Exogenous Nucleation Sites Fail to Induce Detectable Polymerization of Actin in Living Cells

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Abstract. Most nonmuscle cells are known to maintain a relatively high concentration of unpolymerized actin. To determine how the polymerization of actin is regulated, exogenous nucleation sites, prepared by sonicating fluorescein phalloidin-labeled actin filaments, were microinjected into living Swiss 3T3 and NRK cells. The nucleation sites remained as a cluster for over an hour after microinjection, and caused no detectable change in the phase morphology of the cell. As determined by immunofluorescence specific for endogenous actin and by staining cells with rhodamine phalloidin, the microinjection induced neither an extensive polymerization of endogenous actin off the nucleation sites, nor changes in the distribution of actin filaments. In addition, the extent of actin polymerization, as estimated by integrating the fluorescence intensities of bound rhodamine phalloidin, did not appear to be affected. To determine whether the nucleation sites remained active after microinjection, cells were first injected with nucleation sites and, following a 20-min incubation, microinjected with monomeric rhodamine-labeled actin. The rhodamine-labeled actin became extensively associated with the nucleation sites, suggesting that at least some of the nucleation activity was maintained, and that the endogenous actin behaved in a different manner from the exogenous actin subunits. Similarly, when cells containing nucleation sites were extracted and incubated with rhodamine-labeled actin, the rhodamine-labeled actin became associated with the nucleation sites in a cytochalasin-sensitive manner. These observations suggest that capping and inhibition of nucleation cannot account for the regulation of actin polymerization in living cells. However, the sequestration of monomers probably plays a crucial role.

In nonmuscle cells, close to 50% of total actin molecules appear to be present in the unpolymerized form (Bray and Thomas, 1976; Blikstad et al., 1978). The amount of unpolymerized subunits decreases after the stimulation of polymorphonuclear leukocytes (Fechheimer and Zigmond, 1983; Rao and Varani, 1982), Dictyostelium (Condeelis et al., 1988), and platelets (Carlsson et al., 1979; Fox and Philips, 1981), coincident with the appearance of new actin filaments. Thus, it is likely that these subunits may serve as building blocks and become assembled into filaments upon stimulation.

However, one intriguing question is how resting cells maintain the relatively high concentration of unpolymerized actin. Based on in vitro measurements (Bonder et al., 1983), purified actin has a critical concentration of \( \sim 0.3 \mu M \) under physiological ionic conditions. Assuming that the intracellular concentration of actin is 200 \( \mu M \), one would expect \( >99\% \) of actin to be in the filamentous form. The most likely way for maintaining a high concentration of unpolymerized actin is through interactions with various actin binding proteins. Since actin polymerization involves nucleation and subsequent addition of subunits to the nuclei or the ends of filaments, inhibition of polymerization can be achieved in two possible ways. First, monomeric actin can be maintained by binding to a protein that inhibits its polymerization activity. Second, polymerization can be inhibited by a combination of proteins that cap the ends of filaments and proteins that inhibit the formation of active nucleation sites. Thus, even though actin subunits may be active, there are no available sites for the assembly to take place.

A wide variety of actin-binding proteins have been identified in recent years (for reviews, see Stossel et al., 1985; Pollard and Cooper, 1986). For example, gelsolin is a well-characterized protein that caps the barbed ends of actin filaments in a Ca-dependent manner (Yin and Stossel, 1979). Profilin can bind actin monomers and inhibit their polymerization (Carlsson et al., 1977); it may also inhibit the self-nucleation of actin (Pollard and Cooper, 1986). Actobindin is also capable of sequestering actin monomers and inhibiting the formation of nucleation sites (Lambooy and Korn, 1986, 1988). However, serious questions remain concerning the possible roles of these proteins in the regulation of actin polymerization in living cells. For example, a study by Lind et al. (1987) indicates that there may not be enough profilin in platelets to account for the maintenance of unpolymerized actin. In the same study, the extent of gelsolin-actin binding was found to increase, rather than decrease, after platelet activation, contrary to what one might expect if gelsolin were...
involved in the inhibition of polymerization. Furthermore, since the actin binding activity of both gelsolin and profilin can be inhibited by phosphatidylinositol-4,5-bisphosphate (Jainey and Stossel, 1987; Lassing and Lindberg, 1985), a common component of eukaryotic membranes, it is possible that these proteins might be at least partially inactive in resting cells.

In this paper, we attempt to delineate the mechanism of actin regulation by microinjecting living cells with exogenous nucleation sites. If the regulation of polymerization is achieved primarily by the capping of filaments in conjunction with the inhibition of self nucleation, we might observe an increase in actin polymerization after the microinjection. Our results, however, indicate little or no stimulation of actin polymerization, even though the nucleation sites remain capable of binding exogenous, fluorescently labeled actin subunits. The results are thus more consistent with monomer sequestration being the primary mechanism of regulation.

Materials and Methods

Preparation of Nucleation Sites and Fluorescently Labeled Actins

Muscle actin was purified from rabbit back and leg muscles after Spudich and Watt (1971). In some experiments, the actin was further purified by gel filtration chromatography in a Sephadex G-150 column (Sigma Chemical Co., St. Louis, MO), as described by MacLean-Fletcher and Pollard (1980). Nucleation sites were prepared by sonicating fluorescein phalloidin-labeled filaments of muscle actin. Briefly, G-actin was clarified at 25,000 rpm for 20 min in a rotor (42.2 Ti rotor; Beckman Instruments, Inc., Palo Alto, CA), and polymerized in 2 mM Tris-acetate, pH 6.95, 60 mM KCl, 1.4 mM MgCl₂, 0.2 mM ATP, 0.1 mM DTT. Fluorescein phalloidin (Molecular Probes Inc., Eugene, OR) was dissolved in a microinjection buffer containing 2 mM Tris-acetate, pH 6.95, 100 mM KCl, 2 mM MgCl₂, 0.2 mM ATP, 0.1 mM DTT. After clarification, the phalloidin solution was mixed with actin at a phallolidin/actin molar ratio of 0.65:1. The mixture was then dialyzed for 2 hours against the microinjection buffer and sonicated for 15–30 s in a bath sonicator immediately before use. The nucleation sites were microinjected at a concentration of 13.3 μM actin. In some experiments, identical results were obtained with actin polymerized and microinjected in the presence of 2 mM MgCl₂ and no KCl.

N-(1-pyrenyl)iodoacetamide (Molecular Probes Inc.) labeled actin (pyrene actin) was prepared as described by Koyama and Mihashi (1981) with minor modifications (Cooper et al., 1983). The dye-to-protein molar ratio was determined to be 0.8. Termethylrhodamine iodoacetamide (Molecular Probes Inc.) labeled actin (rhodamine actin) was prepared as described previously (Wang, 1984). The dye-to-protein molar ratio was estimated to be 0.7. Rhodamine actin was microinjected in a buffer of 2 mM Tris-acetate, pH 6.95, 0.05 mM MgCl₂, 0.2 mM ATP, and 0.1 mM DTT at a concentration of 4.0–5.0 mg/ml.

Biochemical Assays

The percentage of unpolymerized actin in normal rat kidney (NRK) cells was determined with the DNase I assay at 25°C, essentially according to Bildstok et al. (1978). To inhibit the depolymerization of F-actin after cell lysis, 8 μM phalloidin was included in the lysis buffer as described by Podolski and Steck (1988). Hydrolysis of calf thymus DNA (Sigma Chemical Co.) was followed by the decrease in the fluorescence of ethidium bromide, which was added to the assay mixture at a concentration of 0.4 μg/ml (Laub et al., 1981). Fluorescence measurements were performed with a spectrophuorometer (LS-3; Perkin-Elmer Corp., Norwalk, CT), at an excitation wavelength of 520 nm and an emission wavelength of 602 nm. Compared to the measurements of absorbance at 260 nm (Bildstok et al., 1978), this method offers a higher sensitivity and signal-to-noise ratio.

The assay of the activity of nucleation sites in vitro was performed with pyrene actin, similar to that described by Gershman et al. (1984). Pyrene actin was diluted with unlabeled actin to obtain a molar ratio of 1:20 and a total G-actin concentration of 2.5 μM. At t = 0, 1 vol of a mixture of KCl and MgCl₂, with or without 20 nM actin in the nucleation sites, was added to 8 vol of G-actin to obtain a final KCl and MgCl₂ concentrations of 55 mM and 0.55 mM, respectively. Increase in fluorescence was measured with an excitation wavelength of 365 nm and an emission wavelength of 407 nm.

Cell Culture, Microinjection, and Microscopy

NRK epithelial cells (NRK-52E; American Type Culture Collection, Rockville, MD) were cultured in F12K medium (Hazleton Research Products Inc., Lenexa, KS) supplemented with 5% Nu-serum (Collaborative Research Inc., Bedford, MA), 50 μg/ml streptomycin and 50 U/ml penicillin, and were maintained at 36–37°C with 5% CO₂. In some experiments, subclones that showed a uniform phase morphology were used. Swiss 3T3 cells (American Type Culture Collection) were cultured in DME medium (Hazleton Research Products Inc.) supplemented with 10% calf serum (Sigma, St. Louis, MO). 50 μg/ml streptomycin and 50 U/ml penicillin. Cells were plated on microinjection dishes (McKenna and Wang, 1989) for 12–24 h before microinjection.

Microinjection was performed essentially according to Grussmann et al. (1980). The pressure was generated either with an empty syringe or with an electronic pressure regulation system developed in our laboratory. The cells were maintained on a microscope stage enclosure as described previously (McKenna and Wang, 1989).

Fluorescence images were detected with an ISIT low-light-level video camera (Dage-MTI, Michigan City, IN), coupled to a microscope (IM35 or IM; Carl Zeiss, Inc., Thornwood, NY). The camera was operated below the level of saturation. All observations were made with either a 25×/NA 0.8 Plan-Neofluar objective or a 100×/NA 1.3 Neofluar objective (Cari Zeiss Inc.). The software and hardware used for fluorescence image processing have been described previously (McKenna et al., 1985). A graphics tablet, in conjunction with programs developed in our laboratory, was used to obtain both averaged and integrated fluorescence intensities over specified areas.

Fluorescent Staining of Cells

Cells were rinsed with PHEM buffer (10 mM EGTA, 2 mM MgCl₂, 60 mM Pipes, 25 mM Hepes, pH 6.9; Schliwa and van Blerkom, 1981). NRK cells were extracted and fixed simultaneously with 0.5% Trition X-100, 3.3% formaldehyde (Polysciences Inc., Warrington, PA) in PHEM buffer, pH 6.1, at 37°C for 5 min. 3T3 cells were fixed for 10 min with 2.2% formaldehyde in PHEM buffer, pH 6.1, at 37°C and then extracted for 5 min in an acetone bath chilled in dry ice. The latter procedure yielded a better retention of the nucleation sites. After three rinses with PHEM buffer, pH 6.9, cells were stained for 5 min with rhodamine phalloidin dissolved in PHEM buffer, pH 6.9, following instructions from Molecular Probes Inc.

Immunofluorescence staining was performed essentially as described by Amato et al. (1983). Antibody against the nonmuscle gamma actin was kindly provided by Dr. J. C. Bulinski (Columbia University, NY). This antibody, referred to as the gamma peptide antibody, was prepared using a gamma-actin-specific synthetic peptide as the antigen and has been characterized in detail by Oney et al. (1986, 1988).

Preparation of Cell Models

Extracted cell models were prepared by replacing the culture medium with 0.1% Trition X-100, 8 μM phalloidin (Boehringer Mannheim Biochemicals, Indianapolis, IN) in PHEM buffer, pH 6.1, and incubating at room temperature for 2 min. The coverslip then was carefully rinsed with PHEM buffer, pH 6.9, incubated for 5 min in a nucleation buffer (5 mM Hepes, pH 6.5, 2 mM EGTA, 138 mM KCl, 1 mM ATP, with or without 0.5 μM cytochalsin D) containing 8 μM phalloidin, and then incubated for 20 min with 20 μg/ml rhodamine actin in the nucleation buffer. Unbound actin was rinsed off with the nucleation buffer before observation.

Results

Characterization of Cells and Nucleation Sites

Experiments were performed with NRK epithelial cells and Swiss 3T3 fibroblasts. Under our culture conditions, most 3T3 cells showed prominent stress fibers. Although stress fibers were detectable in NRK cells after staining with...
fluence after staining with fluorescent phalloidin or antibodies. This greatly facilitated the comparison of fluorescence intensities at the nucleation sites and in the surrounding region. In addition, NRK cells were much more uniform in shape and size. For these reasons, NRK cells were used in most experiments, although similar results were obtained with 3T3 cells. We have used the DNase I assay to measure the percentage of unpolymerized actin in NRK cells. The value, 50–60%, is very similar to those reported for other cultured cells (Blikstad et al., 1978).

Nucleation sites were prepared by sonicating muscle actin filaments that had been labeled with fluorescent phalloidin. Fluorescein phalloidin served both to stabilize the nucleation sites, and to label the nucleation sites for detection. As described by Yanagida et al. (1984) and by Kron and Spudich (1986), individual actin filaments labeled with fluorescein phalloidin can be directly observed under a fluorescence microscope (Fig. 1). The sonicated fragments had an apparent length of 0.9 ± 0.8 μm (n = 151).

The ability of the actin filament fragments to nucleate actin assembly in vitro was confirmed with pyrene actin (Gershaman et al., 1984). A lag period of 1–2 min, detected in the absence of nucleation sites, was completely abolished by the addition of nuclei containing 2.2 nM actin (Fig. 2).

Failure of the Microinjected Nucleation Sites to Stimulate Extensive Actin Polymerization

Nucleation sites were microinjected into NRK and Swiss 3T3 cells to determine whether they can induce polymerization of endogenous actin subunits. The injection caused no detectable change in the cell as determined by phase optics. After a 20-min incubation, injected cells were processed for indirect immunofluorescence to reveal the distribution of actin. We have used a primary antibody that reacts only with endogenous nonmuscle actin (Otey et al., 1986), to exclude the staining of microinjected nucleation sites. Rhodamine-labeled secondary antibodies were used to allow distinction from the fluorescein-labeled nucleation sites.

The distribution of actin in injected cells appeared indistinguishable from that in un.injected cells. Furthermore, little or no increase in local actin concentration was observed around the microinjected nucleation sites, as shown in Fig.

3. The averaged signal at the nucleation sites was very close to that in the surrounding region (ratio = 1.04 ± 0.09, n = 17). Similar results were obtained with nucleation sites prepared from column-purified actin (ratio = 1.11 ± 0.12, n = 13), indicating that the trace amount of capping factors in the conventional preparation of actin is not responsible for the lack of stimulated polymerization.

A second way to examine the effect of the nucleation sites was to measure the total amount of filamentous actin in injected cells. To this end, one member of a pair of NRK sister cells was microinjected with fluorescein-labeled nucleation sites. After 20-min incubation, both cells were stained with rhodamine phalloidin (Fig. 4). Because of the specific binding of phalloidin with F-actin, the integrated fluorescence intensity over a cell should be proportional to the total amount of actin filaments. The values from paired sister cells were within 2% of each other (ratio = 1.005 ± 0.23, n = 15), suggesting that there was no significant increase in the extent of actin polymerization after the microinjection of nucleation sites. Similar results were obtained with nucleation sites prepared from column-purified actin (ratio = 1.004 ± 0.15, n = 11).

Interactions between Microinjected Nucleation Sites and Exogenous Actin Subunits

We have performed double microinjection experiments to determine whether the nucleation sites have maintained the ability to interact with exogenous actin subunits. NRK and Swiss 3T3 cells were first microinjected with the nucleation sites, and then incubated for 20 min before the second injection with monomeric rhodamine actin. After further incubation for 20 min, cells were fixed and extracted and the rhodamine fluorescence examined. As shown in Fig. 5, the rhodamine actin clearly became associated with the nucleation sites. For NRK cells the averaged signal at the nucleation sites was 80% higher than that in the surrounding cytoplasm (ratio = 1.80 ± 0.43, n = 16), indicating that the nucleation sites had maintained the ability to bind actin subunits. These results were independent of the site of the second microinjection relative to that of the first injection.
Figure 3. Lack of association of cytoplasmic actin with microinjected nucleation sites. An NRK (a and b) or Swiss 3T3 (c and d) cell was microinjected with fluorescein phalloidin-labeled nucleation sites, incubated for 20 min, and then processed for indirect immunofluorescence using a primary antibody specific for the endogenous actin and a rhodamine-labeled secondary antibody. Fluorescein fluorescence shows a cluster of nucleation sites (b and d). Rhodamine fluorescence indicates that endogenous actin fails to concentrate around the nucleation sites (a and c; arrows). Bar, 10 μm.

Control experiments were performed by using rhodamine-labeled ovalbumin in the second microinjection. Before extraction and fixation, there was no detectable concentration of ovalbumin at the nucleation sites. After extraction, the ovalbumin became dissociated from the cell beyond the level of detection. These results ruled out nonspecific associations through the rhodamine group as a binding mechanism. As a second control, we used buffer alone in the second microinjection, and then processed the cell for actin immunofluorescence. No concentration of endogenous actin at the nucleation sites was detected, indicating that the association of exogenous actin was not caused by perturbations from the second microinjection.

In Fig. 6, rhodamine actin was microinjected into NRK cells 1 h before the microinjection of nucleation sites. In this case, little or no association with the nucleation sites was detected. The results were similar to that obtained by single microinjection and immunofluorescence (Fig. 3), suggesting that cells may be able to regulate the exogenous actin following a period of incubation.

To characterize further the association of exogenous actin with the nucleation sites, extracted, phalloidin-stabilized cell

Figure 4. Similar distribution of actin filaments in injected and uninjected sister cells. One of a pair of sister NRK cells in a subclone was microinjected with fluorescein-labeled nucleation sites. The cells were incubated for 20 min, and then stained with rhodamine phalloidin. The distribution of rhodamine fluorescence was similar for injected (b) and uninjected (a) cells. No concentration of actin filaments was detected near the cluster of nucleation sites (arrow). The nucleation sites themselves were labeled poorly with rhodamine phalloidin since they were already heavily labeled with fluorescein phalloidin. Bar, 10 μm.
models were prepared after the NRK cells had been microinjected with nucleation sites. The cell model was then incubated with rhodamine actin under a condition that was unfavorable for self-nucleation but allowed elongation. As shown in Fig. 7a, the rhodamine actin became associated with both the nucleation sites and the cytoskeleton, with the nucleation sites being the most prominent structure. When 0.5 μM cytochalasin D was included during the incubation, the labeling of both the cytoskeleton and the nucleation sites was significantly reduced (Fig. 7b), even though the nucleation sites remained intact as detected with fluorescein fluorescence.

Discussion

Since actin polymerization involves two discrete components, actin monomers and nucleation sites or filament ends, the reaction may be inhibited by blocking the activity of either component. Thus, two possible mechanisms may be used by the cell to maintain a high level of unpolymerized actin: blocking the activity of monomers, or capping the filaments and inhibiting the formation of nucleation sites. The two mechanisms may be distinguished by determining which component remains active in the cell.

We have microinjected functional nucleation sites, which consisted of fragments of fluorescein phalloidin-labeled actin filaments, into living cells to test whether they can induce polymerization of the endogenous actin subunits. Based on an average length of 0.9 μm or ~300 subunits and an actin concentration of 0.6 mg/ml, one can estimate that 100–1,000 nucleation sites were delivered per 1 × 10⁻⁴ liter. Furthermore, assuming that the microinjected volume corresponded...
To 5% cell volume, the amount of actin microinjected as nucleation sites should correspond to <0.5% of the endogenous actin.

Based on staining with fluorescent phalloidin and on indirect immunofluorescence with antibodies specific for endogenous actin (Fig. 3), the nucleation sites induced no detectable change in the distribution of actin. Furthermore, little or no endogenous actin became associated with the nucleation sites. One possibility is that new actin filaments did assemble at the nucleation sites, but subsequently dissociated and moved to other regions of the cell. However, when injected and uninjected sister cells were stained with rhodamine phalloidin, similar total intensities were obtained, indicating that there was no detectable increase in the total amount of actin filaments after the microinjection of nucleation sites.

Therefore, either endogenous actin subunits were incapable of polymerizing onto the nucleation sites, or the nucleation sites became inhibited after microinjection. This question was examined both by microinjecting rhodamine actin into cells that had previously been microinjected with nucleation sites, and by incubating nucleation sites in cell models with rhodamine-labeled actin subunits. In both cases, exogenous actin became extensively associated with the nucleation sites, indicating that the nucleation sites have maintained their activities. Control experiments indicated that the association was not because of either nonspecific binding through the rhodamine group or perturbations from the microinjection. In addition, the sensitivity of the association of rhodamine actin with nucleation sites to cytochalasin D suggested that at least part of the association represented polymerization at the barbed ends.

Based on these observations, we may conclude that endogenous actin subunits, as compared to exogenous purified actin, are much less capable of interacting with nucleation sites. In the experiment involving the microinjection of rhodamine actin (Fig. 5), assuming that 5% cell volume of rhodamine actin at 100 μM was microinjected, the cytoplasm should contain approximately 5 μM rhodamine actin, which showed extensive incorporation into the nucleation sites. Thus, the concentration of endogenous "active" actin is probably much lower than 5 μM, or ~5% of the unpolymerized actin molecules.

The simplest explanation for this observation is that monomer sequestration proteins play a major role in regulating actin polymerization. Although the regulation of nucleation and the capping of filament ends may also be involved in controlling the rate or location of actin polymerization after cell activation, it is unlikely to be responsible for maintaining the high concentration of unpolymerized actin molecules in resting cells. This conclusion is consistent with previous experiments involving the microinjection of fluorescently-labeled actin into cultured cells (Glacy, 1983). Incorporation into microfilament-rich structures, including lamellipodia and stress fibers, was detected within 5 min of microinjection, indicating that the ends of endogenous actin filaments in these structures are not stably capped.

The protein(s) actually responsible for the sequestration of actin monomers remains to be identified. The candidate must be relatively abundant to inhibit a high concentration of actin monomers (close to 100 μM), and should respond to certain second messengers to allow actin polymerization in response to stimuli. Profilin represents the best characterized monomer sequestration protein, but its affinity for actin and its intracellular concentration may not be high enough to account for the extent of inhibition (Pollard and Cooper, 1986; Lind et al., 1987). Actin depolymerizing factor from chick brain (Bamburg et al., 1980), and similar proteins found in Acanthamoeba and in starfish oocyte (Cooper et al., 1986; Mabuchi, 1983) are attractive alternatives, but the amount of this protein again appears too low to account for the extent of inhibition of polymerization (Cooper et al., 1986; Koffer et al., 1988). Actobindin also binds actin monomers and is present at a relatively high concentration in Acanthamoeba. However, the affinity of this protein for actin monomers appears low (Lambooy and Korn, 1986, 1988). A definitive identification of the monomer sequestration protein and its control mechanism represents the crucial next step in our understanding of the regulation of actin polymerization in living cells.

The authors wish to thank Dr. J. C. Bulinski for the gift of antibodies against nonmuscle gamma actin, and Dr. E. Luna for reading the manuscript.

This study is supported by grants from the National Institutes of Health (GM-32476) and the Muscular Dystrophy Association.

Received for publication 14 July 1989 and in revised form 13 October 1989.

**Figure 7. Cytochalasin-sensitive association of rhodamine actin with nucleation sites and cytoskeleton.** NRK cells were microinjected with fluorescein-labeled nucleation sites. After incubation for 20 min, the cells were extracted with 0.1% Triton X-100 and incubated with rhodamine actin under a condition unfavorable for self-nucleation. In the absence of cytochalasin D (a), there was an extensive association of rhodamine actin with both the nucleation sites (arrow) and the cytoskeleton. In the presence of 0.5 μM cytochalasin D (b), the associations with both the nucleation sites (arrow) and the cytoskeleton were severely inhibited. Images in the two panels were recorded and printed under identical conditions. Bar, 10 μm.
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