Mobility of Microinjected Rhodamine Actin within Living Chicken Gizzard Cells Determined by Fluorescence Photobleaching Recovery

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Summary

Rhodamine-labeled actin microinjected into living embryonic chicken gizzard cells became associated with its characteristic cytoskeletal structures. In these domains the translational diffusion coefficients (D) of rh-actin were determined in vivo by fluorescence photobleaching recovery (FPR) measurements. Two classes of actin molecules with respect to their mobilities were detected: rh-actin with a half-time of recovery of 5–10 min in stress fibers and focal contacts (immobile on the time-scale of FPR measurements) and rh-actin with D = 2–3 × 10^{-9} cm²/sec in the cytoplasm and leading lamellae. The slow recovery on stress fibers exhibited similar kinetics whether a short segment or the entire structure was photobleached, indicating that recovery occurs predominantly by exchange with the surrounding diffusible actin. We propose that a steady-state equilibrium between the soluble and cytoskeletal pool of actin exists in living cells.

Introduction

A prerequisite for many of the different motile activities of living cells is the existence of highly dynamic cytoskeletal networks. The rapid assembly and disassembly of these structures is probably required for the reorganization of intracellular structures, changes in cell shape, formation of cellular contacts and cell locomotion. Many studies over the last several years implicated the actin-associated microfilament system with such dynamic cellular events (Buckley and Porter, 1967; Pollard and Weihing, 1974; Trinkaus, 1976; Korn, 1978). It was shown that nonmuscle cells contain actin, myosin and several additional actin-related proteins that may participate in mechanoochemical processes (Ishikawa et al., 1969; Lazarides and Weber, 1974; Pollard and Weihing, 1974; Weber and Groeschel-Stewart, 1974; Lazarides, 1976; Korn, 1978). Immunolabeling of a variety of cell types with antibodies to actin or its associated proteins indicated that they are assembled in several distinct, structurally defined domains. These include the highly motile ruffling membrane and leading lamella, as well as the stress fibers (see for example Lazarides and Weber, 1974; Lazarides, 1976; Wehland et al., 1979). Electron microscopy has shown that actin in the leading lamella and in the ruffling membrane is largely organized in a three dimensional network of filaments. The stress fibers are tightly packed bundles of microfila-

ments that are associated at their termini with the cell membrane (Buckley and Porter, 1967; Goldman and Knipe, 1972; Heath and Dunn, 1978; Small et al., 1976).

Most of our knowledge of the cellular motile apparatus is derived from observations with nonviable cells. Obviously, electron microscopy (conventional or high voltage) requires adequate fixation of the examined cells. The same is true for immunofluorescence, where permeabilization and fixation steps are required. Furthermore, reconstituted model systems in vitro (Wegner, 1976; Kirschner, 1980; Simpson and Spudich, 1980), provide only limited information concerning the dynamics of the native structures in the living cell.

Fluorescence photobleaching recovery (FPR) measurements have been used for several years to determine the mobility of fluorescent probes attached to lipids or proteins in membranes of living cells (Edidin et al., 1976; Jacobson et al., 1976; Schlessinger et al., 1976; Peters, 1981). Recently, this technique was applied to determine the diffusion coefficients of fluorescently labeled bovine serum albumin (BSA) and goat immunoglobulin G (IgG), which were introduced into the cytoplasm of fibroblasts by red-cell-mediated microinjection (Wojcieszyn et al., 1981).

Several groups have demonstrated that cytoskeletal structures may be visualized within the cytoplasm of living cultured cells (for reviews see Taylor and Wang, 1980; Kreis and Birchmeier, 1982). This was achieved by microinjection of fluorescently labeled cytoskeletal proteins into cells with the use of glass capillaries and by subsequent determination of the distribution of the injected proteins by fluorescence microscopy (Taylor and Wang, 1978; Feramisco, 1979; Kreis et al., 1979; Burridge and Feramisco, 1980; Kreis and Birchmeier, 1980; Wehland and Weber, 1980; Keith et al., 1981). Until now, however, only limited advantage has been taken of this approach for studies on the dynamic features of cytoskeletal structures including their assembly, their interactions with the cell membrane and the generation and transduction of mechanical forces.

The purpose of the present study is to analyze the intracellular dynamics of actin. We tested the incorporation of microinjected actin into the various actin-containing domains and determined in vivo the association of rhodamine actin (rh-actin) with preexisting actin as well as with newly formed filamentous arrays in sessile and in moving cells. Furthermore, we measured the mobility of rh-actin within the various cytoplasmic actin-containing domains by using the method of FPR.

Results

The Distribution of Microinjected rh-Actin in Living Chicken Gizzard Cells

Shortly after microinjection into embryonic chicken gizzard cells, rh-actin became associated with fila-
mentous structures throughout the cells. This incorporation of rh-actin into the cellular networks was observed as early as 10–20 min after microinjection and could still be detected within the cells 24–48 hr later (Figures 1A and 1C).

A comparison between the distribution of microinjected rh-actin and the total cellular filamentous actin (F-actin; visualized by labeling with fluoresceinated phalloidin within the same cell) pointed to an almost identical distribution (Figure 1). Naturally, a significant degree of variability existed in the pattern of rh-actin distribution within individual living cells of any population of cultured cells (Figure 2). Two extreme examples are shown in Figures 2A–2C. The first two show the distribution of rh-actin in a typically motile cell, and the latter shows its distribution in a sessile cell. In motile cells, an intense fluorescence of rh-actin appeared at the edge of the leading lamellipodium (see also arrows in Figures 1A and 1B) and was apparently associated with numerous spots that correspond to small focal contacts (2B). Some motile cells incorporated the labeled protein primarily into the termini of stress fibers near focal-contact areas (see for instance 2A, 2B and 2G), whereas the rest of the actin bundles remained poorly labeled. Double staining of the same cells for total cellular actin with fluoresceinated phalloidin (fl-phalloidin) or with specific antiactin antibodies revealed a homogeneous and

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**Figure 1.** Comparison of the Distribution of Cellular Actin Labeled with Fluoresceinated Phalloidin with Microinjected Rhodamine-Labeled Actin
Chicken gizzard cells were microinjected with rh-actin and fixed either 30 min (A and B) or 24 hr (C and D) later, permeabilized and incubated with fl-phalloidin. (A and C) Distribution of rh-actin; (B and D) distribution of fl-phalloidin in the same cells. Notice the closely related patterns of rh-actin and fl-phalloidin in injected cells and the absence of rh-actin from the neighboring, fluorescein-labeled cells. Bar = 10 μm.

**Figure 2.** Distribution of Microinjected rh-Actin in Living Cultured Chicken Gizzard Cells 60 Min after Injection
(A and B) Apparently motile cell. The injected actin in this cell is primarily associated with small areas corresponding to newly formed focal contacts and with short filament bundles. (C) Typical sessile cell with large, intensely labeled stress fibers. (D) Microinjected cell, which displays several patterns of rh-actin organization, including focal contacts, stress fibers and polygonal perinuclear networks. (E and F) Fluorescence and interference-reflection images of part of the cell shown in (D). Notice the continuous distribution of rh-actin along the filament bundles and their termini, which are associated with focal contacts. Arrowheads in (D–F) point to same area. (G) Distribution of microinjected rh-actin along thin microfilament bundles. Notice the striated pattern of fluorescence along the filaments. (H) Microinjected rh-actin associated with intense, perinuclear cytoplasmic dots and interconnecting filament arrays, corresponding to polygonal actin networks and their vertices. (I) Distribution of rh-actin in elongated cellular process. In this particular cell the tip of the process is >100 μm apart from the site of microinjection. Bars = 10 μm.
Dynamics of Microinjected Rhodamine Actin
continuous labeling along the entire length of the stress fibers (data not shown). In motile cells, usually only a limited fraction of the labeled protein was observed in distinct microfilament bundles. In the sessile cells, however, the microinjected rh-actin became associated with filament bundles that often spanned the entire length of the cytoplasm. Both forms of cells could be detected in essentially every culture, although young cultures (6-24 hr after plating) contained a significantly higher number of motile cells. We often observed cells that exhibited both features; the cell shown in Figures 2D-2F contains a protruding lamellipodium as well as thick stress fibers, and the fluorescent tracer-protein was incorporated into both areas. Furthermore, many cells contained polygonal networks of actin filaments in the perinuclear area (Figures 2D and 2H). Thetermi of the labeled actin bundles were apparently associated with the membrane at cell-substrate focal contacts. This is shown in Figures 2D and 2E, which depict the organization of microinjected actin in the posterior tail area. The corresponding interference-reflection image of the same cell (Figure 2F) indicates that rh-actin is associated both with the focal contacts and with more central regions of the stress fibers (Figures 2E and 2F). Thin filament bundles the appearance of the labeled actin was often striated with a periodicity of about 1 striation/μm (Figure 2G). Note that although the fluorescent actin was usually injected into the cytoplasm at the perinuclear region, it became distributed throughout the entire cell. Reorganization of rh-actin was observed even in distal, elongated processes remote (>100 μm) from the site of microinjection (Figure 2I).

To monitor the assembly of stress fibers and focal contacts and to follow their fate during cell locomotion, the changes in the distribution of rh-actin were recorded for more than 1 hr. Therefore, the cells were visualized by a video-intensifying system (ISIT camera, see Experimental Procedures), which required a very low intensity of illumination and minimized possible light-induced damage to the cells (Schlessinger et al., 1978). We found that microinjected rh-actin participated in the formation of new focal contacts and stress fibers. Focal contacts and stress fibers appeared initially as small spots near the leading edge and became more extensive in size and fluorescence intensity within 10-20 min. Furthermore, although the spatial arrangement of actin in focal contacts is relatively stable, their orientation and exact shape may change significantly during cell locomotion (T. E. Kreis et al., in preparation).

Definition and Characterization of rh-Actin-Containing Structural Domains in Living Chicken Gizzard Cells

As mentioned above, microinjected rh-actin displayed a rather heterogeneous pattern of distribution in cells. Although the different actin-containing structures within cells have been extensively described and discussed over the past several years (see for example Lazarides and Weber, 1974; Lazarides, 1976; Heggenes et al., 1977; Gordon, 1978), we find it necessary to define here the different actin-containing structural domains that we detected in living, microinjected cells. These definitions will be essential for both the quantitative and qualitative evaluation of the cellular dynamics of actin we describe. In the diagram shown in Figure 3, we marked six characteristic actin-containing areas: (one) the interfibrillar space in between stress fibers in flat regions at the cell periphery, where no defined fibrillary structures are resolved by fluorescence microscopy; (two) the perinuclear area, where the actin organization is apparently diffuse and the thickness of the cell is several fold higher than at the periphery; (three) the leading edge, a domain that is most developed in motile cells; (four) the focal contacts that are usually located at the termini of stress fibers and may be defined either by interference-reflection microscopy or by immunofluorescence-labeling for vinculin; (five) the stress fibers or microfilament bundles; and (six) the trailing edge, which usually has a cylindrical geometry, is rich in stress fibers and often contains large focal contacts.

Quantitative Analysis of the Distribution of rh-Actin within the Specific Cellular Domains

We used the optical detection system of the FPR apparatus to evaluate the relative fluorescence intensities of rh-actin in different regions of living chicken gizzard cells. The attenuated laser beam (radius 1.5 μm) was focused on the appropriate cellular areas, and the fluorescence intensities of the areas were measured. The relative intensities of microinjected rh-actin within the six domains characterized in Figure 3

Figure 3. Schematic Drawing of the Different Actin-Containing Domains

Six different domains are marked: (1) interfibrillar space near the cell border; (2) interfibrillar space in the perinuclear area; (3) loading edge; (4) focal contact; (5) stress fiber; and (6) trailing edge.
are summarized in Table 1. It should be emphasized that the fluorescence intensities in each of these regions were compared with those measured in the neighboring interfibrillar space. Hence the increased values of fluorescence intensity measured on stress fibers or focal contacts are not the result of increased cell thickness, but are, rather, proportional to the concentration of actin in these domains.

**Quantitative FPR Measurements of the Mobility of Microinjected Proteins in Living Chicken Gizzard Cells**

Microinjected cells were localized in the microscope by using a silicon-intensified target (SIT) camera with 10–50 fold attenuated excitation light. The attenuated laser beam was then focused on the appropriate cellular domain, and FPR measurements were performed.

Typical FPR curves obtained for rh-actin in the different cellular regions are shown in Figure 4. The six actin-containing domains can be divided into two major classes with respect to the mobility of actin within them: virtually immobile rh-actin was found in stress fibers and focal contacts, while variable amounts of mobile rh-actin were detected in the other cellular domains with a diffusion coefficient (D) of 2–3 x 10^-9 cm^2/sec (Table 2). In stress fibers and in focal contacts more than 80% of the rh-actin was immobile on the time scale of the FPR measurements. The approximately 20% mobile fraction in these areas was most likely owing to rh-actin from the neighboring interfibrillar area, including the space above or below these structures (the diameter of the laser beam is larger than the stress fiber itself). Hence the diffusion of the mobile fraction of rh-actin measured in stress fibers or focal contacts probably does not reflect the mobility of the tracer protein within these structures. There were no apparent differences in the FPR data whether the measurements were performed 1 hr or 12 hr after microinjection of rh-actin (data not shown).

Quantitative analysis by FPR measurements of the mobility of rh-actin is shown in Table 2, and that of fluorescently labeled control proteins (rh-BSA, fl-BSA, rh-goat-IgG and fl-goat-IgG) is shown in Table 3. Partial fluorescence recovery after photobleaching was measured for rh-actin in all the domains investigated (maximal values obtained in the interfibrillar domains were up to 70%). The apparent diffusion coefficients of rh-actin in all areas were close to 3 x 10^-9 cm^2/sec. The only area with somewhat reduced mobility of rh-actin (D = 2 x 10^-9 cm^2/sec) was the leading edge of the cells.

Repeated bleaches on either focal contacts, stress fibers or interfibrillar domains yielded 70%–80% fluorescence recovery after photobleaching with an apparent diffusion coefficient of about 2.5 x 10^-9 cm^2/sec.
This observation also supports the notion that actin immobility in the various areas was not induced by the laser photobleaching pulse itself.

The microinjected control proteins, BSA and IgG,

<table>
<thead>
<tr>
<th>Fluorescently Labeled Protein</th>
<th>Cellular Domain</th>
<th>Mobile Fraction (%)</th>
</tr>
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<tbody>
<tr>
<td>S. or f-BSA (1.6 mg/ml)</td>
<td>Border area</td>
<td>6.2 ± 0.9 (14)</td>
</tr>
<tr>
<td>S. or f-BSA (1.5 mg/ml)</td>
<td>Perinuclear</td>
<td>6.3 ± 0.9 (7)</td>
</tr>
<tr>
<td>S. BSA (8 mg/ml)</td>
<td>Border area</td>
<td>6.3 ± 1.3 (20)</td>
</tr>
<tr>
<td>S. BSA (15 mg/ml)</td>
<td>Border area</td>
<td>5.7 ± 1.0 (10)</td>
</tr>
<tr>
<td>S. IgG (2 mg/ml)</td>
<td>Border area</td>
<td>6.0 ± 1.3 (15)</td>
</tr>
<tr>
<td>S. IgG (2 mg/ml)</td>
<td>Perinuclear</td>
<td>6.6 ± 0.7 (12)</td>
</tr>
</tbody>
</table>

Stress fibers of a living microinjected cell (A) were bleached by a focused laser beam (1.5 μm radius). The photobleached line is indicated by the arrows and the double line in (B). The video-intensified image of the cell was photographed from the screen of the television monitor prior to bleaching (A), and 2 min (B), 10 min (C) and 30 min (D) after bleaching. Bar = 10 μm.

Notice that rh-actin in the two focal contacts and filament bundles indicated by arrows were completely photobleached. Bar = 10 μm.
labeled with either rhodamine or fluorescein, showed essentially complete recovery (>90%) with apparent diffusion coefficients of $\sim 6 \times 10^{-9}$ cm$^2$/sec (Table 3). Similar values were obtained when the measurements were performed within the flat part of the cell at the periphery, or in the thicker perinuclear areas. The apparent diffusion coefficients of either BSA or IgG are approximately two-fold the values obtained for rh-actin in the same interfibrillary areas. Furthermore, in cells microinjected with a mixture of rh-actin and F-BSA, each protein exhibited its characteristic mobility, even when the two were measured at the same spot (data not shown).

Quantitative Analysis of the Long-Term Kinetics of rh-Actin in Focal Contacts and Stress Fibers

FPR measurements indicated that rh-actin that is associated with either stress fibers or focal contacts is "immobile" on the time scale of FPR experiments. Nevertheless, it was possible to detect a slow rate of fluorescence recovery of rh-actin to photobleached areas in these two domains. To evaluate this slow rate of incorporation of rh-actin into preexisting stress fibers, we bleached a continuous line across a parallel array of stress fibers with a focused laser beam (details in Experimental Procedures) and recorded the recovery of fluorescence in the bleached area by video-intensified time-lapse cinematography (Figure 5). The dark line that was clearly apparent 2 min after bleaching (Figure 5B) gradually disappeared and was hardly detectable 35 min later (Figure 5D). Association of rh-actin could also be detected with preexisting focal contacts. To demonstrate this, two lines perpendicular to each other were bleached in a cell 1 hr after microinjection of rh-actin (Figure 6A), as indicated schematically in Figure 6B. This treatment resulted in the complete disappearance of at least two prominent focal contacts and the entire actin bundles that were associated with them (arrows in Figure 6). Ten minutes after bleaching, rh-actin reappeared in both domains, and virtually complete recovery was observed after 30–35 min. During the time of recording the cell moved. This was manifested by protrusions and retractions of the lamellipodium.

To determine the source of unbleached rh-actin that leads to fluorescence recovery in focal contacts and stress fibers, we photobleached segments of variable lengths on these structures with either a vertical or horizontal line (20–50 μm long, 3 μm wide). Subsequently, we focused an attenuated laser beam on the central part of the bleached area and measured the fluorescence intensity as a function of time (Figure 7). The rate of fluorescence recovery was very similar in all experiments, regardless of the length of the bleached segment. The apparent half-time of recovery was approximately 10 min.

Discussion

Microinjection of fluorescently labeled cytoskeletal proteins into tissue culture cells promises to be a very useful tool for the study of the distribution and dynamic properties of these components in vivo (for reviews see Taylor and Wang, 1980; Kreis and Birchmeier, 1982). So far, however, most investigations have concentrated on the incorporation of the injected proteins (including α-actinin, actin, tropomyosin, tubulin and vinculin) into the proper cytoskeletal network and, to a lesser extent, on the dynamic features of these proteins within single living cells. In the present study we applied a combination of several novel techniques to analyze the intracellular dynamics of actin in vivo. These include microinjection of fluorescently labeled actin, image-intensified time-lapse cinematography to follow the fate of the injected tracer protein and laser photobleaching experiments combined with fluorescence photobleaching recovery (FPR) measurements that enabled us to determine the mobilities of the injected protein within the cell.

Does Microinjected rh-Actin Reflect the Dynamic Properties of the Native Protein?

To evaluate the results and determine their relevance
to the dynamics of endogenous actin in unperturbed cells, we should first consider the technical and theoretical aspects of the methodologies employed and the nature of the information that can be generated by them.

One aspect is related to the possibility that an increase of the intracellular pool of actin caused by the injection might have an effect on the distribution of the native protein. This possibility appears highly unlikely, since the increase in the amount of cellular actin owing to microinjection is estimated to be in the order of 1% (Kreis et al., 1979; Kreis and Birchmeier, 1982). Moreover, fluorescence labeling of cells with antiaxin antibodies or with fl-phalloidin indicated that the actin pattern of injected cells was indistinguishable from that of their uninjected neighbors, both qualitatively and quantitatively (Figure 1).

Microinjected rh-actin efficiently labeled all the cellular actin-containing domains with a low background (see Figures 1 and 2) and participated in the de novo assembly and reorganization of cytoskeletal structures. The rh-actin used here could be detected in recipient cells even 48 hr after injection within the typical actin-rich structures. This turnover rate of rh-actin, appreciated from such prolonged incubations, is in line with that reported for the turnover of native actin, appreciated from such prolonged incubations, including the leading lamella. In all the cellular domains investigated by FPR, the translational diffusion coefficient of mobile rh-actin was in the range of 2–3 \( \times 10^{-9} \) cm\(^2\)/sec. Several mechanisms can account for such a low D value of mobile rh-actin (compared to BSA and IgG) inside the cytoplasm. The microinjected monomeric globular actin may rapidly assemble into short F-actin filaments, which diffuse slower because of their size. Or, actin might interact with the intracellular pool of "immobile" actin (cytoskeletal actin) and its associated proteins, which retard its mobility.

The fact that rh-actin appears in two classes of mobility suggests the following type of analysis (Eisen and Reidler, 1979):

\[
A + B \overset{k_1}{\underset{k_0}{\rightleftharpoons}} C
\]

where A defines an immobile actin-binding structure (such as a stress fiber or focal contact area) and B defines mobile rh-actin with an apparent translational diffusion coefficient \( D_B \). After binding to A and formation of C, B becomes immobile. Hence \( D_A = 0 \); \( k_1 \) and \( k_0 \) are the forward and backward reaction constants.

Two processes can be defined. In FPR, the characteristic time of diffusion of B (\( \tau_B \)) is given by \( \tau_B = \omega^2/4D_B \); \( \omega \) is the radius of the area of observation inside the cell. The characteristic time for association of B with A leading to C is defined as \( \tau_{\text{chem}}^A_B = (k_1[C_A + C_B] + k_0)^{-1} \), where \( C_A \) and \( C_B \) represent the concentrations of A and B, respectively. Since \( \tau_{\text{chem}}^A_B \) is in the range of minutes and \( \tau_B \) is in the range of seconds, we can assume that \( \tau_{\text{chem}}^A_B \gg \tau_B \). Therefore, rh-actin molecules B can be observed either freely diffusing with translational diffusion coefficient \( D_B \), or bound to A and therefore immobile (\( D_A = 0 \)).

Moreover, the existence of two mobility classes of rh-actin in cells, rather than a broad spectrum of D values reflecting the various sizes of assembled F-actin, seems to suggest that the assembly of rh-actin...
inside cells involves a cooperative process. The fraction of immobile rh-actin molecules (or the extent of rh-actin immobilization) should depend on the concentration of the preexisting immobile actin or other structures that bind the mobile form. Therefore, the extent of immobilization of rh-actin is expected to depend on the concentration of the microinjected protein. In cases where $\frac{\tau_{\text{actin}}}{\tau_{\text{rh-actin}}} \ll 1$, it is expected that a single diffusion coefficient and 100% recovery will be observed. This could be the case for rh-BSA and rh-IgG injected into the cytoplasm of cultured cells. Actin, either in a globular monomeric form or in short filaments, should appear mobile in FPR experiments. Furthermore, the hypothetical tailoring of actin in microfilaments (steady state equilibrium between G- and F-actin) of living cells also implies the presence of such mobile actin molecules (see for example Weggner, 1976; Kirschner, 1980; Simpson and Spudich, 1980). To date we cannot neither confirm the existence of G-actin in the cytoplasm nor present conclusive evidence for tailoring of actin in vivo.

Unlike BSA or IgG, rh-actin is partially "immobile" in each cellular domain. Approximately 35% rh-actin in the interfibrillary area appears immobile. This might reflect the amount of rh-actin incorporated into the microfilamentous web beneath the plasma membrane, which can be seen by electron microscopy or by immunofluorescent labeling (see for instance Avnur and Geiger, 1981a). Especially intriguing is the dynamic nature of the leading edge. In this area we measured the highest mobile fraction (~70%) and the lowest apparent diffusion coefficient ($D = 2 \times 10^{-9} \text{cm}^2/\text{sec}$; see Table 2). Ultrastructural analysis of the leading lamella indicates that this region contains a dense, three dimensional network of filamentous actin and usually no stress fibers (Duckley and Porter, 1967; Goldman and Knipe, 1972; Heath and Dunn, 1978; Small et al., 1978). It is possible that individual filaments might move within this web, or else that F-actin is in a steady state equilibrium with G-actin in this area. Thus the recovery of fluorescence might be a consequence of complex processes including polymerization and depolymerization of actin, diffusion of individual F-actin molecules as well as possible gelation-solution and contraction.

Rh-Actin from the Interfibrillary Space Incorporates into Cytoskeletal Structures

The specified actin-containing domains are structurally defined entities (see Figure 3). Nevertheless, none of the FPR measurements could be performed on one domain exclusively. Analyses performed on stress fibers or focal contacts could not avoid the interfibrillary actin that was adjacent, below or above. Thus the ~20% mobile fraction measured in these areas represents most likely the relative amount of soluble actin near the filament bundle. This is in good agreement with the relative intensities of rh-actin measured within these domains (see Table 1). The slow rate of relaxation characteristic to focal contacts and stress fibers was at least two orders of magnitude lower than the mobility in the interfibrillary space. It could not be attributed to translational diffusion of rh-actin. This fluorescence recovery probably represents chemical reaction between the stress fibers (or focal contacts) and the diffuse, mobile pool of actin. This reaction occurs at a similar rate whether a small spot of the entire stress fiber is photobleached (see Figure 7), indicating that most of the "recovery" occurs from the surrounding pool of actin and not from linear mobility along the stress fibers themselves. Thus we assume that even in such stable cytoskeletal structures as focal contacts and stress fibers there is a steady state equilibrium of association and dissociation of actin. This dynamic equilibrium might be regulated by a large number of different factors. Among these may be controlled polymerization-depolymerization processes driven by local changes in ionic concentration or specific proteins that promote polymerization or depolymerization (for review see Schliwa, 1981). Furthermore, the interaction of filamentous actin with various additional proteins may result in gelation-solution transitions as well as the reversible formation of bundles. It is expected that further extension of the approach used here, namely the combination of microinjection and FPR measurements, will shed light on the dynamic interactions and rearrangements of cytoskeletal elements in living cells.

Experimental Procedures

Reagents

Rhodamine lissamin B was from Research Organics (USA). The sulphonyl-chloride derivative (RB2000SC) was prepared according to the method of Brandtsae (1973) and stored at $-70^\circ\text{C}$.

Modified Proteins

Actin was isolated from an aceton powder of rabbit psoas muscle according to the procedure of Spudich and Watt (1971). The protein was more than 95% pure as estimated by polyacrylamide gel electrophoresis. F-actin (2 mg/ml) was modified with 0.3 mM RB2000SC (from a 6 mM stock solution in acetone) in a buffer containing 1 mM sodium borate, 2 mM Tris-Cl, 0.4 mM ascorbic acid, 1 mM ATP, 600 mM KCl, 0.2 mM CaCl$_2$ and 2 mM MgCl$_2$ (pH 8.5) at room temperature. After 10 min, the reaction was quenched with 14 mM mercaptoethanol and then F-actin was pelleted for 2 hr at 80,000 $\times$ g. The pellet was resuspended and depolymerized in 5 mM Tris-HCl, 0.2 mM CaCl$_2$, 1 mM ATP and 1.4 mM mercaptoethanol (pH 8.0) for 15 min on ice and then for 45 min at 0°C in the presence of 800 mM KI, 1 mM CaCl$_2$ and 4 mM ATP (pH 7.0). Modified actin was desalted and separated from uncovalently bound fluorochrome over a G50 column (Phastacryl, Pharmacia) equilibrated with actin-depolymerizing buffer A (see Spudich and Watt, 1971). The protein was collected and centrifuged for 2 hr at 60,000 $\times$ g. Aliquots of the supernatant containing modified G-actin (rh-actin, 1 mg/ml with a rhodamine to protein ratio of $\sim 2.0$) were frozen in liquid air and stored at $-70^\circ\text{C}$ up to three months. Before microinjection, rh-actin was cleared from aggregates by centrifugation in a Beckman airfuge for 30 min at.
permeabilized cells were incubated with fluorescein-labeled phalloidin and Singer (1979). For labeling of F-actin with phalloidin, fixed and permeabilized cells were incubated with fluorescein-labeled phalloidin (10 µg/ml) for 10 min and then extensively washed with PBS.

Microinjection Procedure

Microinjection on glass coverslips was performed as described by Kreis et al. (1979). However, thin wall capillaries (type QC 150TF–15, Clark Electromedical Instruments) were used. Injected cells on coverslips were mounted on a Dvorak-Stodtler chamber and incubated in medium without phenol red. The temperature on the microscope stage was kept at 37°C with a Zeiss air-stream incubator.

Fluorescence Microscopy and Image Intensification Microscopy

Fluorescence microscopy was performed on a Zeiss Photoscope Ill equipped with a Plan-Neofluar 25, 0.8; a Planapo 83, 1.4; and an Antiflex-Neofluar 63, 1.25 oil immersion objective, and a filter set for double (fluorescein–rhodamine) fluorescence. Image-intensified microscopy was performed on a Zeiss Universal Microscope (Neofluar 63, 1.25 oil immersion objective) with either a silicon intensifier target camera (SIT, RCA TC 1030/H) or with an intensified silicon intensifier target camera (ISIT, RCA TC 1040/H) connected to a time-lapse videotape (panasonic VTR-NV-8030) and a 9 in TV monitor (Schlessinger et al., 1978). The intensified cellular images were taken from the monitor with a Polaroid Camera on Type 665 Polaroid films with negatives.

Fluorescence Photobleaching Recovery

The diffusion coefficient of the microinjected fluorescently labeled actin in living cells was measured by the fluorescence photobleaching recovery method (Axelrodi et al., 1976). We have added to the FPR apparatus a silicon intensifier target camera (SIT or ISIT camera). Using these cameras we could load cells even faintly staining fluorescent reagents on or inside cells (see also Schlessinger et al., 1978). The sensitive cameras are also used to align and focus the laser beam (argon, 514 nm) in various areas of the labeled cells. The phase and fluorescent images were then time-lapse videotaped.

The diffusion coefficients were derived from the fluorescence photobleaching recovery curves according to the method of Axelrod et al. (1976). The range of diffusion coefficients that can be measured by our FPR apparatus is 10^{-9} to 3 x 10^{-12} cm^2/sec. Incomplete fluorescence recovery was interpreted as an indication that a fraction of the fluorophores is “immobile” on the time scale of the FPR experiments (D < 3 x 10^{-12} cm^2/sec).

Interpretation of FPR Measurements of Cytoplasmic Markers of Cultured Cells

The FPR methodology was originally developed for the measurement of the lateral diffusion coefficient of various membrane components. In most FPR experiments, a laser beam with a Gaussian profile is focused on the plane of the membrane that defines a focal plane W. The direction of propagation of the laser beam is defined as the Z axis and u is the e^{-2} radius of the laser beam at the focal plane W where Z = Z0. For this geometry we are using the two dimensional treatment (Axelrod et al., 1976) for which it was shown that:

\[ D = \frac{u^2}{4t_c} \]

However, when using this geometry for the measurement of diffusion inside the cytoplasm of living cells, we expect also diffusion along the Z axis. A high numerical aperture objective (X63/1.25 N.A.) yields a diverging beam along the optical axis. The e^{-2} radius at distance Z-Zo from the minimal radius uo equals to \( u(2)^2 = u^2 + (Z-Z_0)^2 tan^2 \theta \) (Schneider and Webb, 1981; Petersen et al., 1982), where \( \theta \) is the divergence angle given by \( \theta = \lambda / a s \). An important conclusion from this equation is that the beam width increases by not more than 10% over a distance of about 10 µm on either side of the focal plane (Petersen et al., 1982). Hence in thin areas at the periphery of cultured fibroblasts the beam width is essentially constant over this range, and therefore diffusion along the Z axis does not significantly contribute to the observed recovery and the two dimensional treatment (Axelrod et al., 1976) can be applied.

In our FPR setup, we use an image plane pinhole for recording the fluorescence recovery curves (Koppel et al., 1976). This geometry was shown to effectively restrict the measured fluorescence intensity from a volume extending no more than approximately 10 µm above and below the focal plane (Incenogle, 1981; Petersen et al., 1982).

Since over this range the beam expands by only 10%, the deviation in the measurement of D should not exceed 20% of the value obtained when using the beam size at the focal plane (W0). Indeed, FPR measurements of the diffusion of either rh-BSA or rh-IgG microinjected into living chicken gizzard cells indicated that similar values of D were obtained at cellular domains of different thickness (Table 3). These results provide experimental support to our assumptions.

Moreover, Wojciezyn et al. (1981) measured the cytoplasmic diffusion of similar reagents by FPR with the use of low power objectives in which the beam maintained almost constant width as it traversed the cytoplasm along the Z axis, thus allowing analysis by the two dimensional treatment (Axelrod et al., 1976). The values obtained for the diffusion coefficient in this study are very similar to the values of D reported in our study.

Acknowledgments

Chicken gizzard cells were kindly provided by Zafiros Avnur. We acknowledge stimulating discussions with Elliot L. Elson. T. E. K. is a recipient of an EMBO longterm fellowship. This work was supported by grants from the Muscular Dystrophy Association (B. G.) from the National Institutes of Health (J. S.) and from the U. S.-Israel Binational Science Foundation (J. S.). B. G. is an incumbent of the C. Reveno chair in biology.

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Received January 25, 1982; revised April 12, 1982

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