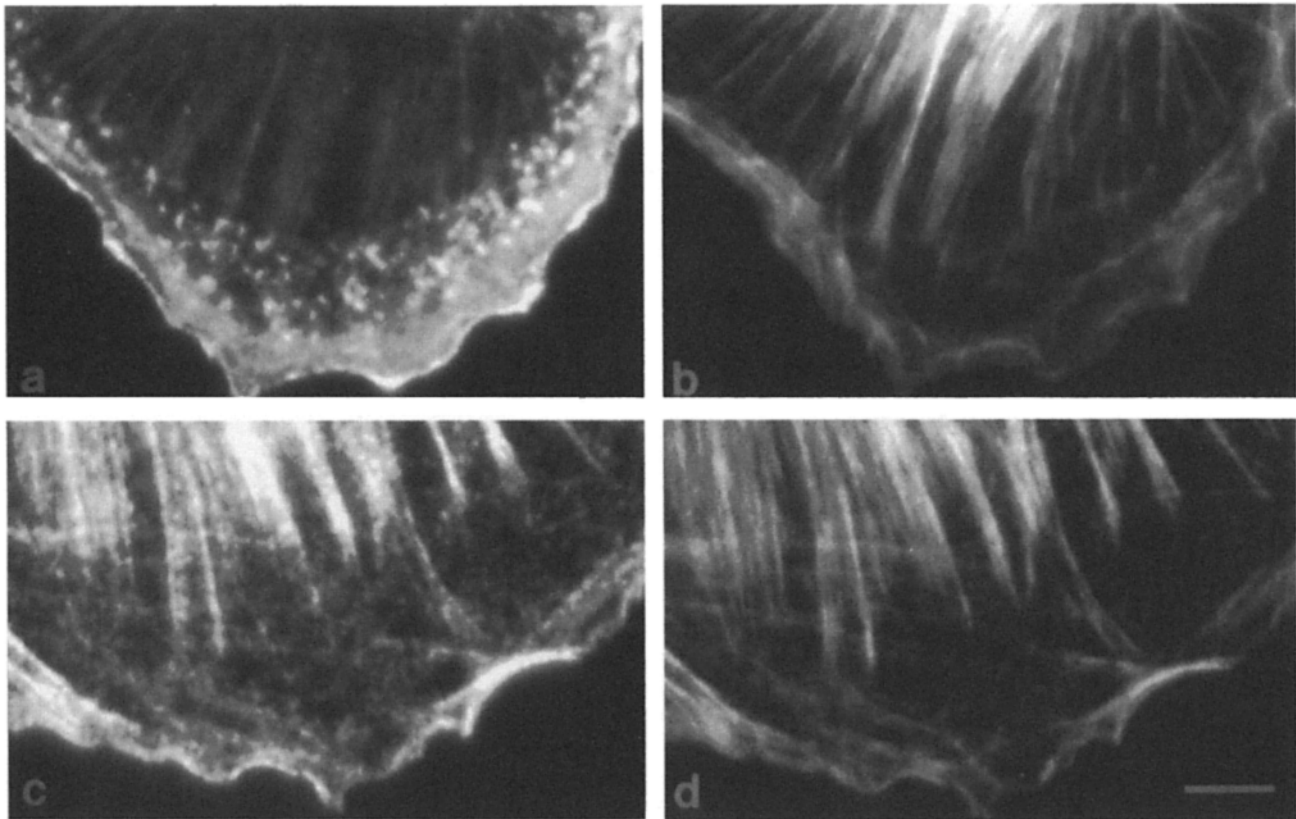


Figure 1. Detection of actin-containing foci with anti-actin antibodies. NRK cells stained with JLA20 anti-actin monoclonal (a) and polyclonal (c) antibodies reveal numerous brightly stained punctate structures behind the leading edge and between lightly stained stress fibers. Corresponding images of fluorescein–phalloidin labeling (b and d) show prominent staining of stress fibers but little or no staining of punctate structures, indicating that actin within punctate structures are predominantly in the nonfilamentous state. Bar, 5 μm .

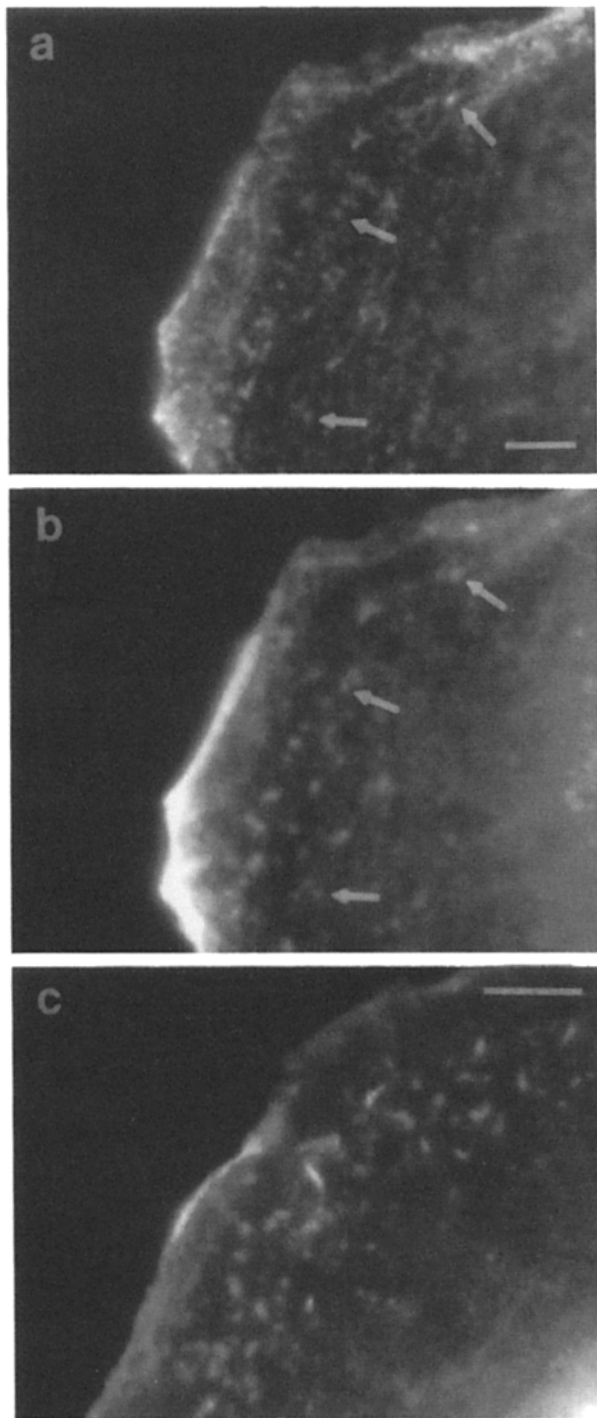


Figure 2. Colocalization of DBP and anti-actin antibodies at actin-containing foci in NRK cells. Fixed NRK cells were first incubated with DBP and then with a mixture of JLA20 monoclonal anti-actin antibody (a) and polyclonal anti-DBP antibody (b). Double-indirect immunofluorescence staining demonstrates that DBP and actin colocalize at the punctate foci (arrows). A similar pattern of distribution is observed in a separate cell stained directly with fluorescently labeled DBP (c). Bar, 5 μm .

anapolis, IN) for 10 min. Immunofluorescence staining of actin was performed with mAbs JLA20 (Lin, 1981; Amersham Corp., Arlington Heights, IL) at a dilution of 1:600 and C4 (Lessard, 1988; Boehringer Mannheim, Indianapolis, IN) at a dilution of 1:40, or with a polyclonal antibody (Biomedical Technologies Inc., Stoughton, MA) at a dilution of 1:30

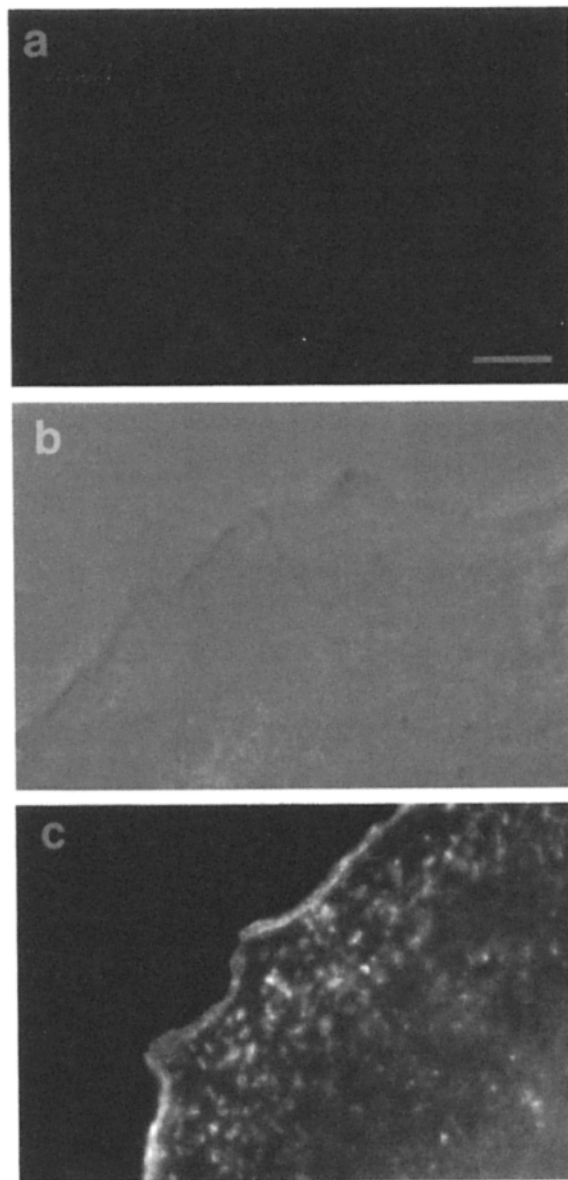


Figure 3. Inhibition of DBP staining by preincubation with G-actin. NRK cells were stained with complexes of fluorescein-labeled DBP and G-actin, prepared by incubating a mixture of actin and DBP at a molar ratio of 25:1 for 60 min at room temperature. There is no detectable staining of actin (a). The corresponding phase image is shown in b. Typical pattern of DBP staining was obtained when actin was omitted from the incubation mixture (c). Bar, 5 μm .

as described previously (Fishkind et al., 1991). Appropriate rhodamine- or fluorescein-conjugated secondary antibodies (Tago, Inc., Burlingame, CA) were used at a dilution of 1:100. Staining with fluorescent phalloidin (Molecular Probe, Eugene, OR) was performed after antibody staining following the procedure recommended by Molecular Probes, with a phalloidin concentration of 220 nM and an incubation period of 20 min.

Staining with DBP was performed with both direct and indirect methods. For the indirect method, cells were incubated with DBP (1–10 $\mu\text{g}/\text{ml}$) in PBS-BSA at room temperature for 60 min. The cells were then washed twice in PBS-BSA each for 10 min and stained with a polyclonal anti-DBP antibody (Dako Corp., Carpinteria, CA; 1:500 dilution) and fluorescent secondary antibodies following the standard immunofluorescence protocol. For the direct labeling, cells were incubated with 10 $\mu\text{g}/\text{ml}$ fluorescein-labeled DBP in PBS-BSA under the same condition as that for the indirect method. Control experiments for the specificity of DBP binding were performed by preincubating fluorescently labeled DBP with 25 \times molar excess

of muscle G-actin in a buffer of PBS-BSA under continuous mixing for a period of 60 min. Subsequent staining of cells was performed as for direct labeling.

Fluorescent Microscopy and Image Processing

All observations were made with an Axiovert-10 inverted microscope (Carl Zeiss, Oberkochen, Germany) equipped with a 100×/NA 1.30 Neofluar and a 40×/NA 1.0 Apochromat objective. A 12 V, 100 W quartz halogen lamp was used as the light source for epi-illumination. The lamp was operated at 7 V for the observation of living cells.

Fluorescence images were acquired with a cooled CCD camera (Star I; Photometrics, Tucson, AZ). Bandpass filters (Zeiss set 487917 for fluorescein and set 487915 for rhodamine) were used for fluorescence observations, and no crossover between fluorescein and rhodamine was detectable. Before generating ratio images, paired video images were placed into registration by rapidly alternating the display between the two images, while moving one of the image relative to the other until no wobbling was observed (Cao and Wang, 1990). Translocation of structures was visualized by loading multiple time-lapse images into the frame buffer, followed by displaying the images in rapid succession. Hardcopies of images were prepared using an image printer (model UP-7100 MD; Sony, Montvale, NJ).

Results

Distribution of Nonfilamentous Actin in NRK Cells

To detect possible sites of nonfilamentous actin, we first examined cells double stained with fluorescent phalloidin and anti-actin antibodies. Structures enriched in nonfilamentous actin should stain poorly with phalloidin but positively with anti-actin antibodies. Three different actin antibodies were used: a mAb originally prepared by Lin (1981; JLA20); a mAb originally prepared by Lessard (1988; C4); and a polyclonal antibody prepared by Biomedical Technologies Inc. (Stoughton, MA). The specificity of these antibodies have been confirmed by us on Western blots.

Phalloidin staining of NRK cells showed the typical strong reaction along stress fibers and lamellipodia (Fig. 1, *b* and *d*). However, immunofluorescence with the two mAbs revealed, in addition to lamellipodia and a relatively weak staining along stress fibers, numerous discrete beadlike structures behind the leading lamellipodia (Fig. 1 *a*). These punctate structures showed no detectable reaction with phalloidin in some cells (Fig. 1 *b*) and weak reactions in others (e.g., Fig. 6). Similar staining of these structures was observed with the polyclonal antibody (Fig. 1 *c*), although the staining was weaker than that obtained with mAbs. Double immunofluorescence with polyclonal and monoclonal antibodies indicates that they recognize the same punctate structures (data not shown).

These results suggest that the punctate foci may be enriched with nonfilamentous actin. To confirm the nonfilamentous state of actin, we stained fixed cells with DBP, a protein that binds tightly and specifically to G-actin (Van Baelen et al., 1980; Goldschmidt-Clermont et al., 1985; Couè et al., 1986), using either an indirect method with antibodies against DBP, or a direct method with fluorescently labeled DBP. As shown in Fig. 2, both methods showed prominent staining of bead structures that colocalize with anti-actin immunofluorescence staining. In addition, some regions of the leading edge showed a strong reaction with DBP. The specificity of labeling toward actin was tested by preincubating fluorescent DBP with excess (1:25) muscle G-actin. As shown in Fig. 3 *a*, the complex failed to bind any detectable structures in NRK cells.

To determine if the punctate structures are present in liv-

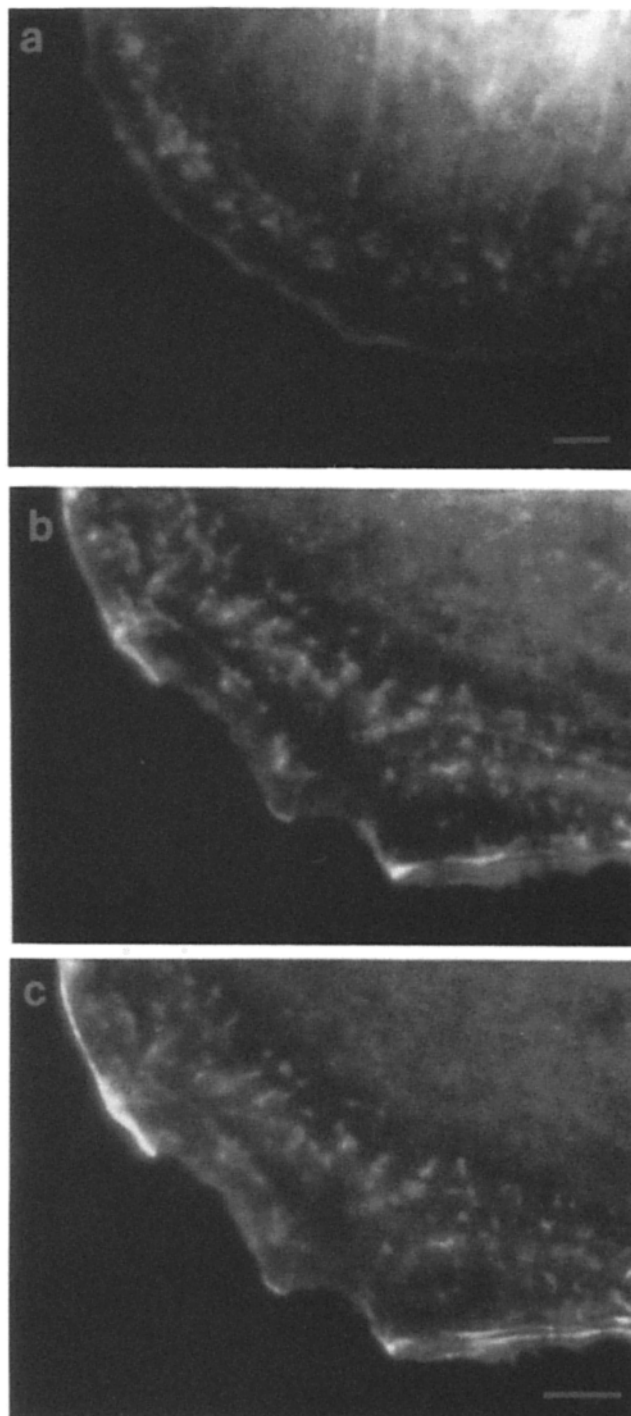


Figure 4. Detection of actin-containing foci in living cells. NRK cells were microinjected with rhodamine-labeled actin and incubated for 2 h. The actin analog incorporates into punctate structures behind the leading edge (*a*). After fixation the actin analog remains associated with bead structures (*b*) and colocalizes with fluorescent DBP staining (*c*). Bar, 5 μ m.

ing cells, we have microinjected live NRK cells with fluorescently labeled actin. As shown in Fig. 4 *a*, the actin analog became incorporated into bead structures, similar to those seen in fixed and stained cells as described above. Subsequent fixation and staining with DBP indicated that actin analogs colocalize with DBP-positive structures (Fig. 4 *b*

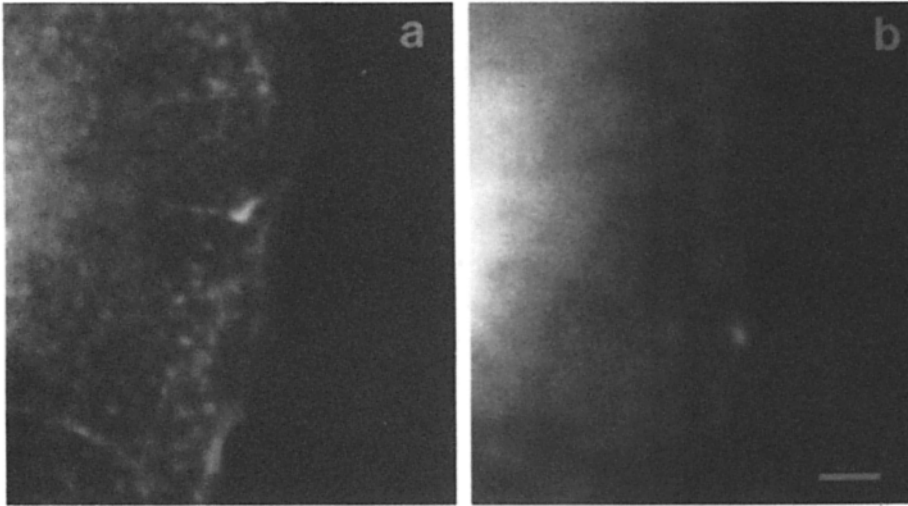


Figure 5. Lack of incorporation of fluorescent dextran into punctate foci. NRK cells were microinjected with a mixture of rhodamine-labeled actin and fluorescein-dextran. The image of actin (*a*) shows many punctate structures near the leading edge and some stress fibers. On the other hand, fluorescent dextran is distributed diffusely throughout the cytoplasm except for a few inconspicuous dense areas (*b*). Bar, 5 μm .

and *c*). The specificity of incorporation was confirmed by microinjecting cells with a mixture of rhodamine-labeled actin and fluorescein dextran. Where discrete bead structures were detected with the actin analog (Fig. 5 *a*), fluorescent dextran showed only a diffuse cytoplasmic distribution (Fig. 5 *b*). Therefore, it is unlikely that the punctate foci represent non-specific accumulation of soluble molecules.

Distribution of Bead Structures in Relation to Cell Locomotion

The specific recognition of filamentous and nonfilamentous actin by phalloidin and DBP, respectively, allowed us to map the relative distribution of G- and F-actin in motile cells. Thus, cells were double stained with rhodamine phalloidin and anti-DBP antibody, and the ratio distribution of non-filamentous actin to filamentous actin was obtained by dividing the images. As shown in Fig. 6, nonfilamentous actin is concentrated at punctate structures behind the lamellipodia and along some segments of the leading edge. In addition, the perinuclear region appears to be enriched in nonfilamentous actin relative to filamentous actin. Filamentous actin, on the other hand, represents the major structural form in the trailing region.

To further investigate the relationship between the distribution of nonfilamentous actin and cell locomotion, DBP staining was performed on confluent monolayers of NRK cells that were wounded by scratching with a Pasteur pipet. In spreading cells located at the wound, the staining with DBP is similar to that seen in spontaneously motile cells (Fig. 7 *a*), with a band of beadlike foci localized behind the lamellipodia. In contrast, no such band of beads was observed in nonmotile cells and those detectable appeared to be randomly distributed (Fig. 7 *b*).

Dynamics of Bead Structures in Living Cells

To investigate the dynamics of punctate structures during cell locomotion, living NRK cells along an experimental wound were microinjected with rhodamine-labeled actin. The dynamics of the bead structures was then examined by time-lapse analysis of image sequences. As shown in Fig. 8, formation of new foci can be detected continuously near the leading edge. At least some of them appear to form at the

base of actin-rich lamellipodia. Once formed, the actin-containing beads move away from the leading edge (Fig. 9), while undergoing concurrent changes in size and shape. The movement occurred at a rate of $0.56 \pm 0.11 \mu\text{m}/\text{min}$, similar to the flow of other membrane/cortical components reported previously (McKenna et al., 1989; Holifield and Jacobson, 1991). Most beads appear to have a relatively short life span, disappearing within 10 min of observation. However, due to the difficulty in maintaining the beads under constant focus, a precise determination of the life span awaits further study.

Discussion

To understand the regulation of actin assembly in living cells, it is important to determine whether nonfilamentous actin molecules are freely diffusible throughout the cytoplasm, or are concentrated at discrete sites. In this study, we have defined the state of cellular actin molecules based on their reactivities toward different probes. Filamentous actin was detected as those reacting positively with fluorescent phalloidin (Wulf et al., 1979; Barak et al., 1980). Actin antibodies and fluorescent analogs of actin were used to examine the overall distribution of both filamentous and nonfilamentous actin (Lessard, 1988). In addition, we have developed direct and indirect methods for staining nonfilamentous actin using DBP as a probe. Although fluorescent DNase I can serve a similar purpose (Wang and Goldberg, 1978; D'Andrea et al., 1991; Knowles and McCulloch, 1992; DuBose and Haugland, 1993), results from such studies may be complicated by the potential binding of DNase I to the ends of actin filaments as well as to DNA (Pinder and Gratzner, 1982; Podolski and Steck, 1987). Since DBP does not appear to bind the ends of actin filaments or DNA (Van Baelen et al., 1980; Couè et al., 1986), it should more accurately reveal the distribution of nonfilamentous actin in cultured cells.

The results of our immunofluorescence and phalloidin staining indicate the existence of discrete beadlike structures that contain actin in a phalloidin-negative form. Similar disparity between anti- β -actin antibody staining and phalloidin binding was clearly shown in images published by Hooek et al. (1991). The consistent results with multiple antibodies, and the ability of microinjected actin to incorporate into these beaded structures in living cells, argue against the pos-

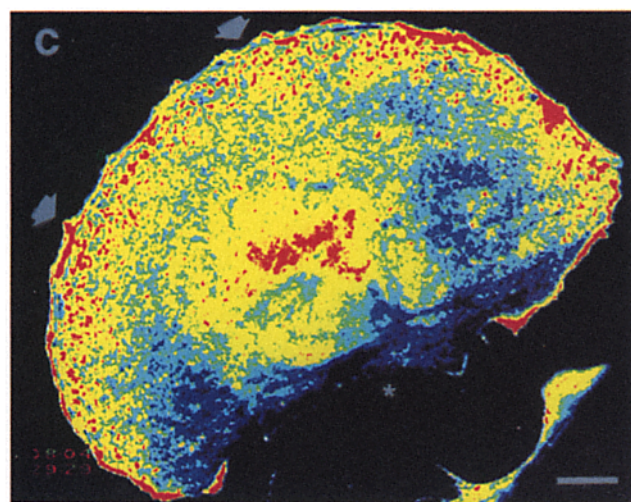
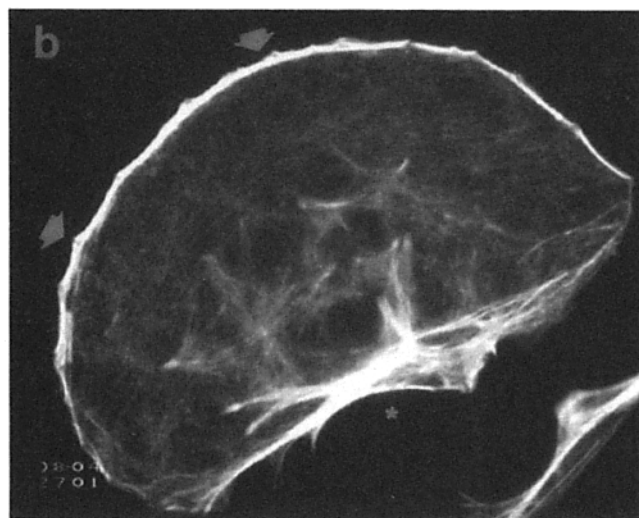
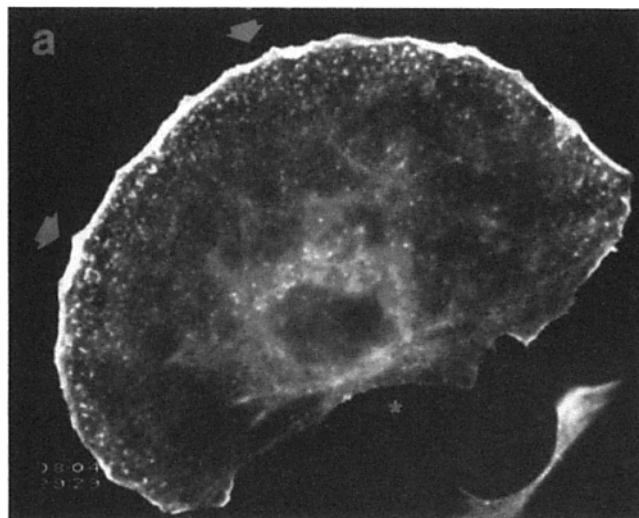


Figure 6. Relative distribution of nonfilamentous actin and filamentous actin in motile cells. Spreading NRK cells were labeled with DBP and anti-DBP antibodies for nonfilamentous actin (a), and with fluorescent phalloidin for filamentous actin (b). The ratio image of the two was then calculated and displayed in pseudo color (c), with red color representing high percentage of G-actin and blue representing high percentage of F-actin. The front of the cell is indicated by the arrows and the tail end indicated by stars. Nonfilamen-

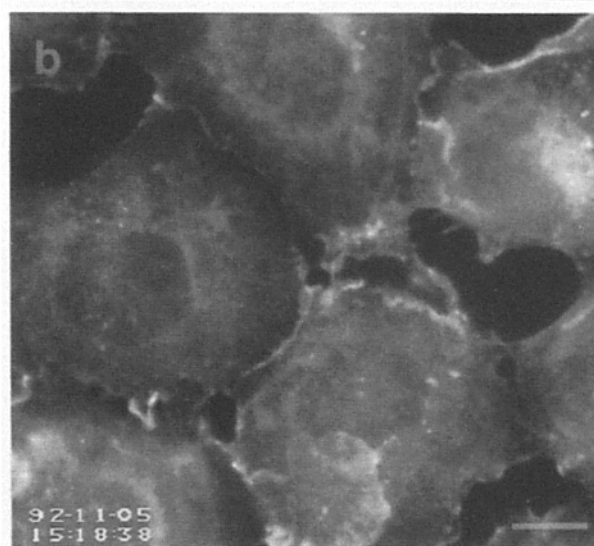
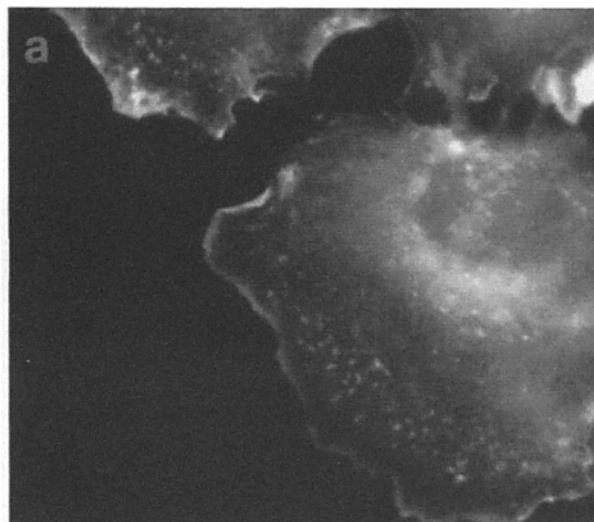


Figure 7. Stimulation of punctate foci behind the leading edge following monolayer wounding. A nearly confluent monolayer of NRK cells was wounded by scratching with a glass Pasteur pipet, incubated for 1 h, and then processed and stained with fluorescent DBP. Migrating cells along the wound edge show prominent nonfilamentous actin aggregates behind the leading edge (a). The punctate foci were randomly distributed in stationary cells located away from the wound edge (b). Bar, 10 μm .

sibility of artifacts caused by either antibody cross-reactivity or fixation. In addition, the strong reaction of the punctate foci with DBP suggests that the lack of phalloidin staining is due to the unpolymerized state of actin, rather than the inaccessibility of some actin filaments to phalloidin binding.

Due to the potential loss of some actin molecules during fixation and extraction (Herman and Pollard, 1978), it is possible that we are examining only a subset of actin molecules in cultured cells. Nevertheless, our results suggest that at least part of the nonfilamentous actin is concentrated at well-

ous actin appears concentrated behind the leading edge and filamentous actin concentrated in the rear portion of the cell. Bar, 10 μm .

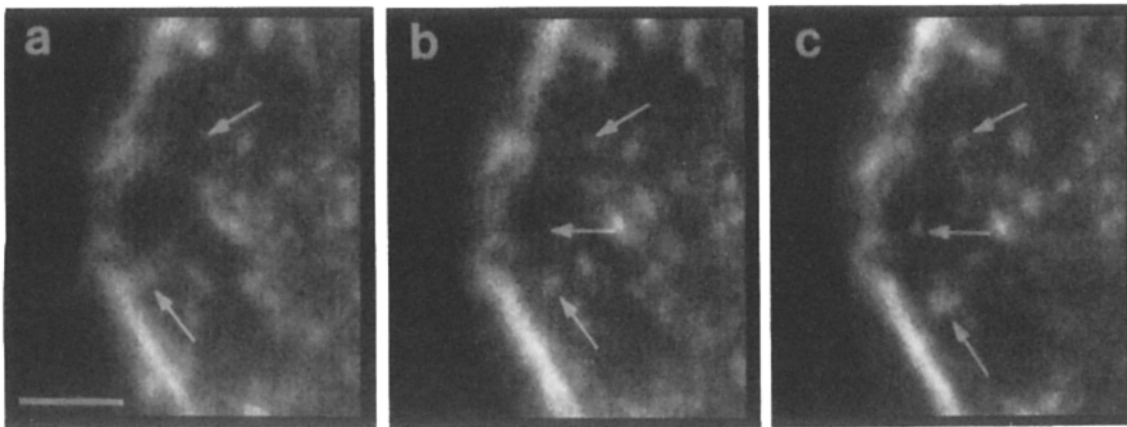


Figure 8. Formation of actin-containing beads behind the leading lamellipodium. Cells were microinjected with a rhodamine-labeled actin analog. After 2-h of incubation, a series of fluorescent images were recorded and the formation of actin foci was detected on motion picture loops. Arrows point to three fixed spots in the cell where formation of new foci was observed. The upper arrow in *a* and middle arrow in *b* point to the positions where new foci appeared in subsequent panels. Bar, 5 μm .

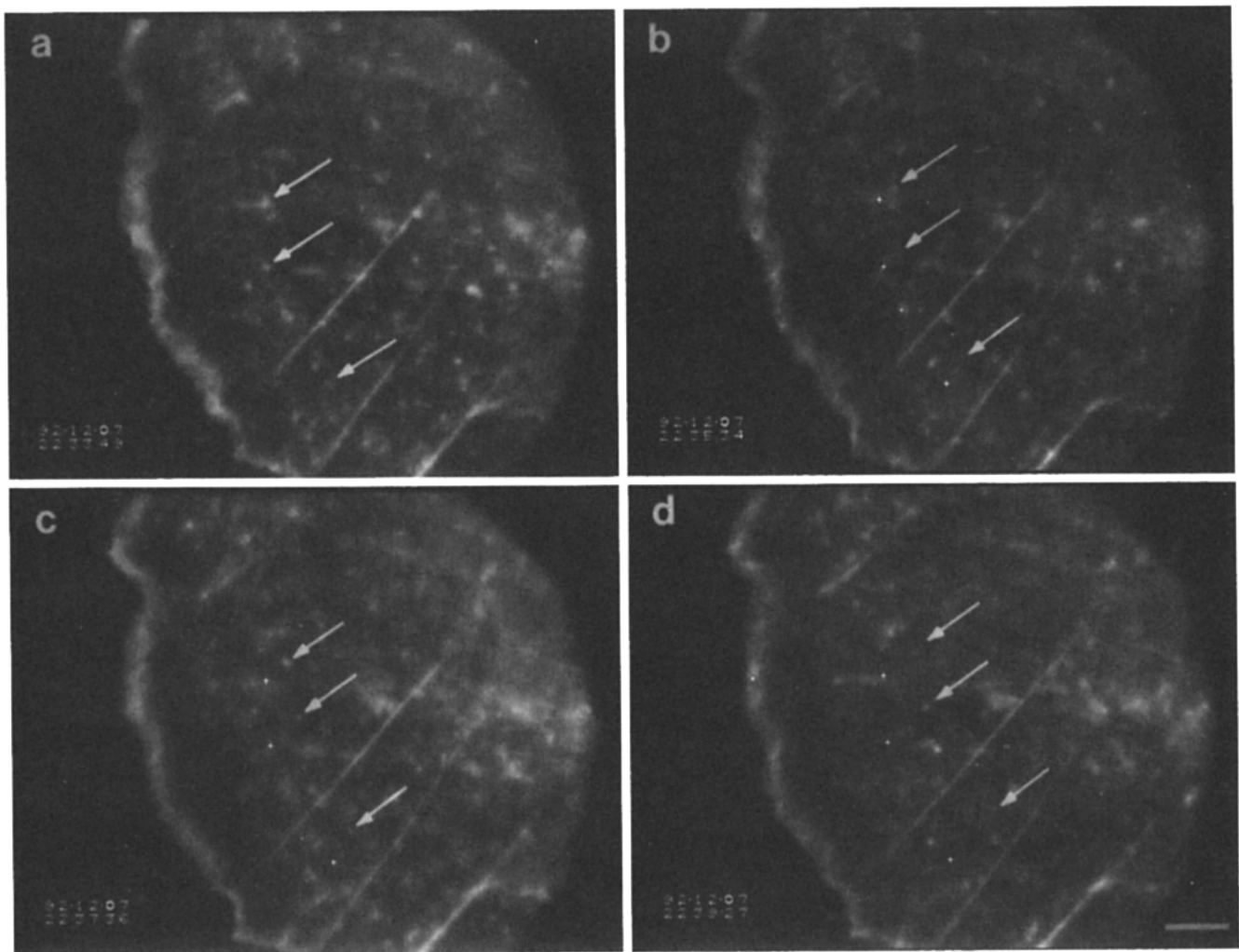


Figure 9. Time-lapse analysis of the dynamics of actin foci in cells injected with a rhodamine-labeled actin analog. After 2 h of incubation, a series of images were recorded over a 10-min period and the dynamics of actin foci was identified using motion picture loops. The retrograde movement of three actin-containing foci are indicated by arrows. The original position of beads is marked by "+". Bar, 5 μm .

defined sites in motile cells. Such structured nonfilamentous actin may affect our understanding of actin assembly in several ways. For example, it is possible that the bead structures may enter the insoluble fraction following cell lysis, thus assays of cellular actin polymerization based on sedimentation may yield an overestimate of filamentous actin (Bray and Thomas, 1976). Since the structured nonfilamentous actin may not inhibit DNase I activities, assays based on DNase I may also be affected (Blikstad et al., 1978).

The punctate foci may play an important role in the regulation of actin assembly in vivo. If the activation of actin assembly is triggered by localized second messengers, then a concentration of sequestered actin at specific sites could alleviate the needs for temporally sustained or spatially widespread signals, making the stimulation much more specific and efficient. In addition, a concentration of nonfilamentous actin would greatly increase the rate of assembly upon activation, by removing the dependence on the diffusion of actin subunits. In the case of sea urchin eggs, where similar structures of nonfilamentous actin have been detected in the cortex, the concentration of actin may be especially important given the large diameter of the cell and the rapid response of actin assembly following fertilization-induced activation of second messengers near the membrane (Burgess and Schroeder, 1977; Spudich et al., 1988; Bonder et al., 1989). Without the concentration of actin, one would expect the assembly of actin filaments to be severely limited by diffusion. Similarly, the extremely high concentration of unpolymerized actin sequestered in the acrosomal cup of Thyone sperm is thought to play a key role in fueling the rapid formation of actin filaments in the acrosomal process following activation by egg jelly (Tilney, 1978).

Two observations suggest a possible role of the bead structures in cell locomotion. First, the structure is concentrated behind the lamellipodia (Fig. 6), where actin has been shown to undergo continuous turnover (Wang, 1985; Theriot and Mitchison, 1991). Second, the formation of bead structures is stimulated upon the activation of cell locomotion (Fig. 7). Thus one attractive possibility is that bead structures may represent a source of actin subunits for the filaments in lamellipodia. In the simplest scenario, one may expect the bead structures to move toward the lamellipodia and disappear near the leading edge of the cell. However, this is opposite to the retrograde movement seen in living cells (Fig. 9).

What, then, may be the relationship between the bead structures and cortical activities near the leading edge? The bead structures are observed to form at the base of lamellipodia, most likely from actin molecules dissociated continuously from the lamellipodia (Wang, 1985), and thus may represent a site of subunit recycling. Individual actin subunits may subsequently be released from the punctate foci, to be reincorporated into filaments at the leading edge of the cell. Alternatively, nonfilamentous actin may be transported into more interior regions of the cell, before releasing actin subunits for incorporation into filaments. Another possibility is that bead structures may represent precursors of cortical actin filaments. Active assembly of a cortical sheath of actin and myosin is known to take place in the region immediately behind the lamellipodia (Giuliano and Taylor, 1990; McKenna et al., 1989; Heath and Holifield, 1991), in addition to the assembly of actin filaments at the leading edge. This process is coupled to a continuous, long-range

flow of cortex toward the perinuclear region. The bead structures show a similar retrograde motile activity, and may play an important role in cortical dynamics by supplying the actin subunits for structural assembly. Finally, it is also possible that bead structures may represent the sites of synthesis of new actin molecules. The distribution of actin mRNA has been shown to concentrate behind the lamellipodia (Lawrence and Singer, 1986; Hooek et al., 1991; Singer, 1992), where most bead structures are localized. Therefore actin subunits may be continuously released from the bead structures for the subsequent assembly into lamellipodia and/or cortex. A direct study of the relative distribution of bead structures, actin mRNA, and protein synthesis machineries should yield additional clues to the function of structural nonfilamentous actin in cultured cells.

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